إقـــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Characterization and Bioassay of Different Commercial Products of *B. thuringiensis* Against Four Larval Stages and Adults of an Insect *Tuta absoluta* in Laboratory

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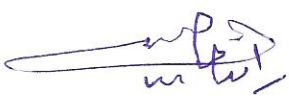

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Characterization and Bioassay of Different Commercial Products of *B. thuringiensis* Against Four Larval Stages and Adults of an Insect *Tuta absoluta* in Laboratory

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نتيجة الحكم على أطروحة ماجستير

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Characterization and Bioassay of Different Commercial Products of *B. thuringiensis* Against Four Larval Stages and Adults of an Insect *Tuta absoluta* in Laboratory

وبعد المناقشة العلنية التي تمت اليوم الاثنين 12 شوال 1434هـ، الموافق 2013/08/19م الساعة الحادية عشرة صباحاً، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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واللجنة إذ تمنحه هذه الدرجة فإنها توصيه بتقوى الله ولزوم طاعته وأن يسخر علمه في خدمة دينه ووطنه.

والله ولي التوفيق،،،

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(قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ)

البقرة 32

DEDICATION

At first I dedicate this work to the souls of all my relatives, especially the martyrs of them in addition to the souls of my ancestors and uncles.

And also dedicate this work to my mother and my father and to my wife and my brothers and sisters who always supported my career and helped me to achieve this degree.

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Characterization and Bioassay of Different Commercial Products of *B. thuringiensis* Against Four Larval Stages and Adults of an Insect *Tuta absoluta* in Laboratory

Abstract

Among the diversity of insect pests affecting on the tomato crop in the Gaza Strip insect (*Tuta absoluta* Meyrick). As the damage caused by the larvae that attack the buds, flowers, and fruits. Larvae prefer clusters leaves and flowers, leading to the loss of these clusters as a whole. For its control, farmers mainly use chemical insecticides, which cause adverse effects to the environment and human health. Biological control of pests comes up as alternative, and among many biological agents, *Bacillus thuringiensis* (Bt) dominate 90% of biopesticide global market.

So it was the aim of this study to characterize and find out the best effectiveness of commercial product effective against insect tomato leafminer *Tuta absoluta*. The first commercial products bacteria *B .thuringiensis var kurstaki* product (Agerin ®) and second bacterium *B. thuringiensis var. israelensis* product (Back tosh ®). The purpose is the protection of tomato crop from danger and threat of use of chemical pesticides on humans and the surrounding environment of Gaza Strip.

The results showed the mean cumulative corrected % mortality After using *B. thuringiensis var kurstaki* bacteria from (Agerin®) product at the highest concentration 5 g\l after six days of exposure it reached 75, 73, 71, 71 % for 1st, 2nd, 3rd and 4th instars, respectively. Also, using of *B. thuringiensis var kurstaki* from (Agerin®) product was affected on the mean cumulative corrected % mortality of adults *T.absoluta* reached 64 % at the highest concentration 5 g\l after three days of exposure. The mean cumulative corrected % mortality after using *B. thuringiensis var. israelensis* from (Back tosh ®) product at the highest concentration 14 ml\l after six days of exposure reached 72, 65, 56, 53 % for 1st, 2nd, 3rd and 4th instars, respectively. Also, the using of *B. thuringiensis var. israelensis* from (Back tosh ®) product was affected on the mean cumulative corrected % mortality of adults *T.absoluta.* It reached 26 % at the highest concentration 14 ml\l after three days of exposure. Therefore, we conclude that the commercial product (Agerin®) is more effective against four larval stages and adult insect *T.absoluta* compared with commercial product (Back tosh®).

الملخص

التشخيص و الفحص الحيوي لأثر بعض المنتجات التجارية المختلفة لبكتيريا *B .thuringiensis*

ضد الأطوار اليرقية الأربعة و البالغات لحشرة Tuta absoluta في المختبر

من بين الآفات الحشرية التي تؤثر على محصول الطماطم في قطاع غزة حشرة عثة الطماطم الأمريكية الجنوبية (***Tuta absoluta* Meyrick**) حيث أن الضرر ناجم عن اليرقات التي تهاجم البراعم و الأزهار والثمار. اليرقات تفضل مجاميع الأوراق و الأزهار ، مما يؤدي إلى فقدان هذه المجاميع بأكملها.
وللسيطرة عليها يستخدم المزارعين بشكل رئيسي المبيدات الحشرية الكيميائية التي تتسبب في آثار سلبية على البيئة و على صحة الإنسان.
والمكافحة الحيوية للآفات تأتي كبديل لها ومن بين العديد من العوامل الحيوية ، *Bacillus thuringiensis* (Bt) و التي تهيمن على 90٪ من المبيدات الحيوية في السوق العالمية.
لذلك كان هدف هذه الدراسة توصيف ومعرفة أفضل تركيز لمنتج تجاري فعال ضد حشرة عثة الطماطم الأمريكية الجنوبية ***Tuta absoluta*** من ***بين منتجين تجاريين الأول بكتيريا*** *B. thuringiensis var kurstaki* من منتج الأجرين (**Agerin** ®) و الثاني ***بكتيريا*** *B. thuringiensis var. israelensis* من منتج باك توش (**Back tosh** ®) بغرض حماية محصول الطماطم من خطرها و خطر استخدام المبيدات الكيميائية على الإنسان و البيئة المحيطة في قطاع غزة.
وأظهرت النتائج الحالية أن متوسط نسبة القتل بعد استخدام بكتيريا *B. thuringiensis var kurstaki* من منتج الأجرين (**Agerin** ®) عند أعلى تركيز5 جرام/ لتر بعد ستة أيام من التعرض وصلت إلى 75، 73، 71، 71٪ للطور الأول و الثاني والثالث و الرابع على التوالي. أيضا، بلغ متوسط نسبة القتل بعد استخدام ذات المنتج ضد بالغات حشرة ***T.absoluta*** عند أعلى تركيز5 جرام/ لتر بعد ثلاثة أيام من التعرض إلى 64٪ .
و أظهرت النتائج أن متوسط نسبة القتل بعد استخدام بكتيريا *B. thuringiensis var. israelensis* من المنتج التجاري باك توش (**Back tosh** ®) عند أعلى تركيز **14** مل /لتر بعد ستة أيام من التعرض وصلت إلى 72، 65، 56، 53٪ للطور الأول و الثاني والثالث و الرابع على التوالي، مقارنة مع معامل السيطرة 0٪ . أيضا، بلغ متوسط نسبة القتل بعد استخدام ذات المنتج ضد بالغات حشرة ***T.absoluta*** عند أعلى تركيز14 مل / لتر بعد ثلاثة أيام من التعرض إلى 26٪ .
و لذلك نخلص إلى أن المنتج التجاري الأجرين هو الأكثر فعالية ***ضد الأطوار اليرقية الأربعة و البالغات لحشرة Tuta absoluta*** من المنتج التجاري الباك توش .

