POTENTIAL OF LOCAL ISOLATES OF BACILLUS THURINGIENSIS IN THE CONTROL OF TUTA ABSOLUTA ON TOMATO PLANTS

By

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NIGERIA.

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DEPARTMENT OF MICROBIOLOGY
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DECLARATION

I declare that the work in this dissertation entitled 'POTENTIAL OF LOCAL ISOLATES OF BACILLUS THURINGIENSIS IN THE CONTROL OF TUTA ABSOLUTA ON TOMATO PLANTS' has been performed 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.

AKINYELURE, Eric Odunayo	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled 'POTENTIAL OF LOCAL ISOLATES OF BACILLUS THURINGIENSIS IN THE CONTROL OF TUTA ABSOLUTA ON TOMATO PLANTS' by Eric Odunayo AKINYELURE meets the regulations governing the award of the degree of Masters in Microbiology of the Ahmadu Bello University, and is approved for its' contribution to knowledge and literary presentation.

Prof. D.A. Machido	_____	_____
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DEDICATION

This dissertation is dedicated to my Parents; Pastor and Deaconess P. A. Akinyelure.

ABSTRACT

The negative impact of chemical pesticides on the environment and the increased resistance of tomato leafminer (*Tuta absoluta*) field populations to chemical pesticides have promoted research on alternative control measures. Biological control with *Bacillus thuringiensis* (Bt) may be an alternative, especially against larval instars of *T. absoluta*. This study involved isolation of indigenous isolates of *Bacillus thuringiensis* (Bt) from the soil, characterization of the isolates using the transcriptional regulator (*XRE*) gene and the crystal proteins *cry1* and *cry2* genes and determination of efficacy of the spore crystal mixtures derived from isolates against larvae of *Tuta absoluta* under screened house conditions. A total of five (5) isolates of *Bacillus thuringiensis* were isolated from soil sampled from different locations in Zaria, Nigeria; and screened for the presence of the *XRE, cry1* and the *cry2* genes using polymerase chain reaction (PCR). Of the five (5) isolates, four (4) showed the presence of the *XRE* genes, two (2) showed the presence of the *cry1* genes while none of the isolates harboured the *cry2* genes. Ten (10) larvae of *Tuta absoluta* were exposed to spore crystal mixture derived from the two isolates found to harbor the *cry1* gene in triplicates at 28^{o}C for the period of three days. Results of the bioassay indicated that, application of each of the spore crystal mixtures derived from isolates harbouring *cry1* genes caused significant mortality of larvae of *T. absoluta* after 72 hours in comparison to the control (sterile distilled water). The mortality of *T. absoluta* larvae when exposed to the spore crystal mixture from isolate C6 for 72 hours ranged from 30 to 73.3% while exposure of the *T. absoluta* larvae to spore crystal mixture derived from isolate R3 under the same conditions for 72 hours was found to be between 33.3 and 96.7%. The observation made in this study strongly suggests that isolates C6 and R3 indigenous to this environment have great potentials for application in the protection of tomato plants against *T. absoluta*.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Tomato (*Lycopersicon lycopersicum* L.) is a fruit that belongs to the family, Solanaceae, even though it is considered as a vegetable in most parts of the planet. Tomato is the most popular and consumed vegetable in the world, accounting for 16% of all vegetable sales (Hepsağ and Kizildeniz, 2021). It is rich in nutrients and its secondary metabolites like, ascorbic acid, folic acid, flavonoids, vitamin E, potassium and lycopene are important for human health (Tehniat *et al*., 2014). The top five major tomato producers are China, the European Union, India, the United States, and Turkey. Global tomato output is approximately around 130 million tons, with 88 million sold fresh and 42 million processed (Bello *et al*., 2016).

Nigeria, with an annual production of 1.8 million tons, is sub-Saharan Africa's leading tomato producer and makes an immense contribution to the generation of numerous other valuable agrarian products as well (Borisade *et al*., 2017). There are various agro-ecological zones in the country, with each zones benefitting from natural environmental features such as favorable climate and the adaptability of soil to specific food crops. Tomatoes are mostly grown in the country's northern states (Etebu *et al*., 2013; Bello *et al*., 2016). Tomatoes are used as sauces in stew, which is a common part of Nigerian meals and accounts for around 18% of the average daily vegetable consumption in the country (Ebimieowei and Ebideseghabofa, 2013).

The invasion of pest among other factors has contributed immensely to the low tomato yield in the country with yearly postharvest losses in the country estimated to be between 45 and 60% (Garuba *et al*., 2018). *Tuta absoluta*, the tomato leafminer, is a major lepidopteran insect pest.

Without effective control actions, it can cause 80-100% loss of both outdoor and greenhouse tomatoes (Urbaneja *et al.*, 2013). The insect typically deposits eggs on undersides of stems, leaves and very rarely on fruits. Once the larvae have hatched, they will infest the leaves and fruits of the tomatoes. They forage and grow on the leaves, creating mines and galleries in the process. The larvae only eat the mesophyll on the leaves, leaving the epidermis alone (Brevault *et al.*, 2014). It has expanded fast across Europe, northern Africa, and the Middle East since its introduction into Europe in 2006, where it quickly reached destructive levels and became a major dangerous pest of tomatoes grown in both outdoor and greenhouse settings (Urbaneja *et al.*, 2013; Brevault *et al.*, 2014). This pest has now been reported in many Middle Eastern, African and Asian countries (Brevault *et al.*, 2014; EPPO, 2015). Before attacking tomatoes in Nigeria, *T. absoluta* was originally recorded in two West African countries: Niger and Senegal (EPPO, 2014; CABI, 2016). It was attributed for tomato infestation in the Nigerian northern states of Jigawa, Kano, Katsina, Kaduna, Plateau, Gombe, and Nasarawa. It has also been spotted in two other states in the South West, Lagos and Oyo, where it has become a big danger to Nigeria's tomato production. (CABI, 2016; EPPO, 2016).

Biological control is the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be (Eilenberg *et al*., 2001). All creatures are susceptible to natural enemies such as predators, parasites and pathogens including bacteria, viruses and fungi, which regulate populations; this is referred to as "natural control". A wide variety of biological control agents exists for the regulation of invertebrate pests; these are classified as pathogens, parasites or predators functionally and as nematodes, mites or microorganisms including bacteria, viruses and fungi,

taxonomically. Hence, managing pests through use of their natural enemies is referred to as biological control (Hajek, 2004).

Bacillus thuringiensis (Bt) is an entomopathogenic soil bacterium capable of generating selective toxic proteins in the form of crystals inside the cell to combat pests (Schunemann *et al.,* 2014). The active ingredient in Bt products is this protein inclusion. It is made up of one or more α-endotoxins in protoxin form. When a susceptible host ingest this inclusion, the protoxin is solubilized, and the protoxin is converted to the active δ-endotoxin form (Crickmore *et al.,* 1998; Palma *et al.,* 2014). The active toxin binds to specific receptor sites on the gut epithelium, causing the gut lining to gradually degrade. Bt products have been shown to slow development, lower survival, and reduces rates of larval feeding in addition to its toxic effects on the muscular and nervous systems of the target larvae culminating in death usually, within 1–3 days (Ware, 2004).

B. thuringiensis produces Crystal (Cry) and Cytolitic (Cyt) protein crystals that are effective against insect pests. (Ibrahim *et al*., 2010). Identification of genes coding for insecticidal toxins is a useful approach for assessing the efficacy of crystals against pests (Nazarian *et al.,* 2009). These endotoxins are specific to their targets; for instance, bipyramidal and cuboidal inclusions encoded by *cry1*, *cry2*, *cry7*, *cry8*, *cry9*, *cry15*, *cry22* and *cry51* genes are effective towards *Lepidoptera* and *Coleoptera* insect pests (Frankenhuyzen, 2009; Jain *et al*., 2017). The spherical, composite, flat and other crystals inclusions are toxic to *Diptera*, *Hemiptera, Hymenoptera*, *Hemiptera, Siphonoptera* insects (Frankenhuyzen, 2009). Furthermore, detection of *cry* genes by PCR method enables discovering genes of novel crystalline toxins (Narzalan *et al.*, 2009).

The use of commercial *Bacillus thuringiensis* (Bt) formulations has had the most success in microbial insecticides. These have been the most efficient biological pest management agents in the world, accounting for 95% of all microbial pesticides sold, with annual sales estimated to be in the $100 million range (Federici *et al*., 2006; Schunemann *et al.,* 2014). *Bacillus thuringiensis* has been utilized as an effective biological pesticide for almost 40 years, and it is a highly precise, safe, and effective tool for controlling a wide range of insect pests (Nester *et al.,* 2002). This study, therefore, is aimed at isolating, characterizing and assessing the poterntials of indigenous isolates of Bt as a biological control tool against *T. absoluta* which is known to be an important insect pest of tomato plants on farmers field.

1.2 Statement of Research Problem

Tomato is grown for the purpose of food as an important nutritious vegetable crop and source of income for many families (FAO, 2014). *T. absoluta*, on the other hand, is a major pest of the tomato crop (Derbalah *et al.,* 2012) in which diseases caused include blights, mildews, cankers and wilts which in turns reduce productivity up to 100% (Noling, 2013). In 2009, the world produced over 152 million tons of tomato, with a total production area of approximately 4.4 million hectares. *T. absoluta* invaded around 1.0 million hectares in 2011, resulting in a considerable reduction in tomato output (Muniappan, 2013).

Since its introduction in Nigeria during the 2016 tomato season, *Tuta absoluta,* often known as "tomato ebola," ravaged the country's northern regions, where tomato production is concentrated (Borisade *et al*., 2017). During the first phase, the pest caused a loss of over 80% of tomato output, which resulted in a lack of raw materials supply to Africa's largest tomato processing company (Dangote Farms Tomato Processing Factory in Kano, Nigeria), and price increases of up to 400% in three months (Bello *et al*., 2016). The larva, which feasts voraciously on tomato

plants from seedling to adult, forming enormous holes in leaves, tunneling in stalks, and devouring apical buds along with green and ripe fruits, is the most devastating stage of the pest (CABI, 2016).

Chemical insecticides have traditionally been used to control *T. absoluta* (Reyes *et al.*, 2012; Konus, 2014). Synthetic pesticides are the most convenient option for farmers to manage these pests and diseases (Birech *et al*., 2006). Synthetic pesticides solve the problem in part, but they also create new ones because they are non-biodegradable, harm the environment, and leave residues in the produce (Bhattacharjee and Dey, 2014). They have an impact on public health, and their persistent use leads to a build-up of resistance among pests and pathogens (Stangarlin *et al*., 2011; Engindeniz and Ozturk, 2013; Terzidis *et al.*, 2014; Wagnitz, 2014; Gontijo *et al.*, 2015; Roditakis *et al.*, 2015; Silva *et al.*, 2015).

T. absoluta is a difficult pest to control, hence efforts to encourage the use of microbial pesticides as alternatives are ongoing, with *Bacillus thuringiensis* (Bt) being one such alternative. Most of the commercially available biopesticides are coming from developed economies which are found to be too costly (beyond the reach of the farmer), not readily available, have poor efficacy and yield inconsistent field performance associated with biopesticides utilization in Nigeria (Caradus *et al*., 2012; Ivase *et al*., 2017). This is mostly due to isolates in the imported formulation are not well adapting to our environment due to differing environmental conditions, our soil contains indigenous microbial community which competes with these imported inoculants and also, the variety of the moths tested over there are not of the same variety here.

Furthermore, the consistent usage of imported organisms may endanger our environment's phytosanitary health (Chethana *et al*., 2012).

1.3 Justification for the Study

In view of the many problems associated with the existing insect control management approaches earlier highlighted, there is need to consider the use of biopesticides as one of the most promising alternative for a more rational and safe crops management practices that has the ability to satisfy consumer needs while also protecting human health and the environment.

There is therefore, the need to conduct investigations with the view to identifying and certifying the capacity of possible biological agents that could provide efficient, cost effective, safe and sustainable means of controlling insect pests of agricultural crops in general.

Bacillus thuringiensis has been shown to be an effective tool in the management of agricultural crop insect pests, with efficacy comparable to synthetic crop protection agents. Products derived from this bacterium have been found to be long-lasting and effective against pests with multiple modes of action, high target specificity, complete absence of harmful residues, cost effective and sustainable.

However, the commercially available Bt products are derived from bacteria isolated from differing environmental backgrounds which could limit the level of efficacy of such products when applied under the local environmental conditions. In addition, such products is often costly to procure and of limited availability to the local tomato farmer. Therefore, the need to search for indigenous isolates with the desired capacity to serve as bioinsecticide cannot be overemphasized.

1.4 Aim of the Study

The aim of this study was to isolate and assess the potential of indigenous isolates of *Bacillus thuringiensis* as means of controlling *Tuta absoluta*, an insect pest of tomato plant.

1.5 Objectives of the Study

The objectives of this study were to:

1. isolate and characterize indigenous isolates of *Bacillus thuringiensis* (Bt) in soils from Zaria, Nigeria.
2. detect the presence of *XRE* genes and lepidoptera-active c*ry1 and cry2* genes in the isolates using PCR.
3. evaluate the larvicidal activity of the generated spore crystal mixture against larvae of *T. absoluta* under screened house conditions.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Genus *Bacillus*

Bacillus is a vast and widespread genus that has garnered recognition among taxonomists for its great phenotypic variety and heterogeneity (Koneman *et al*., 1997). *Bacillus* species are Gram-positive, endospore-forming, chemoheterotrophic rod-shaped bacteria that are usually motile, aerobic or facultatively anaerobic, and catalase-positive. (Waites *et al*., 2008). *Bacillus* genus members are found in a variety of environments, but usually in soil, and have a wide range of physiological properties that allow them to develop in any environment and compete well with other organisms in that environment. The generation of highly resistant endospores and metabolites that have antagonistic effects on other microbes is credited with their long-term survival in many unfavourable environments (Nicholson *et al.*, 2000; Kuta, 2008).

Bacillus species were originally classified into three groups based on the spore and sporangium morphology (Drobniewski, 1993; Emmerson *et al*., 1997). The following are the groups:

• Group 1 – Gram-positive bacteria produce spores that are central or terminal, ellipsoidal or cylindrical, and do not distend the sporangium. It is divided into two subgroups:

- *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus megaterium* are members of the large cell subgroup.
- *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus licheniformis* are members of the small cell subgroup.