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LIST OF ABBREVIATIONS

Bt : *Bacillus thuringiensis*

Bt i : *B.thuringiensis var. israelensis*

Bt k: *B.thuringiensis var. kurstaki*

°C : degrees Celsius

Cry : crystal

Cyt : cytolytic

et al : and others

FAO : Food and Agricultural Organization

h : hour

g : grams

L: litre

µg : microgram/s

mg : milligram/s

mm : millimeter

NB : nutrient broth

LC50 : Lethal concentration that kills 50% of individuals

LC90 : Lethal concentration that kills 90% of individuals

Subsp : Subspecies

sp : Species

U : Unit

UV : Ultra Violet

WHO : World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Overview

Insects are the most abundant groups of organisms on earth. They often negatively affect humans in a variety of ways. They cause massive crop damage and act as vectors of both human and animal diseases, such as malaria and yellow fever **(Glazer and Nikaido, 1994).**

Therefore, human have desired to control insects. As being parallel to development of chemistry, chemical substances had been started to be used for controlling of pests in the mid 1800s. These chemicals were very effective in killing and controlling of many species of pests **(Glazer and Nikaido, 1994).**

A pesticide is defined as the compound used for a wide variety of purposes to control a range of insects **(Rastrelli *et al.*, 2002).** Pesticides that include insecticides, herbicides, and fungicides are employed in modern agriculture to control pests and to increase crop yield. In both developed and developing countries, the use of chemical pesticides has increased dramatically during the last few decades. Since 1950s, organochlorine compounds, organophosphorus compounds, arsenic and mercury compounds, phenoxy acid herbicides, atrazine, pyrethroids, and dithiocarbamates have been the most popular pesticides **(Dich *et al*., 1997).**

However, Control of pests with chemicals results in several problems. They have many direct and indirect adverse effects on ecosystem including accumulation of toxic residues in nature, leading health problems in mammals and development of insect resistance **(Glazer and Nikaido, 1994).** The problems related with chemical pesticides oriented human to find out safer and natural alternative ways of pest control.

Microbial insecticides offer an alternative to chemical insecticides with increased specificity and safety therefore they are used in integrated pest management programmes **(Anagnou-Veroniki, 1996; Pena and Schaffer, 1997).** These insecticides contain microbial or biochemical agents produced by microorganisms. The advantages of using microbial control agents are their efficiency, safety for humans and other non target organisms, reduction of pesticide residues in food,

preservation of other natural enemies, and increased biodiversity in managed ecosystem **(Lacey and Sigel, 2000).** Also, microbial agents are highly specific against target pests so they facilitate the survival of beneficial insects in treated crops **(Meadows *et al.*, 1992).**

In nature, some microorganisms have the potential to produce some biological agents capable of infecting other living organisms including insects. Many of these infectious agents have a narrow host range and, are not toxic to beneficial insects or vertebrates **(Glazer and Nikaido, 1994).**

Therefore, the use of these non-pathogenic microorganisms have been developed as the biological way of pest control. Insect viruses (baculoviruses), some fungi, protozoa and bacteria have been used as biological pest control agents.

Bacillus among all, *Bacillus thuringiensis* is the most important microorganism with entamopathogenic activity against certain insect orders. It is ubiquitous, gram-positive and spore-forming bacterium which produces insecticidal crystal proteins during sporulation. Natural isolates of *B. thuringiensis* have been used as a biological pesticide since the 1950s for the control of certain insect species among the orders *Lepidoptera, Coloeptera and Diptera* as an alternative to chemical pesticides. This feature makes *B. thuringiensis* the most important biopesticide on the world market **(Bernhard *et al.*, 1997).**

Bt preparations account for 80-90% of world biopesticide market **(Kumar *et al.*, 1996).** By contrast, it represents only 2% of the total global pesticide market with 90$ million worldwide sales **(Lambert and Peferoen, 1992; Schnepf *et al.*, 1998).**

Tomato (*Lycopersicon esculentum* Mill.) is a vegetable crop of large importance throughout the world and it an integral part of human diet world wide. It is a member of the *Solanaceae* family and is a watery fruit containing 5-7 % dry matter. Although it contains relatively low concentrations of vitamin C, pro-vitamin A and minerals, compared to other fruit species it is major source of these nutrients because it is consumed in large quantities **(McGlasson, 2003)**. Tomato is the second most important vegetable crop next to potato. Present world production is about 100 million tons fresh fruit produced on 3.7 million hectares. Tomato production has been reported for 144 countries **(FAOSTAT Database, 2004).**

Its annual production accounts for 152.9 million ton with a value $74.1 billion **(FAOSTAT Database, 2009).**Tomatoes are grown both under plastic covered greenhouses and in open field.

The tomato leafminer, *Tuta absoluta Meyrick, (Lepidoptera : Gelechiidae)* is a serious pest of both outdoor and greenhouse tomatoes. The insect deposits eggs usually on the underside of leaves, stems and to a lesser extent on fruits. After hatching, young larvae penetrate into tomato fruits, leaves on which they feed and develop creating mines and galleries. On leaves, larvae feed only on mesophyll leaving the epidermis intact **(OEPP, 2005).** Tomato plants can be attacked at any developmental stage, from seedlings to mature stage.

Originated from South America, *T. absoluta* was reported since the early 1980s from Argentina, Brazil and Bolivia **(Estay, 2000)**, the insect rapidly invaded many European and Mediterranean countries. It was first recorded from eastern Spain in late 2006 **(Urbaneja, 2007),** then Morocco, Algeria, France, Greece, Malta, Egypt and other countries **(Roditakis *et al*., 2010 ; Mohammed, 2010).**

1.2 Aim of the Study

The aim of this study to find out the best effectiveness of commercial products of *Bacillus thuringiensis var*.(*kurstaki* (Btk) *and israelensis* (Bti)) against tomato leafminer *Tuta absoluta* to protect tomato crops and surrounding environment in Gaza Strip.

1.3 Specific Objectives of the Study

The following specific objectives will be achieved:

1) To isolate *B. thuringiensis* strains from different commercial products**.**

2) To characterize the isolates using classical microbial, and biochemical characteristics.

3) To bioassay different commercial products of *Bacillus thuringiensis* against the different larval stages and adults of tomato leafminer *Tuta absoluta*.

4) To select effective commercial product of *Bacillus thuringiensis* against tomato leafminer *Tuta absoluta* for using it as bio-insecticide to protect tomato crops.