• Group 2 – *Bacillus circulans* and *Bacillus coagulans*, both Gram variable (a mix of pink and purple cells) with central or ellipsoidal spores and inflated sporangia. *Bacillus alvei*, *Bacillus*

brevis and *Bacillus macerans* were previously classed as members of this group, but have since been moved to other genera (Todar, 2012).

• Group 3 – *Bacillus sphaericus*, sporangia swollen with terminal or subterminal spores and are Gram variable

In two groups of the genus *Bacillus*, there has been a taxonomic shift in recent years (Fritz, 2004). They are known as the *B. subtilis* and *B. cereus* groups.

2.2 *Bacillus cereus* group

Gram-positive bacteria with a low guanine-cytosine content (GC%) that belong to the phylum Firmicutes make up the *Bacillus cereus* group. At least eight closely related species were found in the *Bacillus cereus* group: *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis, Bacillus cytotoxicus, and Bacillus toyonensis* (Liu *et al*., 2015). Because of their clinical importance, *B. cereus* and *B. anthracis* have gotten a lot of attention, whereas *B. thuringiensis* has been employed to manage insect pests since the 1920s. With the exception of *B. cytotoxicus*, which has a chromosome of 4.085 Mb and is the most diverse of the group (Lapidus *et al*., 2008), the genomes of the *B. cereus* group members are extremely conserved, with genome sizes ranging from 5.2 to 5.5 Mb and 16S rRNA gene sequences that are relatively comparable.

B. thuringiensis has numerous large transmissible plasmids that encode a wide range of insecticidal toxins, including Cry and Cyt, which form the species' parasporal crystal (Rasko *et al*., 2005). *B. anthracis* has two plasmids, pXO1 and pXO2, which contain the structural genes for toxin proteins and the biosynthetic genes for capsule development (Rasko *et al*., 2007). The plasmid pCER270, which encodes enzymatic components essential for the nonribosomal production of the toxin cereulide, is found in emetic *B. cereus* isolates (Rasko *et al*., 2007).

Speciation based on plasmid content as well as morphologic or physiological aspects can be perplexing and lead to subjective judgments (Ehling-Schulz *et al*., 2019). The *B. cereus* group can be classified into five major clades, according to a recent genomic analysis of 224 isolates (Meric *et al*., 2018), which do not correspond to the eight species listed above (Ehling-Schulz *et al*., 2019). This discrepancy between genomic-based phylogenetic analysis and specific phenotypes supports the theory that plasmids encoding toxin genes, such as *B. anthracis*' pXO plasmids and *B. thuringiensis*' Cry plasmids, cannot be used as species identification (Liu *et al.*, 2015).

It is more beneficial to regard the *B. cereus* group as a new species comprising of extremely different isolates whose traits differ due to plasmid presence or gene expression connected with essential regulatory genes, as proposed by Helgason and colleagues (2000) and by Rasko and colleagues (2005). Due to the great genetic relatedness of the three *Bacillus* species, it was suggested that they be placed together as members of a single species, *Bacillus cereus sensu lato* (Helgason *et al.*, 2000; Rasko *et al.*, 2005; Bartoszewicz and Marjanska, 2017).

2.3 History of *Bacillus thuringiensis*

Ishiwata Shigetane isolated *Bacillus thuringiensis* for the first time in Japan in 1901, and it was identified as the bacteria responsible for the silkworm disease sotto *Bombyx mori*. Because of this, the bacteria was given the name *Bacillus sotto*, which refers to the soft and floppy appearance of infected larvae. Young bacterial cultures were not hazardous to larval insects, however old cultures that had sporulation were extremely toxic (Fernández-Chapa *et al*., 2019). However, it was not until 1911 that Ernst Berliner, a German scientist, isolated it from sick

larvae of the flower moth *Anagasta kuehniella*. *Bacillus thuringiensis* is named after Thuringia, the German town where moths were discovered. (Melo *et al*., 2016).

2.4 Biology of *Bacillus thuringiensis*

Bacillus thuringiensis is a Gram-positive, rod-shaped soil bacteria that has been isolated from a wide range of environments around the world, including soil, water, dead insects, grain dust, deciduous tree leaves, various conifers, and insectivorous mammals (Dharmendar *et al*., 2008; Roh *et al*., 2017). It is distinguished by its ability to generate crystalline inclusions (Cry toxins) during sporulation, which contain insecticidal proteins known as δ-endotoxin. *Bacillus thuringiensis* crystalline inclusions have been shown to be toxic to a wide range of insect pests, including Lepidoptera, Coleoptera, and Diptera, as well as hemipterans, as well as other biological activities like molluscicidal, nematicide (human and animal parasites, and free living; Rhabditida), acaricide, and even human cancer cells (Chougule and Bonning, 2012; Van Frankenhuyzen, 2013; Abd El-Ghany, 2017).

Bacillus thuringiensis crystal and produced soluble toxins are extremely selective for their hosts and have become a popular alternative to chemical pesticides around the world. Bacillus thuringiensis toxins currently comprise more than 98 (424 million USD) of developed sprayable bacterial pesticides (Lacey *et al*., 2015), and it is the most widely used and commercialized environmentally friendly insecticide worldwide (Glare *et al*., 2012; Jurat-Fuentes & Crickmore, 2017).

It has a basic life cycle. The spore germinates, forming a vegetative cell that develops and reproduces through binary fission when nutrition and environmental conditions are favorable for growth. Cells keep multiplying until one or more resources, such as carbohydrates, amino acids, or oxygen, are no longer available for vegetative development. The bacteria sporulates in these

conditions, producing a spore and parasporal body, the latter of which is primarily constituted of one or more insecticidal proteins in the form of crystalline inclusions (Figure 2.1 (e) and (f)). These are also known as insecticidal crystal proteins, and they can make up as much as 40% of the dry weight of sporulated culture (Federici *et al*., 2010).

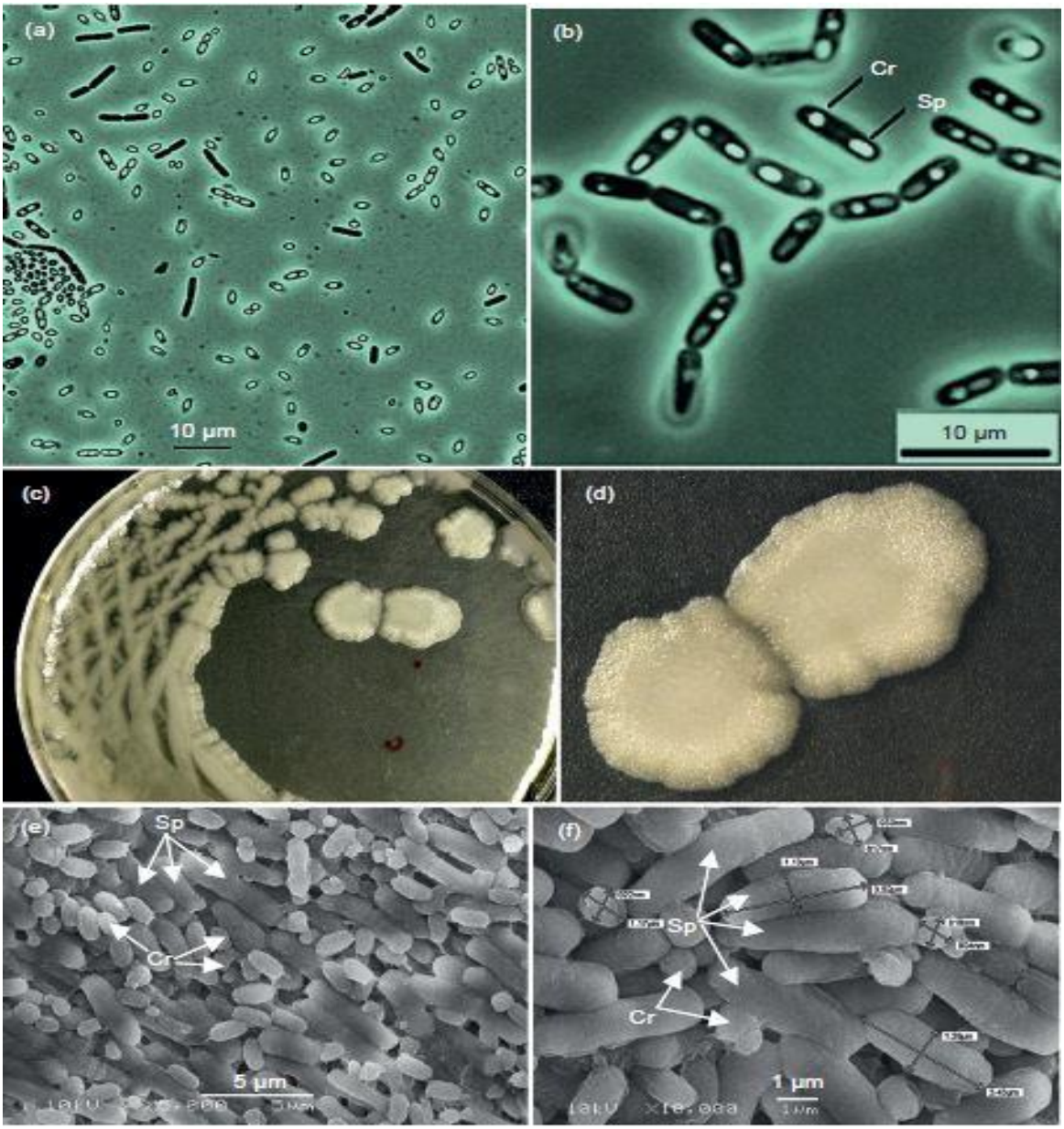


Figure 2.1: A photo plate showing morphological features of a strain of *B. thuringiensis* (Bt).

(a) and (b): Phase Contrast micrographs (×1000) of the Bt isolate showing spores (Sp) and parasporal crystals (Cr), whichappear brighter.
(c): Growing colonies of Bt isolate seeded in nutrient-supplemented agar media. (d): A magnified colony showing its characteristic slightly raised center with fried egg appearance; white, large, nearly circular with fine irregular and glossy margins.
(e) and (f): Scanning Electron Micrographs of Bt isolate showing clearly large numbers of cystals (Cr) and spores (Sp).
Source: Ahmed *et al*., 2021.

2.5 Ecological Distribution of *B. thuringiensis*

Bacillus thuringiensis appears to be native to a wide range of habitats (Roh *et al*., 2017). *B. thuringiensis* isolates have been isolated from a variety of environments, including:

(i) Soil: *B. thuringiensis* spores persist in soil and produce vegetative cells when favourable conditions prevailed (Martin and Travers, 1989; Fayad *et al*., 2019; Heldari and Zeinali, 2019; Riskuwa-Shehu *et al*., 2019).

(ii) Aquatic environments: Many *B. thuringiensis* subspecies were isolated from aquatic habitats (Meadows, 1993; Pushkar *et al*., 2018; Santos *et al*., 2019).

(iii) Plant surfaces: In this habitat *B. thuringiensis* isolates have been isolated extensively from phylloplanes of trees, vegetables, field crops and stored product dust (Meadows *et al*., 1992; Islam *et al*., 2019). Also they were isolated from deciduous and coniferous leaves (Kaelin *et al*., 1994; Thomas, 2019).

(iv) Insects: Many researchers isolated *B. thuringiensis* subsp from dead insect larvae and pupae (Carozzi *et al*., 1991; Santos *et al*., 2019).

(v) Air: There is a certain strain of *B. thuringiensis* that has been isolated from aerial application named *B. thuringiensis var kurstaki* and its presence was demonstrated in air for up to 17 days (Swadener, 1994).

2.6 Characteristics of *B. thuringiensis*

Biochemical typing, flagellar serotyping, profiling plasmid arrays or proteins, use of monoclonal antibodies, and hybridization or PCR amplification based on sequences of known *cry* genes are all methods used to characterize *B. thuringiensis* isolates in any given collection (Keshavarzi, 2008). Other methods for characterizing isolates of *B. thuringiensis* include *in situ* hybridization,

microarray analysis, (Ha *et al*., 2019), nano liquid chromatography followed by tandem mass spectrometry (nano LC/MS/MS), and insilico procedures (Baragamaarachchi *et al*., 2019).

2.6.1 Morphological or preliminary screening of *B. thuringiensis*

The morphology of *B. thuringiensis* colonies on culture media can help identify them from those of other *Bacillus* species. It develops white, coarse colonies that spread very swiftly and can cover the entire plate. The spores of *B. thuringiensis* isolates are unswollen and ellipsoidal, with a subterminal location (Cowan and Steel, 2003; Roh *et al*., 2017).

The greatest criterion for distinguishing *B. thuringiensis* from other *Bacillus* species is the presence of parasporal crystals proximal to the spore in the mother cell. Parasporal inclusion shape, size, and number may differ amongst *B. thuringiensis* isolates.

However, four distinct crystal morphologies are visible: the typical bipyramidal crystal, which is related to cry 1 proteins; cuboidal inclusions, which are related to cry 2 proteins and are usually associated with bipyramidal crystals (Ohba and Aizawi, 1986); amorphous and composite crystals, which are related to cry 4 and cyt proteins (Federici *et al*., 1990); and flat, square crystals, which are related to cry 3 (Lopez-Meza and Ibarra, 1996).

The initial step in building *Bacillus thuringiensis* strain collections is to observe crystal shape, because there is a link between toxic activity and crystal structure, phase contrast microscopy can reveal essential information about crystal morphology. Using electron microscopy, Wasano *et al*. (2000) studied parasporal inclusions of lepidopteron-specific and coleopteran-specific B. thuringiensis. Parasporal inclusions had round to oval bodies, according to the researchers. Lepidopteran larvae are usually poisoned by bipyramidal shaped crystals, cuboidal crystals are poisonous to both lepidopteran and dipteran insects, and flat or irregular shaped crystals are poisonous to coleopteran insects.

2.6.2 Biochemical characteristics for the identification of *B. thuringiensis*

To screen the diverse isolates of *B. thuringiensis*, Eswarapriya *et al*. (2010), Ahmed *et al*. (2015) and Riskuwa-Shehu *et al*. (2019) all performed several biochemical experiments. They performed biochemical tests such as the Indole test, the Methyl red voges proskauer (MRVP) test, the Starch hydrolysis test, the Citrate utilization test, the Catalase test, the Casein hydrolysis test, and the Gelatin test. Except for the methyl red test, all of the *B. thuringiensis* isolates were positive.