1.4 Significance of the Study

Most of the pests giving damage to crops lands belong to *Lepidoptera Coleoptera,* and *Diptera* and orders. In addition, some species of *Arachnida, Orthoptera, Hymenoptera, and Psocoptera* can also cause damage in stored crops products.

So the importance of this study is the ability to characterize and testing different commercial products of *Bacillus thuringiensis* against tomato leafminer *Tuta absoluta* pest to evaluate their efficacy as bio-insecticides to select the best of effectiveness. For using it rather than chemical insecticides against tomato leafminer *Tuta absoluta* because the Long-term exposure to these chemicals can cause cancer, liver damage, immunotoxicity, birth defects and reproductive problems in humans, and animals. Also, they can cause accumulation and persistence of toxic residues in soil, water and food; toxicity against beneficial insects and development of pest resistance.

CHAPTER 2

LITERATURE REVIEW

2.1 Pests in Crop Lands

Human population is estimated to increase to 7.7 billion by the year 2020 **(United Nations, 1996).** This increased population will cause an increase in the demand for agricultural production. However, the land suitable for agricultural production is limited due to restricted water availability, depletion of land sources and already cultivated highly productive soils. Under these limitations, it is important to develop the yield of agricultural production **(Oerke and Dehne, 2004).**

It has been estimated that upto 15% of crops worldwide are lost due to insect damage only **(Boulter *et al.*, 1989).** Therefore, the need to exterminate insects that are destroying crops becomes urgent. Wheat, rice, maize and barley are the primary source for human nutrition worlwide and cover more than 40% of global cropland **(Tilman, 1999).**

Most of the pests giving damage to these grains belong to *Coleoptera* and *Lepidoptera* orders. In addition, some species of *Arachnida, Orthoptera, Hymenoptera, Diptera and Psocoptera* can also cause damage in stored grain products.

2.2 Pesticides

Early pesticides were the chemical substances. Certain properties made them useful, such as long residual action and effective toxicity to a wide variety of insects. However, the use of them may lead to negative outcomes. The chemical insecticides used today are considered as presumably safer than those used in the past, but there are still some concerns.

Long-term exposure to these chemicals can cause cancer, liver damage, immunotoxicity, birth defects and reproductive problems in humans and animals **(Kegley and Wise, 1998).**

Also, they can cause accumulation and persistance of toxic residues in soil, water and food; toxicity aganist beneficial insects and development of pest resistance **(Marrone and Macintosh, 1993; Van Frankhuyzen, 1993; Glazer and Nikaido, 1994).**

Nevertheless, chemical insecticides have a large market volume, and global sales of them are about 5$ billion a year **(Glazer and Nikaido, 1994).**

By contrast, microbial pesticides are safe for ecosystem. They are non-toxic and non-pathogenic to wildlife and humans. The toxic action of them is often specific to a single group or species of insects, so they do not affect the other insect population in treated areas. Because they have no hazardous residues to humans or animals, they can also be applied when crop is almost ready for harvest **(Neppl, 2000).**

In spite of these attractive features, microbial pesticides represent about 2% of global insecticide sales. *Bacillus thuringiensis* based pesticides account major share of the bioinsecticide market with 80-90% **(Glazer and Nikaido, 1994).**

For several reasons, the use of biopesticides as insecticide has grown slowly when compared with chemicals. Microbial pesticides are generally more expensive to produce than many chemicals. Large quantities of toxins have to be applied to the field to ensure that each larvae will ingest a lethal dose. However, the cost can be decreased by increasing demands. Many chemical pesticides have broad spectrum of toxicity, so pesticide users may consider microbial pesticides with a narrower range to be less convenient. In addition, microbial pesticides kill the insects in a slower speed and thus, this contributes users that they are less effective than the traditional chemical agents **(Glazer and Nikaido, 1994).**

Nevertheless, the use of biological pest control agents have been considered to be much safer than chemical ones for the ecosystem. Moreover, the future prospects of them seem to be positive. It is estimated that, the growth rate of usage of biopesticide use over the next 10 years will be 10-15% compared with 2% for chemical pesticides. Also, the cost of development of *Bacillus thuringiensis* insecticides is predicted to be 3-5$ million, compared with 50-80$ million for chemical insecticides. In addition, the use of chemical insecticides seems likely to decline in the future, restrictions for their registration will increase resulting in a smaller chemical pesticide market **(Navon, 2000).**

2.3 Overview of Tomato

Tomatoes *(Lycopersicon esculentum Mill)* are an integral part of human diet world wide. It ranks third in the world's vegetable production, next to potato and sweet potato, placing itself first as processing crop among the vegetables **(BBS, 2007).** It is a dicotyledonous plant and a member of the *Solanaceae* family and is a watery fruit containing 5-7 % dry matter. Although it contains relatively low concentrations of vitamin C, pro-vitamin A and minerals, compared to other fruit species it is major source of these nutrients because it is consumed in large quantities **(McGlasson, 2003).** The yield of tomato is variable according to the growing conditions, crop duration and the variety; it is between 60 – 120 ton ha-1 **(Vural *et al*., 2000).**

The optimum temperature for tomato production is between 20-27 °C. High and low temperatures cause a reduction in fruit setting. Tomato is not selective in terms of soil requirements, and it can be grown in every type of soil however in light soils production will be earlier than the heavy soils **(Hanson *et al.*, 2001).**

2.3.1 Tomato Taxonomic Classification

Kingdom: *Plantae*

Sub kingdom: *Tracheobiota*

Division: *Magnoliopsida*

Class: *Asteridae*

Order: *Solanales*

Family: *Solanaceae*

Genera: *Solanum*

Species: *S. lycopersicum*

2.3.2 Tomato Pests

Tomato is attacked by a large number of insect pests from seedling to harvest the fruits, although there are hundreds of insects and mites that live in the consumption of this plant, few causing considerable damage, and that in many regions or times make it difficult or almost impossible to culture. Five insect pests that limit production and have led farmers to use chemical insecticides deliberately **(Vallejo, 1999).** Are considered important such as tomato leafminer *(Tuta absoluta)*, whitefly *(Trialeurodes vaporariorum)*, the fruit borer *(Neoleucinodes elegantalis Guenee)*, the

Leaf Miner Liriomyza spp , and aphids: *Aphis gossypii*, *Macrosiphum euphorbiae*, *Myzus persicae.*

2.4 The Tomato Leafminer, *Tuta absoluta*

The tomato leafminer, *Tuta absoluta* was first described by entomologist E. Meyrick (1917) from a male specimen collected in Huancayo, Peru, calling absoluta Phthorimaea **(Rojas, 1981).** The tomato borer, *Tuta absoluta* (Meyrick) *(Lepidoptera: Gelechiidae),* is now found throughout South America, where it is considered to be one of the most devastating pests for tomato crops **(Barrientos *et al.*, 1998; Estay, 2000; EPPO, 2005).** Tomatoes destined to fresh market and processing are affected by this pest throughout the growing cycle, with larvae causing losses of up to 100% by attacking leaves, flowers, stems, and especially fruits **(Lo´pez, 1991; Apablaza, 1992).**

In Spain, this pest was first detected at the end of 2006 in the north of Castello´n (Eastern Spain) **(Urbaneja *et al.*, 2008).** During 2007, *T. absoluta* was detected in several locations throughout the Spanish Mediterranean Basin, the most important tomato growing region in the country. Since then, its presence has also been confirmed in Algeria, Canary Islands, France, Italy, Morocco, and Tunisia in 2008, and in Albania, Bulgaria, Cyprus, Germany, Malta, Portugal, Switzerland, the Netherlands, and the United Kingdom in 2009 **(Desneux *et al.*, 2010; EPPO, 2010).** It is detected for the first time in the province of Khan Younis on 2010 and then spread across the Gaza Strip and has recorded in the West Bank and within the Green Line.

2.4.1 Taxonomic Classification of *Tuta absoluta*

The tomato leafminer belongs to the phylum Arthropoda, class Insecta, order Lepidoptera, suborder Glossata, superfamily Gelechioidea, family Gelechiidae, subfamily Gelechiinae, tribe Gnorimoschemini, and species *Tuta absoluta.*

Table 2.1 *Tuta absoluta* taxonomic classification

Phylum	Arthropoda
Class	Insecta
Order	Lepidoptera
Suborder	Glossata
Superfamily	Gelechioidea
Family	Gelechiidae
Subfamily	Gelechiinae
Tribe	Gnorimoschemini
Genus	*Tuta*
Full Name	*Tuta absoluta* (Meyrick 1917)
Preferred Common Name	tomato leafminer

2.4.2 The Current Management

The current management of *T. absoluta* in the Mediterranean Basin is mainly based on treatments with chemical insecticides. Nevertheless, few active ingredients are effective against *T. absoluta* and selective to beneficials and pollinators at the same time. Therefore, integration with other control methods (cultural, biological, and biotechnological methods) becomes imperative, as the continued use of chemical insecticides could harm non-target organisms (beneficials, users, and consumers) and the environment **(Weisenburger, 1993; Desneux *et al.*, 2007; Landgren *et al.*, 2009)**. Also, prolonged use could lead to resistance **(Devonshire and Field, 1991)** as occurred in this pest's area of origin **(Siqueira *et al.*, 2000, 2001; Lietti *et al.*, 2005).** Therefore, strong emphasis has been placed on implementing environmentally safe measures, like biological control, to manage *T. absoluta* in Spain. Since *T. absoluta* was detected in the Mediterranean Basin, some indigenous parasitoids and predators have been reported to prey on this exotic pest (fortuitous biological control) **(Urbaneja *et al.*, 2008; Arno´ *et al.*, 2009a, b; Cabello *et al.*, 2009a; Molla´ *et al.*, 2009).** Trials using these natural enemies in biological control programs targeting *T. absoluta* are currently underway **(Arno´ *et al.*, 2009b; Cabello *et al.*, 2009b; Molla´ *et al.*, 2009).** Few studies have evaluated the efficacy of *Bacillus thuringiensis* on *T. absoluta*, although 3,000 species, belonging to 16 orders of insects, have been reported as susceptible to *B. thuringiensis* **(Huang *et al.*, 2004).** Commercial formulates based on this bacterium have been used for decades to control insect pests as an alternative to chemicals. Such formulates are environmentally friendly, harmless

to humans and other vertebrates **(Entwistle *et al.*, 1993; McClintock *et al.*, 1995; IPCS-WHO, 2000),** and have shown high compatibility with the use of natural enemies **(Sjoblad *et al.*, 1992; Ferre´ *et al.*, 2008; Lacey and Shapiro-Ilan, 2008).** Furthermore, they are also recommended when insect populations have developed resistance to other products or when treatment is required just before harvest **(Charles *et al.*, 2000).**

2.4.3 Life Cycle of *Tuta absoluta*

Tuta absoluta is a holometabolous insect with a high rate of reproduction. It may be able to complete 12 generations per year depending on environmental conditions **(EPPO, 2005).** In the laboratory (at a constant temperature of 25°C and 75 percent Relative humidity), *Tuta absoluta* completes a generation in 28.7 days **(Vargas, 1970).** Given the field conditions in the Arica Valley in Chile, *Tuta absoluta* could complete seven to eight generations per year at that location **(Vargas, 1970).** Since this pest can infest hosts grown in protected situations (such as greenhouses) its rapid reproductive rate should be kept in mind. The species can overwinter in the egg, pupal, or adult stage **(EPPO, 2005).**

2.4.4 Stages of *Tuta absoluta* Life Cycle

During the life cycle, *T. absoluta* goes through four stages: egg, larva, pupa, and adult. The duration of these stages is directly related to the diet throughout development and environmental factors such as temperature. According to Velez (1997) the main characteristics of the different stages of *T. Absoluta* are:

Eggs: are oval in shape, measuring an average 0.383 mm long by 0.211 mm wide **(Vargas, 1970).** Newly-laid eggs are creamy white and turn yellow and then yellow-orange during development **(Estay, 2000).** Preferentially found on the undersides of the leaves, but can be found anywhere in the plant. Eggs are laid singly (rarely in batches) on short distance between them. The duration of this stage is 4-8 days on average. When mature, eggs turn dark and the outline of the larval head capsule can be seen through the chorion; this is called the blackhead stage **(Vargas, 1970).**

Figure 2.1 The tomato leafminer, *Tuta absoluta* and eggs (Judit Arnó and Rosa Gabarra, 2010)