2.6.3 Characterization by bioassay

Bioassays were used by Xia *et al*. (2005) to investigate the toxicities of lyophilized crystal spore mixtures derived from *B. thuringiensis* recombinants against *Plutella xylostella*. The *P. xylostella* colony was a stable susceptible colony that had been kept in the lab for over 100 generations without insecticide exposure. The bioassay was carried out using surface contamination of cabbage leaf disks, with one leaf disk per plate. The plates were incubated at 26^{o}C, with a humidity of 85% and a photoperiod of 12:12 (L: D) hours. For each concentration, four replicates of 10 third-instar larvae were employed. After four days, the deaths were documented.

Legwaila *et al*. (2015) used five different concentrations of *B. thuringiensis* (var. kurstaki) to test the toxicity of *B. thuringiensis* (var. kurstaki) against Diamondback Moth (*Plutella xylostella* L.) eggs and larvae on cabbage in a semi-controlled greenhouse setting. Bioassays were performed at 30°C ± 5°C against DBM eggs and second instar larvae at 2, 4, 6, 8, and 10 g/L. Three times each therapy was carried out. When exposed for long periods of time, Btk was effective against both eggs and larvae. Numerous bioassays of *B. thuringiensis* against mosquito larvae were undertaken by Bello *et al*. (2016), Adeyemo *et al*. (2018), and Rajendran *et al*. (2018), and several isolates of *B. thuringiensis* were found to be toxic.

2.6.4 Molecular methods for characterization of *B. thuringiensis* isolates

This is usually achieved using several methods that include:

2.6.4.1 Protein analysis

There are more than 170 toxin-encoding genes known. *Cry* genes of 25 kDa to 140 kDa code for crystalline or delta endotoxin, while *cyt* genes encoded the non-crystalline endotoxin (Rudd *et al*., 1999).

The protein composition and morphology of parasporal crystals from 121 isolates were studied by Dong *et al*. (2007). Using Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) examination of crystal proteins in *B. thuringiensis* isolates, they described the diversity of the isolates.

Heidari and Zeinali (2019) investigated for parasporal inclusion crystals and SDS-PAGE patterns in 200 soil samples and identified 10 bacterial isolates. SDS-PAGE analysis of crystal-spore mixtures revealed a diverse variety of protein with molecular weight between 11-230 kDa.

2.6.4.2 Molecular markers

Biological diversity refers to the variation among all living organisms found in various ecosystems and ecological groupings on the planet. This diversity is essential for the continued evolution of life forms and, as a result, the biosphere's life-sustaining mechanisms. Biochemical tests, restriction analysis of chromosomal DNA (REAC), restriction fragment length length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PAGE), amplified fragment length polymorphism (AFLP), rep (repetitive extragenic palindrome)-PCR and ERIC (Enterobacterial repetitive intergenic consensus), box-PCR based finger printing, multi locus enzymes electrophoresis (MLEE), and single locus and multi locus sequence typing (MLST) and variable number of tandem repeats (VNTR) are some

of the methods that can be used to study genetic diversity in microorganisms, (Suman *et al*., 2001; Muthukumaraswamy *et al*., 2002).

2.6.4.2.1 Plasmid profile analysis

The *cry* and *cyt* genes encode the majority of delta endotoxins seen in mega plasmids larger than 30MDa. Insecticidal action is related to the type of *cry* genes present in a strain. The presence of plasmids containing genes coding for insecticidal toxins distinguishes *B. cereus* from *B. thuringiensis* (Chao *et al*., 2007). The horizontal spread of plasmids, which affects taxonomy and pathogenicity and has been established for *Bacillus cereus* and *Bacillus thuringiensis* (Williams *et al*., 1998), makes identifying *Bacillus thuringiensis* from *Bacillus cereus* challenging.

2.6.4.2.2 Cry gene profiling

The presence of insecticidal proteins or genes can be detected using a bioassay, but it takes time because all target genes toxic to a wide range of insect orders must be screened. When it was discovered that crystal toxins were specific to different insect orders, alternative approaches were developed. The number of bioassays was reduced as a result of this. Recent technologies, like as the heat cycle-assisted polymerase chain reaction (PCR), provide a precise and rapid tool for identifying novel proteins and predicting insecticidal effectiveness of existing proteins (Yashaswini, 2011).

More than one insecticidal *cry* genes of the principal rank can be found in a single *B. thuringiensis* strain. The cry 1 primary rank is the most well-known, as it contains the most *cry* genes. The current availability of nucleotide sequence information for many *cry* genes has paved the way for microarray-based detection of any isolate's *cry* gene profile. Based on current classification of *cry* genes based on amino acids sequence similarity, over 78 known *cry* genes families with 823 distinct *cry* genes and 3 cyt families with 40 distinct cyt genes are known

(Crickmore, 2020). The discovery of *B. thuringiensis cry* genes by PCR is an excellent method for predicting insecticidal action (Carozzi *et al*., 1999). Exclusive PCR (E-PCR), a two-step procedure, is a suitable method to address the fundamental limitation of multiplex PCR, which is that it cannot detect the presence of a novel cry gene from a *B. thuringiensis* strain whose nucleotide sequence is unknown. The fundamental constraint of multiplex PCR, which could only detect already known sequences, would be efficiently solved by E-PCR (Yashaswini, 2011). Thirteen highly similar primers specific to sites within genes encoding seven different subgroups of *B. thuringiensis* cry 1 proteins were described using the PCR technique. (Ceron *et al*., 1994). Based on the five conserved blocks of amino acids of *B. thuringiensis* toxins and their encoding DNA sequences, an alternative PCR approach to screen *cry7* genes was made possible (Ben Dov et al., 2001). *Cry47*Aa, a novel gene producing a protein with 1134 amino acid residues and a molecular mass of 132.2 KDa, was discovered using PCR technology based on amino acid sequence comparison with known cry delta-endotoxins (Kongsuwan *et al*., 2005).

2.6.4.2.3 XRE gene profiling

Characterization of *B. thuringiensis* is complex and cumbersome using the various *Cry* genes as biomarkers since several translated Cry protein product varies with the different categories of *Cry* gene. *B. cereus* and *B. thuringiensis* have a high similarity (>99 %) index in 16S rRNA gene sequences based on universal primers (Böhm *et al*., 2015), which could not be differentiated using genetic and behavioral testing (Peng *et al*., 2015). To overcome this challenge, the *XRE* gene, a novel biomarker, was developed to identify *B. thuringiensis*, the bacteria that controls the creation of the most common type of crystal protein (Wei *et al*., 2019).

The transcriptional regulators were expected to be potential coding sequences (CDS). In the XRE-like protein family and the MerR family of transcriptional regulators, CDS has a helix-turn-

helix (HTH) motif. HTH proteins, along with sigma factors, function as repressors in a variety of signaling pathways, including protease secretion (Pflughoeft *et al*., 2011), biofilm formation (Colledge *et al*., 2011), and sporulation (Murawska *et al*., 2014). In comparison to *cry2*, the *XRE* gene was found to be more accurate in identifying *B. thuringiensis*. Furthermore, the real-time PCR successfully recognized *B. thuringiensis* and could be used to quantify cell numbers targeting the *XRE* gene (Wei *et al*., 2019).

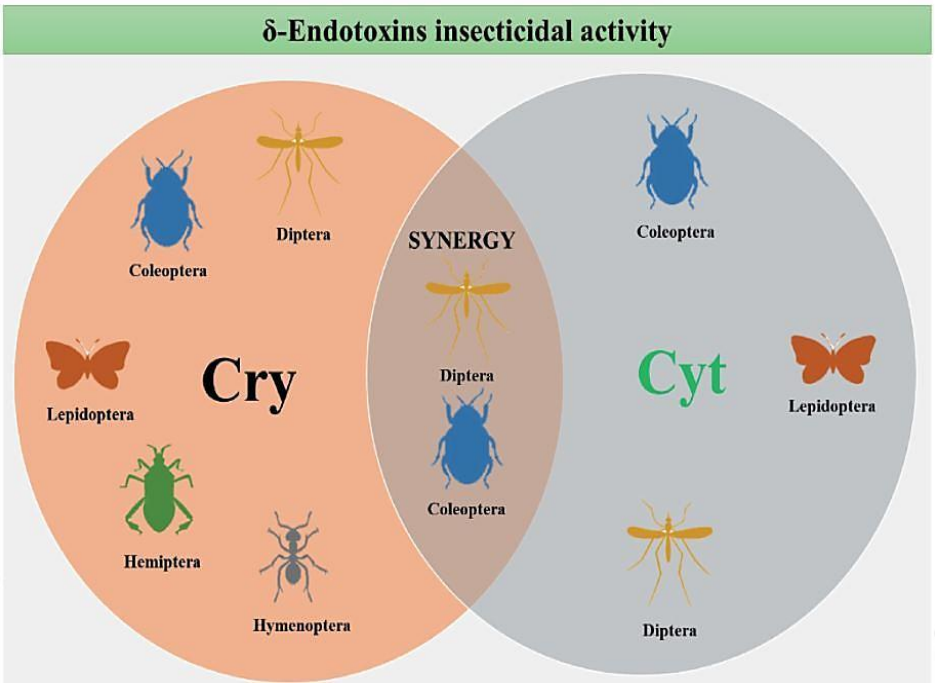
2.7 Bioinsecticidal activity of *B. thuringiensis*

The production of one or more crystalline structures of a protein nature adjacent to the spore during the sporulation process distinguishes *Bacillus thuringiensis* from other closely related bacilli. Certain of these parasporal crystals, known as delta endotoxins (Cry and Cyt), have pathogenic potential against larvae of various insect orders, primarily Lepidoptera, Diptera, Coleoptera, and in some cases other phyla (Palma *et al*., 2014). The bacterium can maintain its survival by generating parasporal crystalline inclusion during sporulation (De Maagd, 2001).

Crystal (Cry) and cytolytic (Cyt) toxins are produced by *Bacillus thuringiensis* isolates at the commencement of sporulation and during the stationary growth phase as parasporal crystalline inclusions. During the vegetative development phase, *Bacillus thuringiensis* isolates can also generate additional insecticidal proteins, which are then secreted into the culture medium as vegetative insecticidal proteins (Vip) (Donovan *et al*., 2006) and secreted insecticidal proteins (Sip) (Warren *et al*., 1998).

Cry proteins are divided into 75 families, each having 800 genes (Sajid *et al*., 2018), whereas Cyt proteins are divided into three families, each with 38 genes (Jouzani *et al*., 2017). Cry proteins are poisonous to Lepidoptera, Coleoptera, Hymenoptera, Hemiptera, Diptera, Orthoptera, and Mallophaga, as well as to nematodes, mites, and protozoa (Figure 2.2). (Ye *et al*., 2012).

Some toxins have a broader range of action, affecting two or more orders or phyla (Van Frankenhuyzen, 2013). Cry1B, for example, is one among those that has a lot of activity against Lepidoptera, Diptera, and Coleoptera larvae. As a result, the spectrum of activity of a strain is determined by the combination of toxins present (Roh *et al*., 2017).



Diptera	Cry1(A-C), Cry2(A-B), Cry4(A-C), Cry10(A), Cry11(A-B), Cry16(A), Cry17(A), Cry19(A-C), Cry20(A), Cry21(A-H), Cry24(A-C), Cry25(A), Cry27(A), Cry29(A-B), Cry30(A-G), Cry32(B-D), Cry39(A), Cry40(A-D), Cry44(A), Cry47(A), Cry48(A),Cry49(A), Cry52(A-B), Cyt1(A-D), Cyt2(A-D), Cyt3(A)
Coleoptera	Cry1(A-I), Cry2(A), Cry3(A-C), Cry7(A), Cry8(A-G), Cry9(D), Cry14(A), Cry18(A-C), Cry22(A-B), Cry23(A), Cry26(A), Cry28(A), Cry34(A-B), Cry35(A-B), Cry36(A), Cry37(A), Cry38(A), Cry43(A-C), Cry55(A), Cyt1(A), Cyt2(C)
Lepidoptera	Cry1(A-K), Cry2(A-B), Cry7(B), Cry8(D) Cry9(A-C,E), Cry15(A), Cry19(A-C), Cry20(A-B), Cry22(A), Cry32(A), Cry51(A), Cry54(A-B), Cry59(A-B), Cyt2(B)
Hemiptera	Cry3(A), Cry5(A), Cry22(A)
Hymenoptera	Cry2(A), Cry3(A), Cry11(A)

Figure 2.2: Spectrum of insect orders susceptible to Cry and Cyt δ-endotoxins

Sources: De Maagd, 2001; Badran *et al*., 2016; Jouzani *et al*., 2017 as cited by (Fernández-Chapa *et al*., 2019).

2.8 Mechanism of action of *B. thuringiensis* against insect larvae

Despite the fact that the mechanism of action of cry toxins against many insects has been extensively studied, there are still many unanswered questions. As a result, there are currently several concepts in the literature that attempt to explain the mechanism of action of these toxins in a more acceptable manner (Zhang *et al*., 2017).

2.8.1 The sequential union model

The classical mechanism is another name for the sequential union model. It has been studied in detail in *Manduca sexta* with the Cry1Ab protein. The toxic characteristics are thought to derive from crystalline inclusions formed during *B. thuringiensis* sporulation. The inert protoxins and their subunits are not physiologically active, and their method of action can be plotted as follows: The delta endotoxins are consumed, the crystals are solubilized by the intestine's alkaline pH, the inactive protoxins are digested by midgut proteases, resulting in an active toxin of about 60–70 kDa that is resistant to proteases, and the Cry toxins come into contact with N-aminopeptidase receptors and cadherin on the surface of the membrane. When toxins bind to specific receptors, the Cry protein is proteolyzed, causing structural alterations in the chains and the formation of oligomers that act as "pre-pores." The N-aminopeptidase receptor anchors the pre-pore in the lipid bilayer, pore formation affects membrane integrity, and electrophysiological evidence and biochemistry suggest that the pores cause an osmotic imbalance that leads to cell death and lysis; the intestine is paralyzed, the insect stops feeding, and diarrhea, total paralysis, and death ensue (Figure 2.3) (Land and Miljand, 2014; Melo *et al*., 2016).

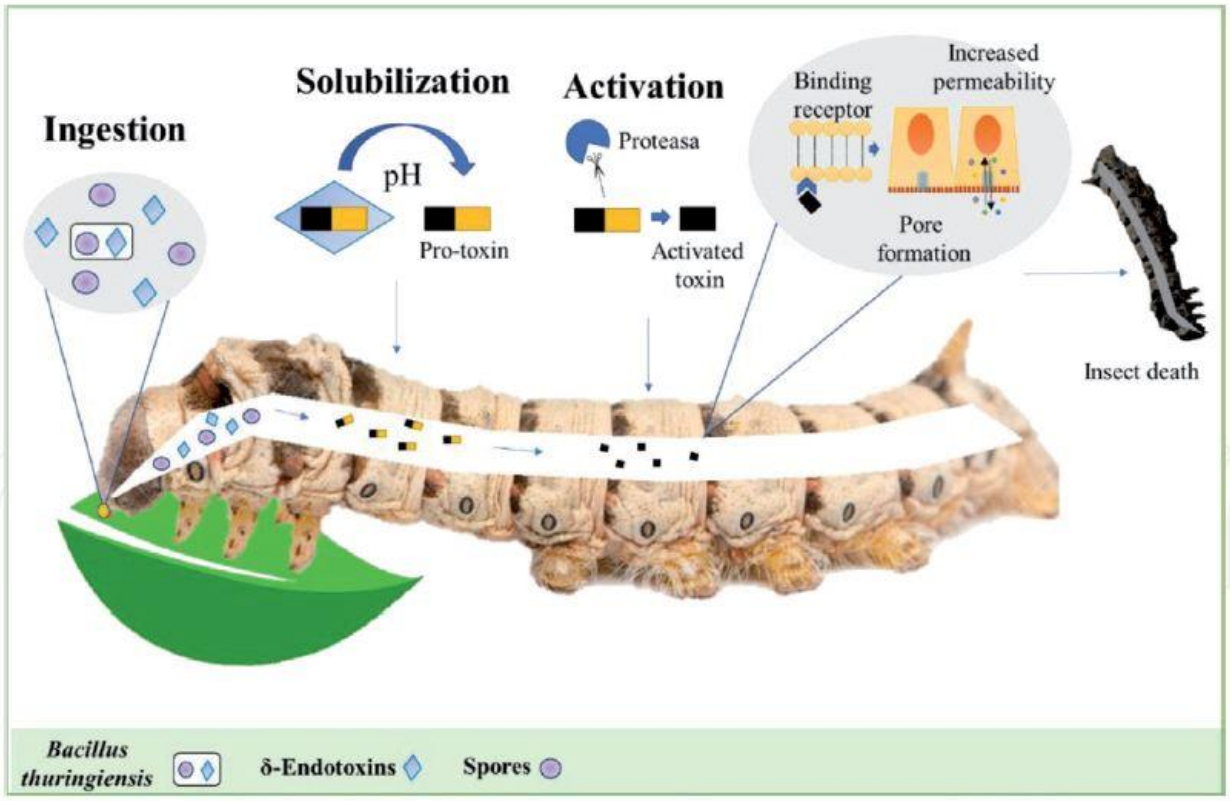


Figure 2.3: Mechanism of action of Cry proteins according to the sequential binding model

Source: Fernández-Chapa *et al*., 2019.

2.8.2 The signaling pathway model

This second hypothesized process is similar to the previous model, but it assigns different causes for cell death. Cry proteins, according to this theory, affect the cell in two ways: first, by forming pores in the membrane, as indicated in the sequential binding model, and second, by causing a series of events that modify cellular metabolism. According to this theory, Cry toxins bind to cadherin receptors, causing an increase in cAMP synthesis through heterotrimeric G protein and adenylyl cyclase. The protein kinase A is activated by cAMP, which induces apoptosis by activating Mg^{2+} channels in the plasma membrane. The activation of these channels promotes aberrant ion flow in the cytosol, which stimulates the apoptotic process (Figure 2.4) (Adang *et al*., 2014; Melo *et al*., 2016).

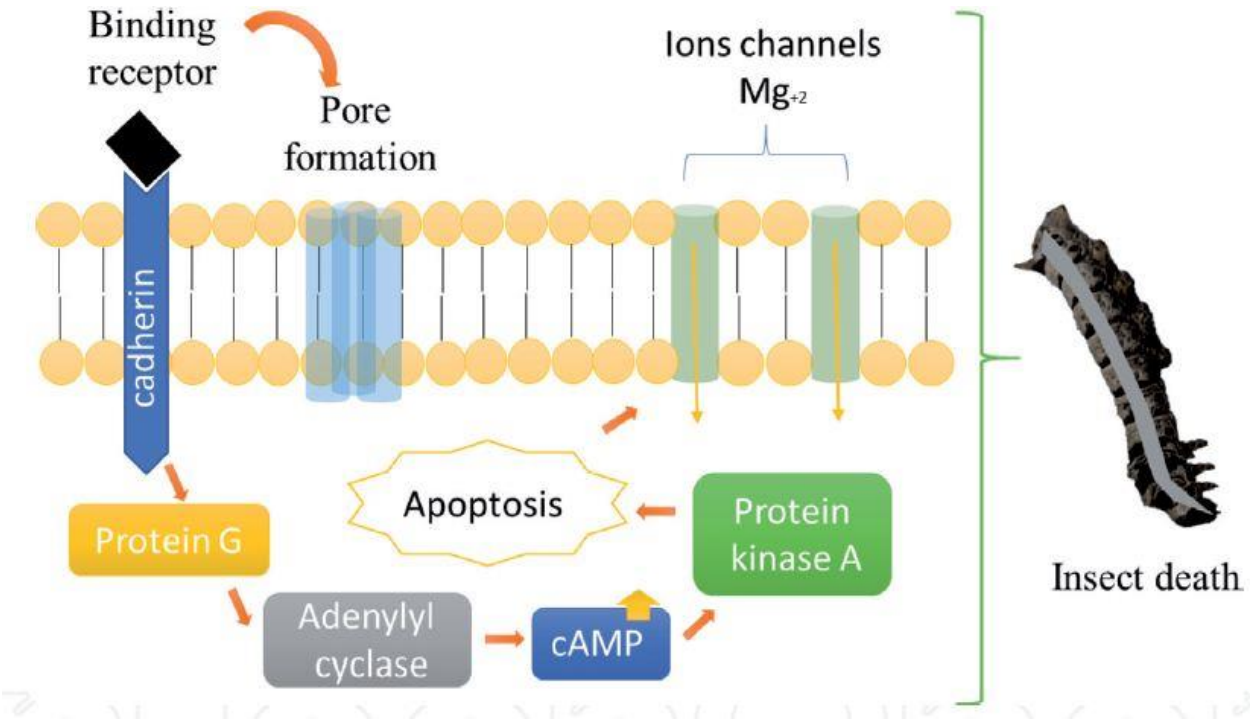


Figure 2.4: Mechanism of action of Cry proteins according to the signaling pathway model

Source: Fernández-Chapa *et al*., 2019.

2.9 Use and efficacy of *B. thuringiensis*

The effectiveness of *B. thuringiensis* is determined by its narrow toxicity spectrum. The majority of *B. thuringiensis* products used in agriculture are designed to kill lepidopteran insects (Abdullah, 2012). In agriculture (e.g., vegetable cultivation, tree fruit and nut crops), forestry, and mosquito control, *B. thuringiensis* has proven to be effective (Marrone, 1994). *B. thuringiensis* applications (which include aizawai and kurstaki isolates) increased dramatically from 2002 to 2009 for crops such as broccoli, cabbage, cauliflower, corn, leaf lettuce, tomatoes, and strawberries, according to California's pesticide use report (2009). Strawberry, tomato, and pomegranate were the three crops in California that received the most *B. thuringiensis* applications in 2009 (which included aizawai and kurstaki isolates). About 32,000 gross pounds of *B. thuringiensis* were applied to strawberry crops, followed by 30,000 gross pounds for tomatoes (fresh and for processing), and 27,000 gross pounds for pomegranate. *B. thuringiensis* efficacy has improved as a result of research and development activities such as improving isolates and formulations and developing synergists (Sanahuja *et al*., 2011).

Insecticide resistance control also relies heavily on *B. thuringiensis*. Pesticide and miticide resistance management (IRM) solutions aim to reduce the selection for resistance to a single insecticide or miticide. In reality, a sustainable and effective strategy to IRM can be achieved by alternating, sequencing, or rotating compounds from various groups with diverse modes of action (i.e. where resistance develops from altered target sites in the insect).

Pests that were once successfully controlled by pesticides are making a comeback. *Spodoptera litura* (also known as the oriental leafworm moth, cluster caterpillar, cotton leafworm, tobacco cutworm, tropical armyworm, taro caterpillar, tobacco budworm, rice cutworm, and cotton cutworm) (Kranthi *et al*., 2001; Ahmad *et al*., 2007), *Plutella xylostella* (diamondback moth) (Bommarco *et al*., 2011), *Helicoverpa armigera* (cotton bollworm) (Wu and Guo, 2005) and

Tuta absoluta (tomato leafminer) are examples of pesticide-resistant insects (Vacas *et al*., 2011). Broad-spectrum pesticides eliminate natural predators and other helpful insects as well. When pesticide control fails due to a lack of natural predators, resistant pest populations can swiftly return.

The increased public awareness about the negative environmental effects connected with the widespread use of chemical insecticides has fueled research into alternative insect pest control approaches. The use of entomopathogenic bacteria like *B. thuringiensis* is one of the viable possibilities. Due to differing mechanisms of toxicity, insect pests that are resistant to chemical pesticides are not cross-resistant to *B. thuringiensis*. As a result, *B. thuringiensis* products have been used to control insect resistance. It is ideal to integrate *B. thuringiensis* in IRM and integrated pest management (IPM) programs to extend *B. thuringiensis*' value. Finally, strong economic rewards and environmental safety will drive the use of safe pest control methods, with *B. thuringiensis* playing a key role.

2.10 Advantages of appliocation of *B. thuringiensis* products over chemical agents

With their specific insecticidal effect on insect pests belonging to the orders lepidoptera (butterflies and moths) (Baig *et al*., 2010; Darsi *et al*., 2010), diptera (flies and mosquitoes) (Pérez *et al*., 2007; Roh *et al*., 2010), hymenoptera (bees and wasps) (Garcia-Robles *et al*., 2001; Sharma *et al*., 2008) and coleoptera (beetles and weevils) (López-Pazos *et al*., 2010; Sharma *et al*., 2010), and to non-insect species such as nematodes (Cappello *et al*., 2006; Hu *et al.*, 2010), toxins produced by *B. thuringiensis* have replaced chemical insecticides as the primary biological control agent (George and Crickmore, 2012).

Various studies have been conducted to determine the safety of *B. thuringiensis* toxins emitted by sprays or transgenic plants to non-target species in the environment, and it has been found to

be mainly ecologically friendly without significant side effects (Kapur *et al*., 2010; Walter *et al*., 2010; Chen *et al*., 2011; Randhawa *et al*., 2011).

The increased popularity of biological control agents over synthetic insecticides is due to the latter's non-selective lethal effects (Moser and Obrycki 2009; Kristoff *et al*., 2010; Shah and Iqbal, 2010; Eriksson and Wiktelius, 2011; Stevens *et al*., 2011) and the rapid development of insect pest resistance to synthetic insecticides (Moser and Obrycki 2009; Kristoff *et al*., 2010; Shah and (Ahmad *et al*., 2008; George and Crickmore, 2012).

2.11 Overview of Tomato

2.11.1 Tomato Description

Tomatoes (*Solanum lycopersicum* L.) are members of the Solanaceae family and have been consumed by people in Central and South America since prehistoric times (Choudhoury, 1967). Tomato plants are of two types of growth habits, namely indeterminate and determinate. Plants with an indeterminate growth habit have main stem which extend regularly while issuing a cluster of flowers. On average, leaves are produced at an interval of every three leaves for indeterminate, while in the determinate it's one to two leaves after each inflorescence. The determinate types have main stems that stop growing and produce a terminal inflorescence after having issued two to six trusses (Raemaekers, 2005).

Although some cultivars have 30 or more flowers per clusters usually 4-12 flowers develop up to maturity. Self-pollination is commonly observed. Fruits of most cultivars are globoid while some are elongated. Ripe fruits colours are usually solid red, pink and orange or yellow (Rubatzky, 1996). There are three main types of tomato based on fruit size: the cherry type has small fruit weighing 10 to 20 g and 20 to 30 fruits; the standard type has medium-sized fruits weighing 80

to 120 g and five to six fruits per truss; and the beefsteak type has fruits weighing up to 250 g and one to three fruits each truss. (Mike and Hilmi, 2009).

2.11.2 Tomato Distribution

Tomato plants originated from the tropics of Central and South America, extending from Mexico, Ecuador, through Chile. Tomato was transported to Europe, and improved further before reaching the United State of America and Asia (Mahmud *et al.*, 2009). It is presently the world's most widely produced vegetable crop, giving grower's income, expanding export potential and improving the supply of vitamins and minerals in human nutrition (Rajkumar, 2007).

Tomato is grown throughout the tropical and the temperate region of the world. It is regarded as one of the most adaptable cultivated plants, requiring a wide range of environmental conditions for seed germination, seedling growth, flavour and fruit quality. The crop grows well in fertile, well drained soils with pH of about 6 and an average monthly temperature of 21°C to 23°C. It may be cultivated commercially at temperatures ranging from 18°C to 27°C (Ssekyewa, 2006).

2.11.3 Importance of tomato

Tomato is grown for its commercially important fruits. It is grown for fresh market in the field or greenhouse and for processing as whole pack, juice, or puree (Decoteau, 2000). Tomato contain significant amounts of vitamins A and C , although levels of both varies in fruit from shaded plants as in those in strong sunlight, the carotenoides are effected by temperature (light intensity), but vitamin A (β-Carotene) is relatively stable (Peirce,1987). Besides being eaten fresh, tomatoes can be boiled, stewed, fried, juiced, or pickled and used in soups, salads, and sauces. Tomatoes are an important in salsa along with onions, garlic, peppers, cilantro, cumin

and lime juice. Cherry tomato (cultivar for fresh market) producing many small fruits, cherry tomatoes are often served as appetizer, are generally considered the best suited for cooking in it sauces and ketchup due to their lower water contained (Decoteau,2000).