Larvae: The first two larval instar correspond to the critical phase of the species, which has a high mortality rate (79.8%), this mortality is due mainly to predators and chemical control. larvae have a cycle from 13-23 days. The tomato moth has four larval instars well defined and different in size and color **(Estay, 2000; Fernandez and Montagne, 1990).** The first instar has white and dark brown head. The shape is

cylindrical, slightly flattened dorsoventrally than your head is prognathous (protruding jaw), has five pairs of pseudopodia **(Vargas, 1970).** After hatching, the larvae seek a point of entry into the leaves penetrating the epidermis of the leaf, and in its progress, producing galleries consume cloud **(Fernandez and Montagne, 1990).** The gallery size increases as the larva grows and subsequently oxidize and tissue necrosis **(Larrain, 1992).** The larvae bore into buds, flowers and fruits, but prefer the leaves and flower clusters formation, resulting in loss of a whole cluster **(Lopez, 1991).** Larvae complete four instars that are well-defined and are of different size and color **(Estay, 2000),** but variation in the number of instars is well-documented within any species of *Lepidoptera* . After hatching, larvae enter the plant tissue and begin feeding, thus creating mines. In tomato, young larvae can mine leaves, stems, shoots, flowers, and developing fruit; later instars can attack mature fruit **(Vargas, 1970).** Larval mines increase in length and width as the larva develops and feeds. In cases of

severe attack, all leaf tissue is consumed leaving behind a skeletonized leaf and large amounts of frass. Larvae spin silken shelters in leaves or tie leaves together **(Vargas, 1970).** Head capsule diameter is the best character to differentiate between larval instars. Larvae are dorso-ventrally flattened and their color changes from creamy white to deep green during development . The last instar takes on a pinkish coloration. When larvae are ready to molt they stop eating and purge their stomach contents, causing their coloration to return to creamy white.

Table 2.2 Larval measurements for *Tuta absoluta*

Instar	Body Length, mm		Head Capsule Diameter, mm		Number of Specimens
	Mean	Range	Mean	Range	
1	1.61	1.40–1.90	0.153	0.15–0.18	44
2	2.80	2.45–3.10	0.253	0.24–0.28	37
3	4.69	3.85–5.65	0.399	0.35–0.43	53
4	7.72	5.50–9.20	0.834	0.70–0.98	37

1 Vargas (1970)

First larval stage

Second larval stage

Third larval stage

Forth larval stage

Figure 2.2 All larval stages of *Tuta absoluta* (Judit Arnó and Rosa Gabarra, 2010)

Pupae: The chrysalis or pupa is obtecta type (can differentiate legs and wings) has a cylindrical shape, is wider at the rear than the front end. Its color is green at first and is become a dark brown as it approaches the emergency and they have smooth, shiny texture **(Velez, 1997).** Its dimensions reach 4.35 mm long and 1.10 mm horizontal **(Vargas, 1970).** Most of the time, is covered with white, silky cocoons **(Apablaza, 1992).** At this stage the larva stops eating and forms a cocoon, dropping to the ground by a silk thread to develop the pupal period **(Vargas, 1970),** lasts for 8-15 days after which adults emerge fully formed **(Velez, 1997).**

Mature larvae purge themselves of food and build a silken cocoon where the larva transforms into a pupa. Newly formed pupae are greenish and turn dark brown as they mature **(Estay, 2000).** Male pupae are lighter (3.04±0.49 mg) and smaller (length 4.27±0.24 mm and width 1.23±0.08 mm) than female pupae (4.67±0.23 mg; 4.67±0.23 mm and 1.37±0.07 mm) **(Fernandez and Montagne, 1990).**

Figure 2.3*Tuta absoluta* pupa (Judit Arnó and Rosa Gabarra, 2010)

Adults: are micro *Lepidopteran* about 6 mm long. Their wings are narrow and long, threadlike antennae. The scaly cryptic coloration is dark gray, brown and cream. Adults are nocturnal but have a limited daytime activity. Mating occurs at night after emergence. The oviposition period lasts an average of 4 days. The average longevity of adults is 8.6 days. The sex ratio of males and females is 1 to 3 and the average number of eggs per female is 52, although there is little sexual dimorphism. The males have a gray belly and thin while females have a creamy white belly and wider than that of males. The wing span of females is 9.0 to 13.0 mm while that of males is 8.5 to 12.0 mm **(Vélez, 1997).** The sex ratio in field-collected populations in

Venezuela was 1 male to 1.33 females **(Fernandez and Montagne, 1990).** Adult males live longer than females. In the laboratory, mated males lived 26.47±7.89 days while virgin males lived 36.17±6.55 days. Mated females lived 23.24±5.89 days while virgin females lived 27.81±10.78 days **(Fernandez and Montagne, 1990).** Both genders mate multiple times. The first mating usually occurs the day after adults emerge. Mating occurs at dawn **(Vargas, 1970).** Studies in Chile revealed that the greatest number of males were captured in pheromone traps during the period 7 to 11 a.m., suggesting that this is the time when males are searching for calling females **(Miranda-Ibarra, 1999).**The average preoviposition period for females was 2.4±0.61 days **(Fernandez and Montagne, 1990).** Female fecundity can range between 60 to 120 eggs **(Torres *et al.*, 2001)** but each female can lay up to 260 eggs in a lifetime **(CABI, 2011).** Oviposition studies in laboratories showed that females can lay eggs for more than 20 days, however, 72.3 percent of the eggs were deposited during the first 5 days and 90 percent in the first 10 days **(Fernandez and Montagne, 1990).**

Table 2.3 Wing length of *Tuta absoluta*

Gender	Wing Length (mm)		
	Mean	Range	Specimens
Male	10.10	8.00–11.60	25
Female	10.73	8.00–12.40	27

1 Vargas, 1970.

Table 2.4 Average length of the life cycle of *Tuta absoluta* at different temperatures (Estay, 2000)

Pest Stage	Temperature and Duration (Days)		
	14 °C	20 °C	27 °C
Egg	14.1	7.8	5.13
Larva	38.1	19.8	12.2
Pupa	24.2	12.1	6.5
Total Egg - Adult	76.4	39.7	23.8

2.4.5 Natural Enemies of *Tuta absoluta*

There are three species of *Hymenoptera* that control different stages of the pest: *Trichogramma petiosum* (Riley) and are *exiguum Trichogramma* egg parasitoids Apanteles while *gelechiidivoris* (Marsh) parasitic larvae presenting a preference for

the third instar **(Escobar *et al.*, 2004).** The use of these controllers has been very effective. However, the most used and known entomopathogenic for controlling *lepidopteran* bacterium is *B. thuringiensis*, because the majority of pathogenic strains possess activity against larvae of this order, among other things no damage to the environment.