2.11.4 Nutrient value content of tomato

Tomatoes are a good source of vitamins and minerals like vitamin C, vitamin B, and riboflavin, which are crucial for skin health and growth. The vitamin C content of the fruit's exterior is high (Tehniat *et al*., 2014).

2.11.5 Medical uses of tomato

Tomato is known for its medical value, it is important in protection from cancer, use in constipation, disorder in liver and kidney function. However, it is important in protection and cure due to containing anti-oxygenate substance such like, vitamin C, glutathione, or β-carotene, as well as it rich in lycopene which result in activation of human body and mind (Tehniat *et al*., 2014).

2.11.6 Tomato insects

Several insects infest tomatoes. Some of those are white fly (*Bemisia tabaci*), Flea Beetle (*Epitricx cucumeries),* Colorado potato beetle (*Leptinotarsa decimlineata)* the green peach aphids (*Myzus persicae*), potato aphid (*Macrosiphum solanifolliare*), leaf hoppers (*Circulifor tenellus*), fruit fly (*Dorsphila melanogaster*) and stink bugs (Decoteau, 2000; Snyder *et al*, 2005). Recently tomato leaf miner *T.absoluta* became a pest of great economic importance in Nigeria. The pest has spread from Latin America, to Middle East, to North Africa, West Africa, then Nigeria in 2016 (Bello *et al*., 2016; CABI, 2016; EPPO, 2016).

2.12 Overview of *Tuta absoluta*

2.12.1 *Tuta absoluta* morphology

The pest adult is a moth, which is grey–brown in colour, approximately 6 mm in length and has a wing–span of about 10 mm (Figure 2.5) (Miano, 2017).

Figure 2.5: Adult stage of *Tuta absoluta*.
Source: *Tuta absoluta* (2016).

The male has a deeper complexion and a slimmer abdomen than the female (Koppert, 2008). These adults are usually active at night and conceal between leaves and fly low during the day. Males and females mate multiple times, with the first mating taking place a day after adults emerge from pupa (Vargas, 1970; USDA–APHIS, 2011) and taking place between 6 a.m. and 11 a.m. (Miranda-Ibarra, 1999). Males have a life expectancy of 18 to 43 days, while females have a life expectancy of 17 to 38 days (Fernandez and Montagne, 1990; Tropea Garzia *et al.,* 2012). Males and females who have never mate live longer than those who have mated. In her lifetime, the female can lay 250–300 eggs. Females deposit eggs singly or in batches on all vegetative parts of the host plant above ground. The pest can reproduce between 10 and 20 times per year, with a life cycle ranging from 21 to 34 days from hatching to adulthood, depending on temperature. The larva, which feeds voraciously on tomato plants from seedlings to adult plants, forming enormous galleries in leaves, burrowing in stalks, and consuming apical buds as well as green and ripe fruits, is the most devastating stage of the pest (Ndung'u, 2014).

2.12.2 Life cycle of *T. absoluta*

Tuta absoluta has a high reproductive rate. Depending on climatic conditions, it can perform 10–20 generations per year, with a minimum action temperature of 9°C (Urbaneja *et al.*, 2013). At the start of photophase, mating happens once a day (Vargas, 1970; USDA–APHIS, 2011). The average pre-oviposition period is 2.4 ± 0.61 days (Fernandez and Montagne, 1990). Oviposition occurs throughout the day, with a peak at night (USDA–APHIS, 2011) and on the first and second days after adults mate (Uchoa-Fernandes *et al*., 1995).

The oval-cylindrical eggs are 0.4 mm in length and 0.2 mm in diameter (Muniappan, 2013). They start out creamy white and gradually turn yellow–orange as they mature. At a temperature of 24.6°C, the egg takes 4-6 days to hatch (Fernandez and Montagne, 1990). They then turn dark

before hatching (Estay, 2000). In most cases, the eggs hatch in the morning. The larvae formed penetrate plant tissue and begin feeding (Muniappan, 2013).

The larval stage consists of four instars that differ in size and colour (Estay, 2000). Larval instar is best determined by measuring the head capsule diameter. Measurements of larval instars (body length) are 1.6, 2.8, 4.7 and 7.7 mm for first, second, third and fourth instars respectively (Estay, 2000; USDA–APHIS, 2011). First instar larvae are cream in colour with a dark head. Larval colour then changes to a deep green colour; the fourth instar changes to a pinkish colour followed by creamy white again as they purge their stomach contents towards pupation (USDA–APHIS, 2011).

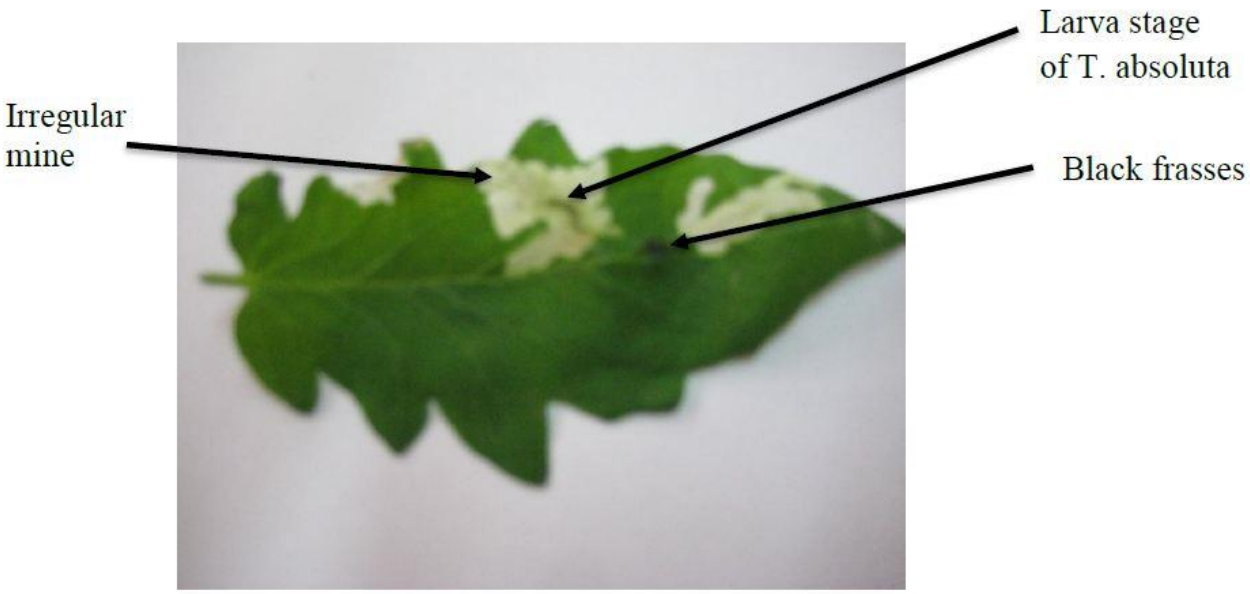


Figure 2.6: Larvae of *Tuta absoluta* feeds on tomato leaf
Source: *Tuta absoluta* (2016).

Larvae typically emerge in the morning and roam around for 5- 40 min before they start mining (Fernandez and Montagne, 1990; Tropea Garzia *et al.,* 2012). The larva takes an average of 8 days to fully develop (Muniappan, 2013). The larvae feed on the stem, leaves, shoots, flowers, and early fruits. They feed by constructing irregular galleries that grow longer as they feed, leaving a dark frasses behind (Fig. 2.5). *T. absoluta* is in its most destructive stage at this point. They subsequently bury themselves in the dirt to pupate (Muniappan, 2013).

Pupae are brown in color and measure approximately 4.3 mm long and 1.1 mm wide. Pupation can occur in the soil, on withered leaves or stems, or on both. On average, it takes 6–8 days for the adult to emerge (Muniappan, 2013). Males emerge in 7–8 days, whereas females take 6–8 days (Fernandez and Montagne, 1990).

T. absoluta's life cycle is influenced by temperature, with a minimum temperature of 9°C required for activity. It takes 76 days at 14°C, and 24 days at temperatures over 20°C (Muniappan, 2013). This suggests that *T. absoluta* will find a new habitat in Africa. At the pupa stage, the male and female *T. absoluta* can be recognized and isolated in the laboratory utilizing their reproductive organs.

2.12.3 Migration and distribution of *T. absoluta* in Africa

T. absoluta is thought to have originated in South America and spread throughout Europe, the Middle East, and the Mediterranean region before reaching Africa (Koppert, 2008). *T. absoluta* was proven to be found in Sudan, Ethiopia, Niger, and Senegal in 2012. (Muniappan, 2013). It was found to be present in Kenya in 2014. It was also reported in Tanzania in late 2014, however unlike Kenya, Russell IPM created and introduced sustainable and affordable biological remedies to empower farmers against insect harm, with encouraging results (Agripest, 2015). Mozambique, Malawi, Zimbabwe, Zambia, Botswana, and South Africa have all been affected.

The problem of *T. absoluta* has worsened as a result of indiscriminate use, overuse, and application of banned pesticide products, promoting early resistance development, polluting the environment, and exposing customers' health to danger (Agripest, 2015).

T. absoluta was first detected in Nigeria in May of 2016. It devastated 80% of Nigeria's tomato farms, resulting in a loss of over one billion naira (about US$3.5 million) (Borisade *et al*., 2017). Figure 2.7 depicts how *T. absoluta* was expected to expand across Africa, with the majority of regions afflicted by the end of 2016.

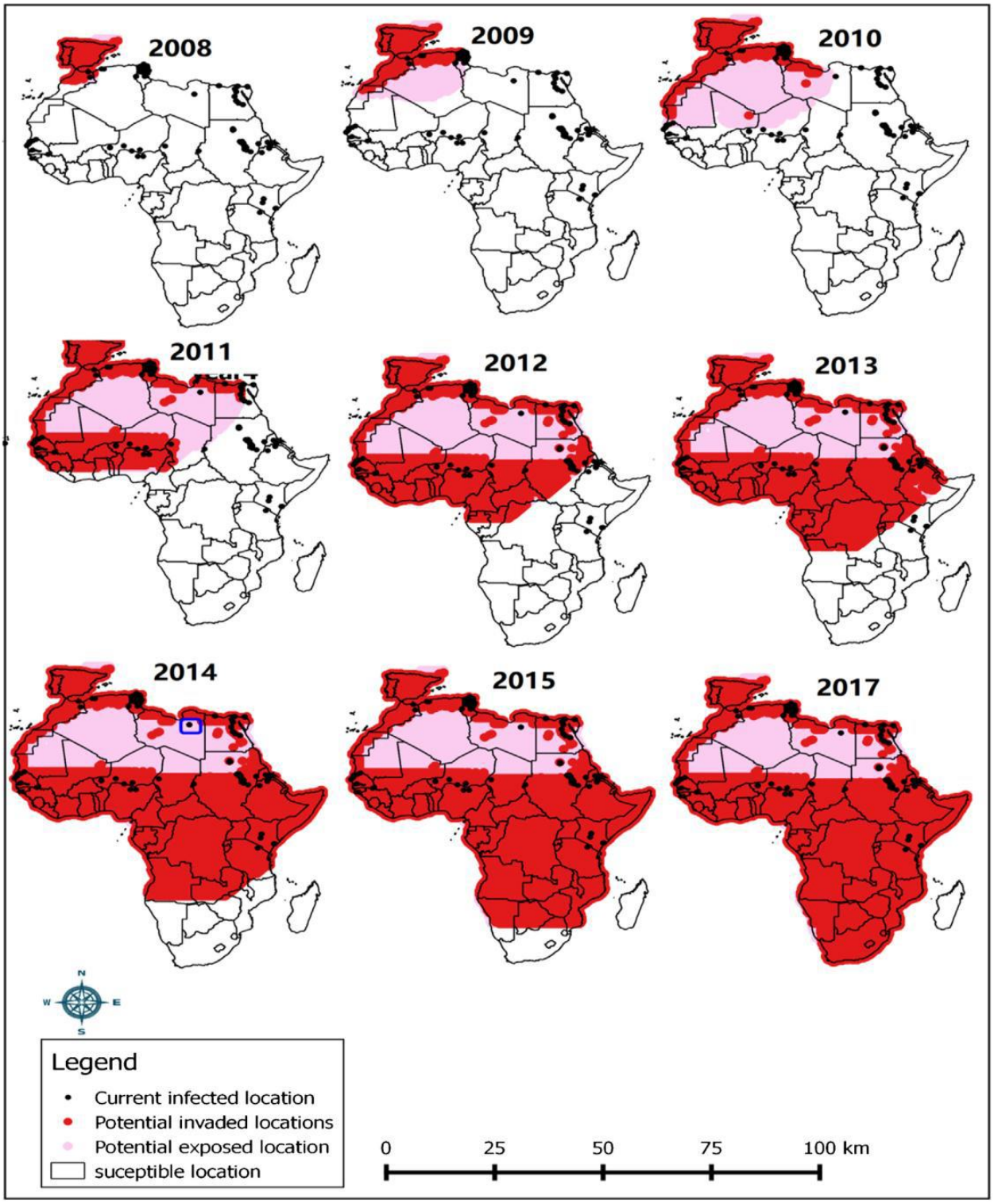


Figure 2.7: Predicted spread of *T. absoluta* in Africa obtained through a 10 year simulation

Source: Guimapi *et al*. (2016)

T. absoluta can reproduce between 10 - 20 generations each year in Africa, with a life cycle of 21 – 26 days from hatching to adulthood (Ndung'u, 2014).

Several approaches have been developed to assess the presence and severity of infestation. Physical counting of eggs on leaves, counting of larvae, mines on leaves, or adult males taken on pheromone traps are only a few examples (Gomide *et al*., 2001). Counting larvae and mines in the leaves is the most successful of these methods (Benvenga *et al.*, 2007). Overnight, the *T. absoluta* moth can fly a distance of 0.4 km (Salama *et al*., 2015).

2.12.4 Control methods of *T. absoluta*

2.12.4.1 Cultural methods

These involve:

(a) The pest and infested parts of the crop, or the entire crop, are physically removed and either burned or buried deep in the soil.

(b) Weed control before and during the cropping season, particularly of any other alternative host plants such as black nightshades, potatoes, datura, solanum, and nicotiana (Gitau, 2014; Ndung'u, 2014).

(c) Crop rotations with non-host crops assist in pest management (Markovi, 2013),

(d) If a greenhouse has been infested with the pest, it is recommended that the produce be removed and the greenhouse be closed to prevent the adults from migrating to the open field; and

(e) ploughing, over–head irrigation, soil solarisation, pest-free seedlings, and manuring (Balzan and Moonen, 2012; Jehle, 2015).

2.12.4.2 Chemical methods

Due of the cryptic character of the larvae and the insect's strong biotic potential, insecticides advised for the management of *T. absoluta* are of low to moderate efficiency. Pyrethrin, carbaryl,

deltamethrin, spinosin, indoxacarb, abemectin, emamectin benzoate, and cyromazin are some of the pesticides used. Insecticide resistance to organophosphates, pyrethroids, abamectin, cartap, permethrin, and spinosad has been observed (Siqueira *et al*., 2001; Haddi *et al*., 2012, Ndung'u, 2014).

In all areas where *T. absoluta* has been introduced, the immediate result has been an increase in the use of various insecticides and the number of times these chemicals have been used. This has put tomato consumers' health at danger, as well as the environment as a whole. It has also resulted in higher production costs, higher tomato prices, trade bans on tomato goods, and disruption of integrated pest control programs for other tomato pests (Ndungu, 2014).

2.12.4.3 Semiochemicals

Sex pheromones have been routinely employed to forecast, monitor, and/or reduce the numbers of moth pests (Prasad and Prabhakar, 2012). They are chemical signals emitted primarily by female adult creatures in order to attract the opposing sex species for mating (Cocco *et al*., 2013; Megido *et al*., 2013).

The synthetic pheromone blend has been successfully used in: a) pheromone-baited traps, b) mating disruption based on synthetic pheromone atmospheric saturation to reduce mating chances, and/or c) lure and kill techniques using a combination of a low amount of T. absoluta synthetic sex pheromone and an insecticide to reduce the male population (Cocco *et al*., 2013).

Other *T. absoluta* traps use a pheromone lure positioned above a sticky surface that can be removed to catch the attracted insects.

Traps containing water and detergent are also employed instead of sticky surfaces. Male *T. absoluta* are attracted to the lure and then drown as they fall into the water. Water traps capture a large number of adult males while remaining insect-free (Clarke, 2005). Pheromone-based traps

are only effective against male *T. absoluta*, which does not seek tomatoes according to study. The main concern should be mated *T. absoluta* females, as these are the ones who find oviposition locations.

2.12.4.4 Sterile males

The use of sterile males as an alternate technique for controlling *T. absoluta* has been advocated (Cagnotti *et al*., 2012). *T. absoluta* reproduces only through amphimixis, according to this mechanism. Female polyandry may obstruct this strategy and may have ramifications for IPM programs that rely on pheromones (Clarke, 2005). Polyandry has an impact on fecundity, fertility, genetic diversity, and other pest characteristics (Torres-Vila *et al*., 2004).

2.12.4.5 Light Traps

T. absoluta has a robust phototactic reaction in both sexes (Vargas, 1970). In greenhouses, light traps can be employed to catch these pests at twilight and sunset. Ferolite-TUA is a light trap that employs a mix of a very appealing light frequency for *T. absoluta* and pheromone lures (Russell IPM, 2009). Light traps are 200 to 300 percent more effective than traditional pheromone traps (Cocco *et al*., 2012). Both males and females are drawn to them. The distribution of energy in Nigeria, and Africa in general, is a barrier to the use of light traps.

2.12.4.6 Host – Plant Resistance

Host–plants high in zingiberene (a repellant) and/or acylsugar (which prevents the larval stages from fully developing) are now being tested for protection against tomato pests. This could lead to the creation of tomato types that do not attract females for oviposition and/or do not allow the larvae to fully develop (Maluf *et al*., 2010).

2.12.4.7 Biological methods

Mired bugs (*Nesidiocoris tenuis*) and *Macrolophus pygmaeus* are predators of *T. absoluta*. They are commonly used throughout Europe and North Africa and are commercially available (Balzan and Moonen, 2012). *T. absoluta* is controlled by insecticide formulations based on *Bacillus thuringiensis* in both native and invaded areas. Molla *et al*. (2011) found that they are largely utilized to control first-instar larvae and have no negative impacts on beneficial arthropods. Azadirachtin, a component of neem, is also efficient against *T. absoluta*. For *T. absoluta*, it works as a systemic and contact pesticide (Goncalves-Gervasio and Vendramin, 2007). Fungal species such as *Metarhizium anisopliae* and *Beauveria bassiana* have been documented to affect *T.absoluta* eggs, larvae, and adults (Pires *et al*., 2009, 2010).

Trichogramma pretiosum, Pristomerus, Dineulophus phthorimaeae, Cremastus, Copidosoma, and *Apanteles* are natural enemies of the *T. absoluta* moth (Berti and Marcano, 1991). These parasitoids are employed as parasites. Chilocorus (Vasicek, 1983) spiders, carabids, earwigs, hemipterans, wasps, ants, lace wings, and *Steinernema carpocapsae* are among the moths' predators (Jimenez *et al.,* 1989). *Bacillus thuringiensis* isolates are also used as biopesticides because they are environmentally friendly, effective, long-lasting, and safe (Adeyemo *et al*., 2018; Rajendran *et al*., 2018; Fernández-Chapa *et al*., 2019).

2.12.5 Economic importance of *T. absoluta*

Many subsistence farmers rely on tomatoes and potatoes as main staples. They are the world's most frequently farmed horticultural crops. *T. absoluta* attacks both of them, posing a major nutritional threat to the entire population (Agripest, 2015). *T. absoluta* larvae feed on stems, leaves, buds, calyces, flowers, and young and ripe tomatoes. It has the potential to destroy 90–100 percent of open-field tomatoes (Estay, 2000; USDA-APHIS, 2011). Pesticides were

administered more than 15 times per season in the first year in all places where *T. absoluta* was introduced. When *T. absoluta* spreads over the world, the expense of managing it is expected to rise by $500 million every year (Muniappan, 2013). This will have an impact on tomato consumer health, ecosystem devastation in general, high production costs, increased tomato prices, trade bans on tomato products, disruption of other tomato pest integrated pest management programs, and the overall human diet. (Zappalà *et al*., 2012; Zlof and Suffert, 2012).

2.12.6 Environmental impact

Increased use of a wide number of pesticides and their frequency have been the direct effects of *T. absoluta* infestation. In most cases, farmers utilize trial and error tactics to control the pest, spraying any agro-chemical in the process, resulting in high residue levels on tomato fruits, jeopardizing consumers' lives and health, as well as hurting the ecology (Ndung'u, 2014; Jehle, 2015). *T. absoluta* has developed resistance to synthetic insecticides, and the pest has expanded rapidly, leading to the discovery of novel pesticides and their growing use without a thorough understanding of their environmental impact (Belmain *et al*., 2013). Integrated pest management efforts for other tomato insect pests have also been disrupted as a result (Arno´ and Gabarra 2011; Biondi *et al*., 2012, 2013)

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Collection of Soil Samples

Soil samples were collected from fields at three different sites in Zaria namely, Tomato farmland, Cow rangeland and Refuse dump site. One sample of 10g each was collected from the surface layer (5 to 10cm) at ten different spots per site. The ten samples from each site were bulked and thoroughly mixed to obtain representative composite samples (Atta, 2009; Stefani *et al*., 2015). The three composite samples from the three sites were stored in polythene bags and brought to the Environmental Research Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria and stored at ambient temperature for analysis.

3.2 Isolation of *Bacillus thuringiensis*

Bacillus thuringiensis were isolated according to sodium acetate selection method described by Travers *et al*. (1987) with some modifications. For each sample, 10g soil was added to 90mL of distilled water and incubated at 28^{o}C for 45 mins on a rotatory shaker operating at 250 rpm. Thereafter, 2 mL of the suspension was added to 20 mL of freshly prepared Luria Bertani (LB) (Tryptone 10g/L, yeast extract 5g/L, NaCl 5g/L) broth buffered with 0.25 M sodium acetate (pH 6.8) in 50 mL Erlenmeyer flask and incubated at 28^{o}C for 4 hours on a rotatory shaker at 250 rpm. After incubation, 5 mL aliquots from each culture were placed in hot water bath operating at 80^{o}C for 3 mins. And then 0.1 mL was spread on LB agar (Tryptone 10g/L, yeast extract 5g/L, NaCl 5g/L and Agar 15g/L) and incubated at 28^{o}C for 24 hr. Colonies that showed cultural morphology typical of *Bacillus thuringiensis* (cream coloured, dry surface with entire margin) were selected and purified by subculturing on freshly prepared T3 agar (Tryptone 3g/L, yeast

extract 1.5g/L, Peptone 2g/L, $MnCl_2$ 0.005g/L, Sodium phosphate 0.05M and Agar 15g/L). The isolates obtained were then stored in a refrigerator operating at 4^{o}C for further studies.

3.3 Identification of isolates

The cultural characteristics and biochemical properties of isolates presumed to be *Bacillus thuringiensis* were determined (Cowan and Steel, 2003; Bergey, 2004).

3.3.1 Cultural and Morphological characterization on T3 agar medium

The cultural characteristics of the isolates were determined on the basis of the colour, form, margin and elevation of the colonies.

The morphological characteristics of the pure isolates were determined by:

3.3.1.1 Gram staining

This was carried out by preparing thin smear of each of 24-hour cultures on clean grease-free slides and heat-fixing the smear by passing over Bunsen burner flame. The heat-fixed smears were then stained by addition of two drops of crystal violet solution for 60 seconds followed by rinsing with water. The stained smears were flooded with Lugol's iodine for 30 seconds, rinsed with water, followed by decolourization with 70% alcohol for 15 seconds and rinsed with water. The smears were counter-stained using two drops of Safranin for 60 seconds and finally rinsed with water and allowed to air dry. The stained slides were examined under the microscope using oil immersion objective lens. Presence of Gram positive, long rods with endospores were presumed *Bacillus* spp. (Fawole and Oso, 2004).

3.3.1.2 Endospore Staining

This was carried out to detect the presence of bacterial endospores. Heat-fixed smears from 48-hour culture of the isolates were prepared on slides and flooded with 5% Malachite green solution and steamed for a minute. The stain was washed off with water and counter stained with 2 drops of safranin solutions for 20 seconds. The slides were then allowed to air dry and examined under oil immersion objective. Endospores stained green while vegetative cells stained pink (Cheesbrough, 2006). Isolates that showed the presence of endospores were further confirmed to be *Bacillus* spp.

3.3.1.3 Motility test

This was carried out by inoculating the motility medium with a pinch from colonies of 24 h culture of the isolates by stabbing with a straight wire to a depth of one third the total depth of the medium. The cultures were then incubated at 37^{o}C for 24 h. Cultures that turned turbid (cloudy) as a result of migration of the inoculated cells were putatively identified as *Bacillus thuringiensis* (Cowan and Steel, 2003).

3.3.2 Biochemical Characterization Tests of the Isolates

Isolates presumed to be *Bacillus* spp. On the basis of their cultural and morpohological characteristics were further characterized by subjecting them to the following biochemical tests:

3.3.2.1 Catalase test

A small quantity of 24 h culture was transferred into a drop of 3% Hydrogen peroxide solution on a clean slide with the aid of a sterile inoculating loop. Evolution of frothy gas by an isolate further confirmed it to be *B. thuringiensis* (Cowan and Steel, 2003).

3.3.2.2 Oxidase test

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a suspected *B. thuringiensis* colony and smeared on the filter paper. If the organism is oxidase-producing, the phenylenediamine in the reagent would be oxidized to a deep purple colour. The emergence of a purple colour by an isolate further confirmed it to be *B. thuringiensis* (Cheesbrough, 2006).

3.3.2.3 Methyl Red and Voges-Proskauer tests

Test tubes containing 5 mL of MR-VP broth each was inoculated with the suspected *B. thuringiensis* colony using wire loop. The tubes were then incubated at 37^{o}C for 48 hrs. After incubation, about 2ml of the cultured broth was transferred to a small test tube to which three drops of methyl red indicator was added. Formation of red colour on addition of the indicator signified a positive methyl red test and a yellow colour signified a negative test

To the rest of the broth, 2 drops of 40% potassium hydroxide was added followed by 2 drops 5% α Naphtol 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 indicated a VP positive test. No colour change indicated VP negative test. Development of a red colour further confirmed it to be *B. thuringiensis* (Cowan and Steel, 2003).

3.3.2.4 Starch hydrolysis

This was carried out by inoculating suspected *B. thuringiensis* colonies on starch agar. The inoculated plates were then incubated at 37^{o}C for 24 hrs. After incubation, the plates were flooded with Lugol's iodine and examined for zone of hydrolysis. Observation of zone of hydrolysis around an isolate further confirmed it to be *B. thuringiensis* (Cowan and Steel, 2003).

3.3.2.5 Citrate utilization

The ability to utilize citrate as sole source of carbon and energy was tested on Simmon's citrate agar medium. This was carried out by inoculating suspected *B. thuringiensis* colonies on the Simmon's citrate agar medium. The inoculated McCartney bottles were incubated at 37°C for 48 hrs. After incubation, the bottles were examined for colour from green to blue. The change of colour of the medium from green to blue by an isolate confirmed it to be *B. thuringiensis* (Cowan and Steel, 2003).

3.3.2.6 Casein hydrolysis

Overnight grown culture of *Bacillus thuringiensis* isolate was spotted on skim milk agar plates and incubated at 28^{o}C for 24 hrs. The production of halo zone around the colony was taken as positive for the test (Cowan and Steel, 2003).

3.3.2.7 Arginine dihydrolysis

The overnight grown culture was spotted on nutrient agar medium supplemented with 3% L-arginine and incubated for 24 hrs at 37^{o}C. The plates were observed for the zone of clearance after incubation and culture showing clear zone around the colony were scored as positive for the test. Observation of no zone of clearance around an isolate further confirmed it to be *B. thuringiensis* (Cowan and Steel, 2003).

3.3.3 Screening the isolates for capacity to produce Crystal toxin

Single colonies from overnight cultures on LB plates were inoculated into 10 mL of T3 sporulation medium and incubated at 28^{o}C for 5 days on a rotatory shaker operating at 250rpm. Smears of the cultures were heat fixed on the glass slide and stained with Coomassie brilliant blue 0.25% (w/v) in 60% ethanol (v/v) and 7% of acetic acid (v/v), with the aim of determining the presence and morphology of the crystals (Fakruddin*, et al*., 2012). The stained smears were

then examined under a light microscope. Presence of crystals confirms the capacity of the isolates in production of crystal toxins.