2.5 Microbial Insecticides

Microbial insecticides offer an alternative to chemical insecticides with increased specificity and safety therefore they are used in integrated pest management programmes **(Anagnou-Veroniki, 1996; Pena and Schaffer, 1997).** These insecticides contain microbials or biochemicals produced by microorganisms. The advantages of using microbial control agents are their efficiency, safety for humans and other nontarget organisms, reduction of pesticide residues in food, preservation of other natural enemies, and increased biodiversity in managed ecosystem **(Lacey *et al.*, 2001).** Also, microbial agents are highly specific against target pests so they facilitate the survival of beneficial insects in treated crops **(Meadows *et al.*, 1992).**

Microbial insecticides are being developed as biological control agents during the last three decades. The widely known and used bacteria as insect pathogens belong to *Bacillus* genus and these are *B. thuringiensis*, *B. lentimorbus*, and *B. sphaericus* **.**The most common biopesticides applied in many agroecosystems are the commercial formulations of Bt **(Entwistle *et al.*, 1993).**

2.6 The Genus *Bacillus*

The genus *Bacillus* is composed of many saprophytic bacteria capable of producing an endospore **(Slepecky and Leadbetter, 1994).** The bacteria belong to this genus are rod-shaped, usually Gram-positive, catalase-positive, and aerobic or facultatively anaerobic **(Thiery and Frachon, 1997).** Most Gram-positive endosporeforming bacteria are soil microorganisms **(Slepecky and Leadbetter, 1994).**

Bacillus has been divided into three morphological groups based on spore shape and swelling of the sporangium **(Gordon *et al.*, 1973).** Group I is characterized by the presence of ellipsoidal spores that do not swell the mother cell **(Priest, 1993).** This group comprises a large number of species living in soil such as *B. thuringiensis*, *B. sphaericus*, *B. subtilis*, *B. anthracis*, and *B. cereus*. Some of these species are very

closely related and form different groups within the group I. One of these subgroups includes the *B. cereus* group.

2.6.1 The *Bacillus Cereus* Group

This group includes *B. cereus, B. mycoides, B. thuringiensis, B. anthracis, B. pseudomycoides,* and *B. weihenstephanensis* **(Chen and Tsen, 2002; Helgason *et al.*, 2000).** Systematists consider the former three species as subspecies of *B. cereus* because they are closely related **(Leonard *et al.*, 1997).**

The genetic and phenotypic characteristics of *B. thuringiensis* are very similar to *B. cereus* **(Toumanoff and Vago, 1951; Priest, 2000).** The only difference between these two species is the formation of large proteinaceous parasporal inclusions observed in *B. thuringiensis.* These inclusion bodies, crystals have unique toxic activities against certain insects and some other invertebrates **(Charles *et al.*, 2000)**, against human cancer cells **(Mizuki *et al.*, 1999, 2000),** and human pathogenic protozoa **(Kondo *et al.*, 2002).**

2.6.2 Bacillus thuringiensis (BT)

Bacillus thuringiensis (BT) is an aerobic, Gram-positive, rod-shaped, spore-forming bacterium. This bacterium has filamentous appendages or (pili) on the spores **(Des Rosier and Lara, 1981; Smirnova *et al.*, 1991; Zelansky *et al.*, 1994).** Colonies have a dull or frosted glass appearance and often undulate margin from which extensive outgrowths do not develop **(Sneath, 1986).**

Under aerobic conditions, Bt grows in a simple culture medium such as nutrient broth. After nutrients are depleted, it produces a spore along with one or several parasporal crystals. There are seven stages during the sporulation. Parasporal protein synthesis starts at about stage II or III of sporulation, and the crystal reaches its maximum size (approximately spore size) by stage V. The crystals are made of proteins varying in size. These crystal proteins are called as δ-endotoxins or insecticidal crystal proteins. When the spore matures, cells lyse. Then, free spores and crystals are released into the environment **(Aronson *et al.*, 1986; Asano *et al.*, 2003).**

2.6.2.1 *Bacillus thuringiensis* Classification

The classic work for identification of Bt began in the early 1960's as a member of family *Bacilliceae*. The classic bacteriological, biochemical and serological methods in addition to insecticidal activity were used to catalog Bt into different subspecies and differentiated between closely related subspecies.

Bt strains are classified into different subspecies, on the basis of their flagellar H-antigen (**de-Barjac and Bonnefoi, 1962**). However, this method was not completely satisfactory especially between Bt subspecies that shared the same serotypes (**Dulmage and Aizawa, 1982**).

The advent of molecular based techniques (total DNA, fingerprinting, plasmid profiling, protein profiling, random amplified DNA PCR-RAPD) have provided new approaches into bacterial classification (**Laemmli, 1970; Ward and Ellar, 1983; Gill *et al*., 1987)**, as all phenotypic characters (morphological, biochemical and others) are the result of gene expression. Strains of Bt are also classified into five pathotypes on the basis of their insecticidal range: *Lepidopteran*-specific (e.g., *var. berliner*); *Dipteran*-specific (e.g., *var. israelensis*); *Coleopteran*-specific (e.g., *var. tenebrionsis*); active against both *Lepidoptera* and *Diptera* (e.g., *var. aizawai*) (**Hofte and Whiteley, 1989**). Recently many reports about the nematicidal activity of Bt have been published (**Schnepf *et al*., 1998; Mozgovaya *et al*., 2002).**

2.6.2.2 Morphological Features of *Bacillus thuringiensis*

B. thuringiensis forms white and rough colonies which spread out and can expand over the plate very quickly. The spores of the organism are elipsoidal, unswollen and lie in the subterminal position in the cell. The best criteria to distinguish *B. thuringiensis* from other *Bacillus* species is the presence of parasporal crystal inclusions which can be easily observed under phase contrast microscope. Morphology, size and number of crystal inclusions may vary among *B. thuringiensis* strains. There are five distinct crystal morphologies: bipyramidal crystals, related to Cry1 proteins; cuboidal crystals, related to Cry2 proteins; amorphous and composite inclusions, associated with Cry4 and Cyt proteins; flat-square crystals, typical of Cry3 proteins; and bar-shaped inclusions, related to Cry4D proteins **(Lopez-Meza and Ibarra, 1996; Schnepf *et al*., 1998).**

2.7 Commercial *Bacillus thuringiensis* Products

2.7.1 Cry Proteins for the Control of Pest Insects and Crop Protection

Bacillus thuringiensis is at present considered to be the prevailing form of biologically produced pest control, and is commonly referred to simply as Bt **(Smith *et al*., 1996).** Back in 1995, worldwide sales of Bt reached $90 million **(Smith *et al*., 1996),** prompting the motion towards a natural alternative to hazardous synthetic pesticides. In 1998, the number of registered *Bacillus thuringiensis* products in the United States alone had almost exceeded the 200 mark. Although time consuming, it has become well recognized that Cry-based pesticides generally have low costs for development and registration. Astoundingly the cost of Bt pesticides is estimated at 1/40th that of a comparable novel synthetic chemical pesticide **(Becker & Margalit, 1993).** The United States is still leading the way with Bt pesticide programs already implicated in areas of forestry. Bt pesticides are used in particular in this field to combat the *gypsy* moth **(Machesky, 1989).** These pesticides are based primarily on the strain *Bacillus thuringiensis HD-1* subsp. *kurstaki* **(Dulmage *et al.*, 1970),** which produces CrylAa, CrylAb, CrylAc, and Cry2Aa toxins. The huge success that was achieved by these projects were reflected in results throughout the forestry world, encompassing more than one pest species. *Bacillus thuringiensis* subsp. *israelensis* has become one of the most effective and potent biological pesticides in attemps to combat mosquitoes and blackflies, insect pests capable of spreading fatal human diseases. Mosquitocidal activity has been identified through tests conducted with Cry2Aa, CrylAb and Cry1Ca **(Haider *et al*., 1986).** Many new uncharacterized isolates containing uncharacterized *cry* genes have also been shown to display mosquitocidal activity **(Ragni *et a*l., 1996).**