3.4 Detection of Transcriptional Regulator Gene (*XRE*) and Lepidoptera-active c*ry1* and *cry2* genes using Specific Primers

3.4.1 DNA extraction and gene amplification

DNA extraction from the isolates of *B. thuringiensis* was carried out using Qiagen DNeasy extraction kit according to the manufacturer's instructions. The PCR conditions for the c*ry* genes and *XRE* gene were according to Ben-Dov *et al.* (1997) and Wei *et al*. (2019) respectively.

PCR reactions were carried out in 25 µL reaction mixture containing 8 µL template DNA, 150 mM dNTPs, 20 pM of each of the six primers (Table 3.1) and 0.5U of *Taq* DNA polymerase. Amplification of *XRE* gene was carried out in a DNA thermocycler with the program: one initial denaturing cycle at 94ºC for 3 minutes, 35 cycles containing: denaturing at 94ºC for 30 seconds, annealing at 49ºC for 30 seconds and extension for 30 seconds at 72ºC and then the reaction being terminated by a final extension for 10 minutes at 72ºC while the Amplification of *cry*1 *and cry*2 genes were carried out in a DNA thermocycler with the program: one initial denaturing cycle at 94ºC for 3 minutes, 35 cycles containing: denaturing at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension for 1 minute at 72ºC and then the reaction being terminated by a final extension for 10 minutes at 72ºC.

The *XRE* gene and *cry* genes banding patterns were visualized following agarose gel electrophoresis. An aliquot (15 µL) of each amplification product was loaded onto 1.5% agarose gel and run in TAE buffer (40mM Tris-Acetate, 1 mM EDTA) at 100volts for 1 hour. The gels were stained with ethidium bromide and documented with a 100 bp molecular weight marker.

Table 3.1: Primer sets used in the amplification of *cry* and *XRE* genes

Genes	**Primer Sequence (5'-3')**	**Product size (bp)**	**Reference**
cry1	F CATGATTCATGCGGCAGATAAAC	274	Ben-Dov *et al*. (1997)
	R TTGTGACACTTCTGCTTCCCATT		
cry2	F GTTATTCTTAATGCAGATGAATGGG	689	Ben-Dov *et al*. (1997)
	R CGGATAAAATAATCTGGGAAATAGT		
XRE	F AAGATATTGCAAGCGGTAAGAT	246	Wei *et al*. (2019)
	R GTTTTGTTTCAGCATTCCAGTAA		

3.5 Infesting Screened House Tomato Plants with *Tuta absoluta*

3.5.1 Raising Tomato Plants in the Screened house

Tomato seeds (Roman VF variety) were acquired from Institute of Agricultural Research (IAR), Ahmadu Bello University Zaria. Seedlings of the specified tomato variety were initially raised in nursery beds, composed of mixture of loamy soil and composed manure in a screened house of department of Microbiology, Ahmadu Bello University Zaria. Two weeks after seedling emergence, the seedlings were transplanted into plastic pots (20 cm in diameter) each containing 4 kg of the soil mixture at the rate of one seedling per pot (Appendix V). The potted plants were placed in the screened house to prevent attack by herbivores and other pests of tomato plants. Watering was done regularly to prevent wilting (Legwalia *et al*., 2015).

3.5.2 Collection of *Tuta absoluta* Larvae and Infestation of Tomato Plants

Tuta absoluta larvae were collected from tomato fields in Marabanguga, Zaria using a glass jar with perforated lids and were taken to Entomology laboratory, department of Crop Protection, Ahmadu Bello University, Zaria to confirm the identity, and the second instar stage of the larvae. Tomato seedlings at the five-leaf stage were infested with the confirmed *T. absoluta* larvae. Pots bearing the tomato plants were placed in the two cages measuring 45 cm long, 45 cm wide, and 40 cm high, and was covered with clear lumite netting of 32 mesh size to prevent pest infestation from natural populations (Appendix V). Each cage had a door with a metal sleeve that was used during watering of plants, artificial infestation of the plants, the application of sprays, and the removal of plants during pest larva assessments.

3.6 Efficacy of Promising Native *Bacillus thuringiensis* Isolates Against *Tuta absoluta*

3.6.1 Generation of Spore Crystal Mixture

Spore crystal mixtures of *Bacillus thuringiensis* were generated as described by Sahin *et al*. (2018) with some modifications. Single colonies from overnight LB plates were inoculated into 10mL of T3 sporulation medium and cultured for 5 days at 28^{o}C on a rotatory shaker at 250rpm. Spore crystal mixtures were harvested by centrifugation at 4000xg for 25 minutes. The pellets were resuspended in 5 mL distilled water and centrifuged again (4000xg for 10 minutes). This process was repeated twice. Then, the pellets of the spore crystal mixtures were resuspended in 5 mL distilled water and kept at -4°C until required (Appendix IV).

3.6.2 An Assessment of Potency of the spore crystal mixtures derived from the isolates under Screened house conditions

The spore crystal mixture generated (section 3.8.1) was used to spray the larvae on the infested tomato plants to determine the potency of the cry toxins derived from the isolates. This was carried out by spraying plants infested with 10 larvae each. Treatments were applied in triplicates using small hand-held trigger sprayer that produced a fine spray of a relatively narrow range of droplet sizes (Appendix VI). The test was conducted on second instar larvae which are also known as the leaf miners usually known to be resistant to pesticide (Legwalia *et al*., 2015).

The larvae mortality was assessed at intervals of 24, 48, and 72 hours after treatment. Any larvae that did not show signs of life after prodding with a needle were considered as dead (Legwalia *et al*., 2015) and the numbers of dead larvae were recorded.

3.7 Data Analysis

Data obtained from the study were subjected to statistical analysis using the SAS JMP Pro 14 statistical package. Two-way analysis of variance (ANOVA) was used to compare differences

between the mean toxicity of the crystal spore mixture derived from each isolate tested. Averages were separated using Tukey's Honestly significant difference test (Zar, 1984) where significant effects were observed. Results are then presented in tables, graphs and charts where applicable.

CHAPTER FOUR

4.0 RESULTS

4.1 Occurrence of *Bacillus thuringiensis* in the study sites

Five isolates were Gram positive and rod shaped. Result from the endospore test revealed that the five isolates are spores-producing organisms. Colonies that appeared creamy white, circular, dry, with flat elevation and wavy margin on LB agar were regarded putatively as *Bacillus thuringiensis*. All the five isolates were positive to Catalase, Oxidase, Citrate and Voges-Proskauer tests while they are all negative to Arginine dihrolysis (Table 4.1). Cow rangeland had the highest occurrence of 50%, followed by refuse dump site (40%), while, tomato farmlands had no occurrence of *Bacillus thuringiensis* (Table 4.2).

4.2 *XRE* gene and *cry1* and *cry2* genes profiles of the isolates

The five isolates identified using cultural, microscopic and biochemical characterizations were screened for the presence of the *cry1*, *cry2* and the *XRE* genes using specific primers by PCR. The amplicons of the *XRE* gene (246 bp) were detected in four of the isolates of *Bacillus thuringiensis* (Plate I). The *cry1* gene (274 bp) amplicons were detected in two of the isolates of *Bacillus thuringiensis* (Plate II) while the *cry2* gene (689 bp) was not amplified in all the isolates.

Table 4.1. Microscopic and Biochemical characterization of *Bacillus thuringiensis* isolated in the study

	C1	**C2**	**C6**	**R3**	**R4**
***Colonial morphology**	+	+	+	+	+
Gram reaction/Cellular morphpology	+/R	+/R	+/R	+/R	+/R
Cellular arrangement (in chains)	+	+	+	+	+
Presence of Endospore	+	+	+	+	+
Motility	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Methyl Red	-	-	-	-	-
Voges Proskauer	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+
Arginine hydrolysis	-	-	-	-	-
Utilization of citrate	+	+	+	+	+
Crystal production	+	+	+	+	+
Inference	Bt	Bt	Bt	Bt	Bt

C1, C2 and C6 = Isolates from Cow rangeland

R3 and R4 = Isolates from Refuse dump site

*** =** colonies that appear creamy white, circular, dry, flat elevation and with wavy margin on LB agar

+ = positive reaction. **-** = negative reaction. R = rod.

Bt = *Bacillus thuringiensis*.

Table 4.2. Occurrence of *Bacillus thuringiensis* isolates in soils from various sites sampled

Sources of soil samples	*Number of Bacillus like isolates	Number of Bt isolates	[a]Bt index	Frequency of distribution of Bt (%)
Agricultural farmland	5	0	0.00	(0)
Cow rangeland	6	3	0.50	(50)
Refuse dump site	5	2	0.40	(40)
Total	16	5	0.31	(31)

***** Creamy white, circular, dry, flat elevation, with wavy margin

[a] Bt Index: The isolation index of Bt was calculated by dividing the number of Bt isolates by the total number of Bacillus-like colonies obtained.

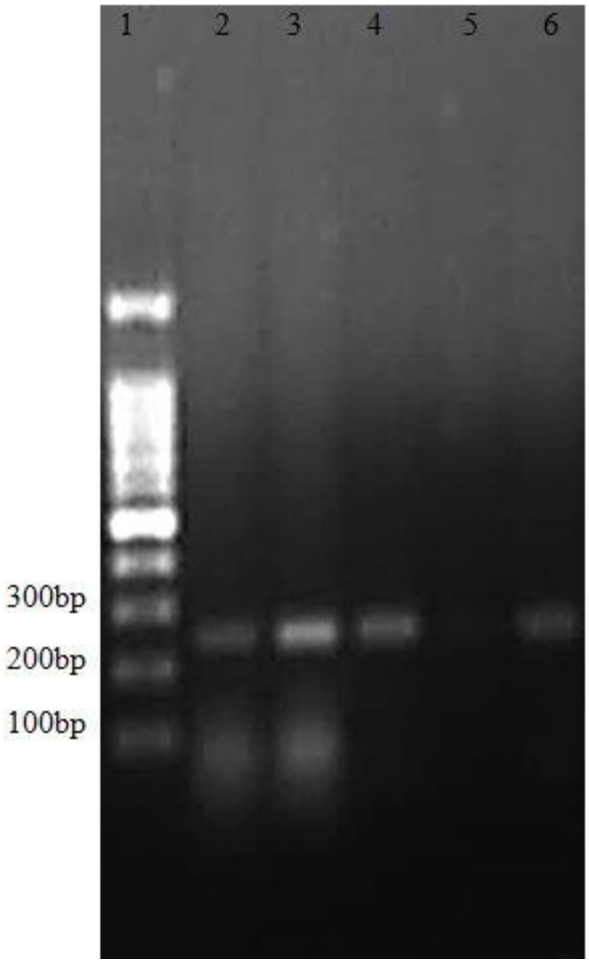


Plate I. *XRE* gene (246 bp) profiling of the isolates

Key:

Lane 1: DNA ladder (100bp)

Lanes 2 - 4: Bt isolated from Cow range (C2, C6, C1, respectively)

Lanes 5 and 6: Bt isolated from Refuse dump (R4 and R3, respectively)

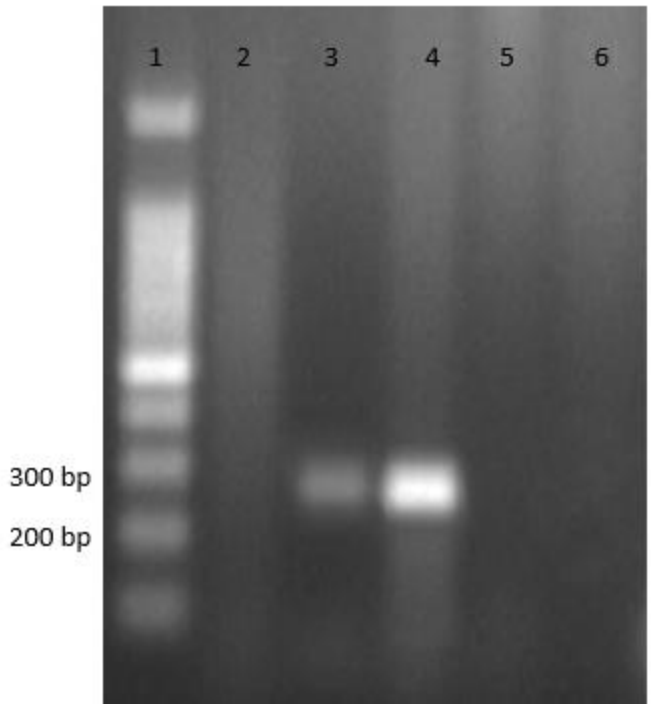


Plate II. *cry1* gene (274 bp) profiling of the isolates

Key:

Lane 1: DNA ladder (100bp)

Lanes 2 and 3: Bt isolated from Refuse dump (R4 and R3, respectively)

Lanes 4 - 6: Bt isolated from Cow range (C6, C2, C1, respectively)

4.3 Larvicidal activity of spore crystal mixtures derived from the isolates

Results in Table 4.3 show that both isolates and time period after spore crystal mixture treatment significantly affected the average mortality of *Tuta absoluta* larvae per plant (ANOVA, $P < 0.05$). The interactions were also significant. The greatest mortality (96.7% per plant) occurred 72 hours after treatment of the larvae with spore crystal mixture derived from isolate R3, while the lowest mortality (30%) occurred 24 hours after treatment with spore crystal mixture derived from isolate C6. The results also show that the least mortality (6.7–16.7% per plant) occurred in the control treatment throughout the study period. Mortalities that occurred 48 hours after exposure of the larvae to spore crystal mixture derived from C6 were similar to those achieved 72 hours after exposure of the larvae to spore crystal mixture derived from C6 and 48 hours after exposure of the larvae to spore crystal mixture derived from R3 (Tukey, $P < 0.05$).

The overall isolate averages show that the spore crystal mixture derived from the different isolates has a significant toxicity on the mortality of larvae. Overall larval mortalities did not differ significantly from each other on plants treated with the spore crystal mixture derived from isolate C6 and R3. The overall exposure period results were significantly different and increased in the order 23.3 < 44.4< 62.2% when assessment was done 24, 48 and 72 hours after application.

Table 4.3. Larvicidal Effects of Spore Crystal Mixtures Derived from *Bacillus thuringiensis* Isolates on *Tuta absoluta*.

PERIOD OF EXPOSURE AFTER APPLICATION (HOURS)	ISOLATE C6 (%)	ISOLATE R3 (%)	CONTROL (STERILE DISTILLED WATER) (%)
24	30.0^{cd}*	33.3^{c}	6.70^{e}
48	56.7^{b}	63.3^{b}	13.3^{de}
72	73.3^{b}	96.7^{a}	16.7^{cde}

Notes: *Interaction averages in the body of the table followed by the same letters are not significantly different (Tukey's Honestly significant difference test ($P < 0.05$).

CHAPTER FIVE

5.0 DISCUSSION

The isolation procedure similar to the one used for the isolation of five indigenous isolates of *Bacillus thuringiensis* in this study was also utilised by Bello and Hussaini (2017) and Adeyemo *et al*. (2018) in which numerous isolates of *Bacillus thuringiensis* were isolated from different soil samples. The biochemical characteristics observed in this study are similar to those reported by Kaur *et al*. (2002), Eswarapriya *et al*. (2010), Ahmed *et al*. (2015) and Riskuwa-Shehu *et al*. (2019); which show that, in addition to producing parasporal crystal bodies, the isolates of *Bacillus thuringiensis* have positive reactions for catalase production, oxidase activity, citrate utilization, Voges-Proskauer, starch and casein hydrolysis. This study is of great importance and relevance since it identified indigenous isolates of *Bacillus thuringiensis* which represent an alternative for the control of insect pest *Tuta absoluta*.

The higher occurrence of *Bacillus thuringiensis* isolates in Cow rangeland could be explained by the higher organic matter concentration of this soil type, which favors the growth and multiplication of *Bacillus thuringiensis*, which naturally thrives on saprophytic plants and feeds on decaying organic matter. This research supports Bello and Hussaini's (2017) findings, which found a higher prevalence of *Bacillus thuringiensis* in the Cow rangeland soil type. While, the non-occurrence of *Bacillus thuringiensis* isolates in tomato farmland observed in this study could be as a result of the use of synthetic chemicals such as fertilizers and pesticides on the farmland. Also, intensive cultivation of a farmland for a long period of time could lead to reduction of organic matter availability which in turn reduces heterotrophic population of microorganisms.

The estimated value of successful *Bacillus thuringiensis* isolation (Bt index) varies depending on soil type, with an average Bt index of 0.31 in this study. The average Bt index differs among soil

samples around the world, as previously observed (Vilas-Boas and Lemon, 2004; Lone *et al*., 2016; Lone *et al*., 2017). The difference in Bt index could be due to differences in terrain, nutrient availability, humidity, isolation source, geography, and bacterial-pest interactions, which could have a considerable impact on their abundance in different environments.

The amplification of the *XRE* gene in this study revealed that out of five isolates of putative isolates of *B. thuringiensis*, the gene was detected in four (80%) when end-point PCR was used which only detects the gene and does not quantify it; while Wei *et al*. (2019) utilised a real-time PCR which quantifies the number of genes targeted in the amplification of the *XRE* gene in identifying *B. thuringiensis* with a specificity of 94% achieved. According to report by Wei *et al*. (2019), the *XRE* gene is more dominant than the *cry2* gene when compared in isolates of *B. thuringiensis*. While in this study, the *XRE* gene is more dominant than both the *cry1* and the *cry2* genes. The main reason for the efficiency of *XRE* gene over the two *cry* genes could be due to the fact that the former is a transcriptional regulator, it regulates the main type of crystal protein production (Wei *et al*., 2019) and there are currently 78 known *cry* genes families available as biomarkers since several translated cry protein product vary with the different categories of the *cry* genes. This study represents the first characterization of indigenous *Bacillus thuringiensis* isolates diversity from Nigeria, in terms of *XRE* gene profile.

Owing to the speed and reproducibility of the PCR-based approach, it has been widely used since its introduction by Carozzi *et al*. (1991) for the detection of known and new *cry* genes in *B. thuringiensis*. For the five isolates isolated in this study, 2(40%) of the 5 isolates were found to be potential candidate for biocontrol of *T. absoluta* since they harbor the lepidopteran- active

cry1 genes. The *cry2* gene was not amplified in the 5 isolates, implying that the isolates have not acquired the plasmids harbouring the *cry2* gene. The main reason for toxin genes diversity could be attributed to the fact they are mostly borne on plasmids which are easily acquired partially or completely by *B. thuringiensis* isolates (Fiuza, 2015; Liu *et al*., 2015). This agrees with the report of (Bello and Hussaini, 2017; Jain *et al*., 2017) which indicates that a large number of isolates do not carry the *cry2* genes. However, the predominance of *cry2* gene has also been reported (Liang *et al*., 2011; Lone *et al*., 2017; Wei *et al*., 2019). The high frequency of *cry1* gene observed in this study is consistent with the reports of Cinar *et al*. (2008); Sahukhal *et al*. (2008) and Khojand *et al*. (2013), who posited that the observed high frequency of incidence of *cry1* genes could be attributed to the fact that, *cry1* gene-containing isolates of *Bacillus thuringiensis* are possibly the more abundant isolates in nature. Clear understanding of toxin encoding gene in an isolate of Bt can be used to predict the potential pathogenicity of the isolates against the target pests (Baig and Mehnaz, 2010; Konecka *et al*., 2012). The presence of *cry1* gene in isolates R3 and C6 observed in this study strongly suggests the potential of the isolates against lepidopteran to which *T. absoluta* belongs.

The results obtained from bioassay procedure shows that the spore crystal mixtures derived from the two isolates of *Bacillus thuringiensis* isolated from cow rangeland and refuse dump site respectively suggests they could be used to effectively protect tomato plants from *Tuta absoluta* damage as strain C6 and R3 achieved over 70 and 90% larval mortality respectively after 72 hours of exposure as opposed to the control with only 16.33% larval mortality after 72 hours of exposure. These observations are consistent with those made by Youssef and Hassan (2013), who found that the second larval instars of *T. absoluta* were highly susceptible to continuous exposure (120 h) to *Bacillus thuringiensis* species indigenous to Egypt using Petri dish

bioassays. These authors reported over 60% larval mortality. The results of this study are also in accord with those obtained by Alsaedi *et al*. (2017), who reported 50% larval mortality when suspensions of *Bacillus thuringiensis* were used against second instar larvae of *T. absoluta* under laboratory conditions. Similar reports were also made by Legwaila *et al*. (2015) while testing other insect pests from the same order but different families. They demonstrated that exposing the second larval instar of Diamond-back Moth (*Plutella xylostella* L.), to *Bacillus thuringiensi*s (var. kurstaki) (*Btk*) results in 70% mortality under greenhouse conditions.

Isolates C6 and R3 of *B. thuringiensis* examined in this study exhibited significant variability in terms of potency against *T. absoluta* between the two isolates. The toxicity of R3 and C6 was observed to be 96.7% and 73.3% respectively. The high larvicidal potency observed for strain R3 indicates the likelihood of higher effectiveness of the strain being the most potent strain of the two. This could be suggesting that the differences in the insecticidal activities of the two isolates may be due to the differences in the carbohydrate affinity of the cry proteins domain II, which results in different binding specificities with the insect larvae brush border membrane receptors, resulting in differences in the toxicity of the cry proteins (Smedley & Ellar, 1996). The outcome of this present investigation are in agreement with those of Youssef and Hassan (2013), who conducted studies on different indigineous isolates of *Bacillus thuringiensis* and observed that strain B1 has more potency than the other isolates B2, B3 and B4.

Longer exposure periods are also required to achieve 90%–100% *Tuta absoluta* larval mortality as observed from the study. The main reason for the longer exposure time required to achieve higher efficacy of the spore crystal mixtures derived from the *Bacillus thuringiensis* isolates may be due to the fact that *Bacillus thuringiensis*-affected larvae die of starvation, which may take

several days. Because *Bacillus thuringiensis* cannot kill rapidly, potential users may mistakenly believe that it is ineffective if treatments are evaluated one or two days after use (Legwaila *et al*., 2015; Melo *et al*., 2016; Zhang *et al*., 2017; Fernández-Chapa *et al*., 2019).

The findings of this study show that, in addition to the *Bacillus thuringiensis* strain that should be employed against a pest species, information on an insecticide's label should contain the periods of exposure necessary to achieve the desired level of pest control. Farmers would be able to decide on the intervals between spray applications based on this knowledge. Therefore, the finding that the use of the spore crystal mixture derived from the strain R3 after 72 hours of exposure achieved 96.7% *Tuta absoluta* larval mortality in this study shows that indigenous *Bacillus thuringiensis* strain is efficient in the adequate control of the pest and protect tomato crop from serious damage in a sreened house.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Cow rangeland and refuse dump sites in Zaria are good reservoirs of isolates of *Bacillus thuringiensis*. Five presumptive isolates of *Bacillus thuringiensis* were isolated from the soils sampled and the average *Bacillus thuringiensis* index was 0.31.

Bacillus thuringiensis indigenous to soils in Zaria commonly possessed the *XRE* and the *cry1* genes but not the *cry2* genes. Of the five (5) isolates, four (4) showed the presence of the *XRE* genes, two (2) showed the presence of the *cry1* genes while none of the isolates harboured the *cry2* genes.

The spore crystal mixture derived from isolate R3 is more potent as it achieved 96.7% efficacy against *T. absoluta* than that derived from isolate C6 which achieved 73.3% at 72 hours of exposure. Thus, isolate R3 has a better potential for application in protecting tomato plants from *T. absoluta* infestation on farmers' field.

6.2 RECOMMENDATIONS

1. This study investigated the potentials of wild isolates of *Bacillus thuringiensis*. Thus, further investigations should be conducted to confirm the effectiveness of the toxins produced by strain R3 against *T. absoluta* under field conditions.

2. Further investigations should also be conducted with the view to enhancing the production of the toxins by the promising strain.
3. In this study, it was demonstrated that time is a factor in determining the efficacy of Bt spore crystal mixture. However, further investigations should be carried out to determine the effects of relative humidity, temperature, and adjuvants on the performance of the promising strain.

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APPENDICES

Appendix I: **Scientific Classification of *Bacillus thuringiensis***

Kingdom: *Eubacteria*

Division: *Firmicutes*

Class: *Bacilli*

Order: *Bacillales*

Family: *Bacillaceae*

Genus: *Bacillus*

Species: *thuringiensis*

Binomial name: *Bacillus thuringiensis*

This nomenclature was reported by Berliner (1915).

Appendix II: **Scientific Classification of Tomato**

Kingdom: Plantae

Subkingdom: Tracheobionia.

Phylum: Magnoliopsida.

Sub class: Asteridae.

Order: Solanales.

Family: Solaneceae.

Genus: Lycopersicon.

Species: *Esculentum* Mill (Edrees, 2014)

Appendix III: **Scientific Classification of *Tuta absoluta***

Kingdom: Animalia

Division: Arthropoda

Class: Insecta

Order: Lepidoptera

Sub order: Glossata

Super family: Gelechioidea

Family: Gelechiidae

Sub family: Gelechiinae

Tribe: Gnorimoschemini

Species: *Tuta absoluta* (USDA–APHIS, 2011)

Common Name: tomato leaf miner or tomato ebola

Appendix IV: Raw Data of the Mortality Assessments of the Isolates and Control (Sterile Distilled Water)

ISOLATES	24 HOURS	48 HOURS	72 HOURS
C6	3	5	7
C6	3	5	7
C6	3	7	8
R3	4	7	10
R3	3	6	9
R3	3	6	10
CONTROL	0	1	1
CONTROL	1	2	2
CONTROL	1	1	2

Appendix V: Analysis of Variance (ANOVA) of the Mean Toxicity of the Crystal Spore Mixture Derived from Each Isolate Tested

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	226.66667	28.3333	69.5455
Error	18	7.33333	0.4074	Prob > F
C. Total	26	234.00000		<.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	4.3333333	0.122838	35.28	<.0001*
TEST ORGANISMS[C6]	1	0.173719	5.76	<.0001*
TEST ORGANISMS[R3]	2.1111111	0.173719	12.15	<.0001*
TIME PERIOD[TIME 24]	-2	0.173719	-11.51	<.0001*
TIME PERIOD[TIME 48]	0.1111111	0.173719	0.64	0.5305
TEST ORGANISMS[C6]*TIME PERIOD[TIME 24]	-0.333333	0.245676	-1.36	0.1916
TEST ORGANISMS[C6]*TIME PERIOD[TIME 48]	0.2222222	0.245676	0.90	0.3777
TEST ORGANISMS[R3]*TIME PERIOD[TIME 24]	-1.111111	0.245676	-4.52	0.0003*
TEST ORGANISMS[R3]*TIME PERIOD[TIME 48]	-0.222222	0.245676	-0.90	0.3777

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
TEST ORGANISMS	2	2	136.22222	167.1818	<.0001*
TIME PERIOD	2	2	68.22222	83.7273	<.0001*
TEST ORGANISMS*TIME PERIOD	4	4	22.22222	13.6364	<.0001*

Appendix VI: The Connecting Letter Report after Tukey's Honestly Significant Difference Test

Level	Letters	Least Square Mean	Standard Error	Lower 95%	Upper 95%
R3,TIME 72	A	9.666666667	0.368513866	8.892447764	10.44088557
C6,TIME 72	B	7.333333333	0.368513866	6.559114431	8.107552236
R3,TIME 48	B	6.333333333	0.368513866	5.559114431	7.107552236
C6,TIME 48	B	5.666666667	0.368513866	4.892447764	6.440885569
R3,TIME 24	C	3.333333333	0.368513866	2.559114431	4.107552236
C6,TIME 24	CD	3.000000000	0.368513866	2.225781098	3.774218902
SDW,TIME 72	CDE	1.666666667	0.368513866	0.892447764	2.440885569
SDW,TIME 48	DE	1.333333333	0.368513866	0.559114431	2.107552236
SDW,TIME 24	E	0.666666667	0.368513866	0.107552236	1.440885569

Appendix VII: The extracted spore crystal mixture of Isolates C6 (L) and R3 (R) suspended in sterile distilled water

Appendix VIII: Infested Tomato plants

A. The cage; B. Tomato seedlings after two weeks; C. Tomato seedlings after three weeks; D. Artificial infestation of tomatoes plants

Appendix IX: The spraying of the tomatoes leaves

A. Upper surface

B. Lower surface

Appendix X: Toxicity effects of the spore crystal mixture on the larvae of *T. absoluta*

A. Isolate C6
B. Isolate R3