2.7.2 Formulations of *Bacillus thuringiensis* Preparats

Commercially available *B. thuringiensis* preparats (Bt preparats) contain both spore and toxic crystal protein (δ-endotoxin). In the production, spores and crystals obtained from fermentation are mixed with the additives including wetting agents، stickers, sunscreens and synergists **(Burges and Jones, 1999).** It is excepted that UV inactivation of the crystal toxin is the major cause for the rapid loss of *B. thuringiensis* activity. Several approaches such as the use of some chromophores to shield Bt preparats against sunlight **(Dunkle and Shasha, 1989; Cohen *et al.*, 1991)** and

enhancing the melanin-producing mutants of the organism, increase UV resistance and insecticidal activity **(Patel *et al.*, 1996).** Besides, encapsulation of *B. thuringiensis* in biopolymers reduce washing of the product from the plant by rain **(Ramos *et al.*, 1998).** In the development of new formulations and optimization of the utilization of biopesticides, knowledge of insect feeding behaviour is a fundamental requirement **(Navon, 2000).** Some formulations used to stimulate feeding, such as the use of a phagostimulant mixture or a yeast extract in a dustable granular form have been proposed to increase residual toxic activity and to attract to the feed selectively on the *B. thuringiensis* product than the feed on the plant **(McGuire and Shasha, 1995; Navon *et al.*, 1997).** These approaches can help to increase the effectiveness of the new *B. thuringiensis* formulations.

2.7.3 Applications of *Bacillus thuringiensis* Preparats

In agricultural use, Bt preparats are mostly applied with ground sprayers. Since high volumes of aqueous spray per unit area are needed for adequate coverage of the plant, ground spraying can be impractical in some cases. In recent years, air spraying have been applied from a helicopter have reduced spray volume and made more effective and beter controlling of the droplets **(Wysokis, 1989).** Also the use of air assisted sleeve boom has increased spray penetration, plant coverage and reduce the drift **(Navon, 2000).** Low persistence of the spore-crystal product on the plant is an important problem in *B. thuringiensis* applications. When the products of *B. thuringiensis* were applied to cotton **(Fuxa, 1989)** and potato **(Ferro *et al.*, 1993),** persistance was observed as 48 hours. Therefore, timing is the major factor for determining the effectiveness of *B. thuringiensis* applications. Application early in the season, according to monitoring egg hatching and after sunset instead of in the morning can increase the persistance of Bt preparats **(Navon, 2000).** Laboratory and field assays have showed that younger larvae are more susceptible to Bt preparats than older ones **(Navon *et al.*, 1990; Ferro and Lyon, 1991).**Therefore, larval age is an important aspect in *B. thuringiensis* applications.

2.7.4 Safety of *Bacillus thuringiensis* Products

The primary advantage of *B. thuringiensis* products is their safety resulting from their selectivity which is affected by several factors. The δ-endotoxins are activated by

alkaline solutions and different varieties may require different pH values. Also, crystals need to be broken down to toxic elements by certain enzymes that should be present in the insect's gut. In addition, certain cell characteristics in the insect gut encourage binding of the endotoxin and leading to pore formation **(Gill *et.al.*, 1992).** Therefore, each strain is capable of producing toxic proteins effective on one or few specific groups of insect. Non-target species such as beneficial insects and wildlife pets are not affected by these toxins. According to oral mammalian toxicology and in vitro digestibility studies which are demanded by the Environmental Protection Agency (EPA), cry proteins (cry1Ab، cry1Ac, cry3A) have not shown toxicity to mammals and they are rapidly degraded in simulated gastric fluid **(EPA, 1998).** Additionally, *B. thuringiensis* toxins are biodegradable and do not persist in the environment **(Van Frankenhuyzen, 1993).**

2.8 Ecological Role of *Bacillus thuringiensis*

B. thuringiensis is mainly a soil bacterium living as both saprophytic, digesting organic matter derived from dead organism , and parasitic, colonizing within living insects **(Glazer and Nikaido, 1994).** It can be present naturally in many different habitats such as soil, stored product dust, insect cadavers, grains, agricultural lands, olive tree related habitats, different plants, and aquatic environments **(Martin and Travers, 1989; Meadows *et al.*, 1992 ; Ben-Dov *et al.*, 1997; Theunis *et al.*, 1998; Bel *et al.*, 1997; Mizuki *et al.*, 1999 ; Iriarte *et al.*, 2000).** The true ecological role of *B. thuringiensis* is poorly understood. Meadows *et al*., 1992 has analyzed *B. thuringiensis* as an entomopathogen, as a phyloplane inhabitant and a soil microorganism. Although it is known that *B. thuringiensis* produces different toxic proteins effective against many different insect orders, some strains show no toxicity **(Maede *et al.*, 2000).**

2.9 Bacillus thuringiensis δ-Endotoxins

Two types δ- endotoxin are produced by Bt strains. They are named Cry and Cyt proteins. Each insecticidal crystal protein is the product of a single gene. The genes synthesize these endotoxins are often located on large, transmissible plasmids. Cry and Cyt proteins differ structurally. The most important feature of these proteins is their pathogenicity to insects and each crystal protein has its distinct host range. The

number and type of δ-endotoxin produced determine the bioactivity of a *Bt* strain **(Crickmore *et al.*, 1995 ; Kumar *et al.*, 1996 ; Schnepf *et al.*, 1998 ; Höfte and Whiteley, 1989).** Based on the amino acid homology, over 300 *cry* genes have been classified into 47 groups and 22 *cyt* genes have been divided into two classes **(WEB_2, 2005).**

2.9.1 The Cry Proteins

Cry proteins are the predominant type. The crystal proteins are encoded by *cry* genes. The accumulation of Cry protein in a mother cell can make up 20-30% of the dry weight of the sporulated cells **(Agaisse and Lereclus, 1995 ; Baum and Malvar, 1995).** Each crystal protein has its own insecticidal spectrum. Therefore, Cry proteins have been classified on the basis of their host specificity and their amino acid compositions. **(Höfte and Whiteley, 1989 ; Schnepf *et al.*, 1998 ; Jensen *et al.*, 2003).** The crystal proteins have different forms such as bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A) **(Schnepf *et al.*, 1998).**

Cry1, Cry2, and Cry9 proteins show strongest toxicity to *Lepidopterans* **(Crickmore, 2000).** Proteins belonging to the class Cry4 and Cry11 are specifically toxic to *Dipterans*. Cry3, Cry7, Cry8, Cry14, Cry18, Cry34, and Cry35 proteins show insecticidal activity against *Coleopterans* **(Ellis *et al.*, 2002 ; de Maagd *et al.*, 2001).** Some Cry proteins on the other hand display toxicity to more than one insect order. For example، Cry1I is both active against *Lepidopterans* and *Coleopterans* **(Tailor *et al.*, 1992)**، whereas Cry1B shows toxicity against *Lepidoptera*, *Coleoptera*, and *Diptera* **(Zhang *et al.*, 2000).**

Table 2.5 Cry proteins with specific activity against an insect order

Cry protein	Susceptible organisms
Cry1	*Lepidoptera*, some have dual activity
Cry2	Dual activity against a *Lepidoptera* and *Diptera*
Cry3	*Coleoptera*
Cry4	*Diptera*
Cry5	*Nematodes* , *Mites* , *Hymenoptera*
Cry6	*Nematodes* , *Mites*
Cry7	*Coleoptera*

Cry8	Dual activity against *Coleoptera* and *aphids*
Cry9	*Lepidoptera*
Cry10	*Diptera*
Cry11	*Diptera*
Cry12	Dual activity against *Diptera* and *Coleoptera*
Cry13	*Nematodes*
Cry14	Dual activity against *Diptera* and *Coleoptera*
Cry15	*Lepidoptera*
Cry16	*Diptera*
Cry17	*Diptera*
Cry18	*Coleoptera*
Cry19	*Diptera*
Cry20	*Diptera*
Cry21	*Nematodes*
Cry22	*Hymenoptera*
Cry23	*Coleoptera*
Cry24	*Diptera*
Cry25	*Diptera*
Cry26	*Diptera*
Cry27	*Diptera*
Cry28	*Diptera*
Cry29	*Diptera*
Cry30	*Diptera*
Cry31	*Diptera*

(Barloy *et al.*, 1996; Zhang *et al.*, 1997)

2.9.2 The Cyt Proteins

Beside Cry proteins, some Bt strains also synthesize cytolytic proteins encoded by cyt genes. Cyt means a parasporal inclusion (crystal) protein from Bt that exhibits hemolytic activity, or any protein that has obvious sequence similarity to a known Cyt protein **(Crickmore *et al.*, 1998).** This class of δ-endotoxins differs in amino acid composition and action mechanism from Cry toxins **(Thomas and Ellar, 1983 ; Höfte and Whiteley, 1989 ; Butko *et al.*, 1997).** These toxins act synergistically with mosquitocidal Cry toxins **(Poncet *et al.*, 1994).** Cyt toxins differ from the Cry toxins;

the protoxin mass of Cyt toxins (30 kDa) is smaller than the Cry toxins **(Thomas and Ellar, 1983 ; Du *et al.*, 1999).**

The Cyt toxins are only found in *Dipteran* specific strains, while the Cry toxins are present in many Bt strains with wide host range. One Cyt toxin is found in a given Bt strain, but two or more subclasses of Cry toxins can exist in a strain. Although both the activated forms of these toxins can lead to pores in lipid bilayers, only the Cyt toxins cause the cytolysis of various eukaryotic cells including erythrocytes **(Gill *et al.*, 1992 ; Knowles *et al.*, 1989 ; Slatin *et al.*, 1990).**

Cyt toxins may be used to overcome insecticide resistance and to increase the activity of microbial insecticides **(Guerchicoff *et al.*, 2001).** Cyt1 and Cyt2 are two cytolytic classes of Cyt toxins that have been identified on the basis of the amino acid identity and are divided into 22 subclasses **(WEB_2, 2005).** Among these subclasses، Cyt1Aa and Cyt2Aa display the highest mosquitocidal activity **(Koni and Ellar, 1994).** Cyt1A may be used as a practical tool to manage resistance against *B. sphaericus*, which is also a mosquitocidal bacterium. Also other Cyt proteins may increase the insecticidal activity of non-Cyt proteins to other insects **(Wirth *et al.*, 2000).**

2.9.3 Classification of Insecticidal Crystal Proteins

In previous nomenclature, crystal proteins are classified as CryI (*Lepidoptera* specific), CryII (*Lepidoptera*- and *Diptera*-specific), CryIII (*Coleoptera*-specific), CryIV (*Diptera*-specific), and CytA for cytolytic proteins (*Diptera*-active) based on their structure and host range **(Höfte and Whiteley, 1989).** This nomenclature replaces with the one proposed by Crickmore (1998). In this nomenclature, Cry proteins are named on the basis of the amino acid similarity to established holotype and Cry proteins showing similar amino acid sequences are grouped together. For example, Cry proteins with the same Arabic number share at least 45% amino acid sequence identity for example Cry4; the same Arabic number and upper case letter at least 75% sequence identity (Cry4B), the same arabic number and upper and lower case letter 95% sequence identity (Cry4Ba).

2.9.4 Toxin Structure

X-ray crystallography has been used to solve the three-dimensional structure of the activated forms of Cry3A, Cry1Aa and Cyt2A toxins **(Schnepf *et al.*, 1998).** Cry

toxins share a similar conformation and a common three-domain structure. The N-terminal domain I, is a bundle of seven α-helices. Six of these helices are surrounded by a central core helix, α-5. Domains II and III contain β-sheets in different conformations. Domain II is formed by three antiparallel β-sheets and has the most variable structure between the Cry toxins. The C-terminal domain III is a β -sandwich of two antiparallel β -sheets. The β -sandwich region provides the structural integrity of the toxin. Domain I is responsible for membrane insertion, structural stability, and pore formation. Domains II and III have functions in receptor recognition and specific binding. Furthermore, domain III is also responsible for modulating ion channel activity **(Schnepf *et al.*, 1998 ; Shimizu and Morikawa, 1996 ; Li *et al.*, 1991; de Maagd *et al.*, 2001).** The C-terminal region of the protoxins contains lysine and cysteine residues that are essential in the assembly and solubilization of the crystals **(Choma *et al.*, 1990).** N-terminal region has carbohydrate functionality that provides the binding of the Cry toxins to glycoproteins like receptors such as N-aminopeptidases or E-Cadherins in the insect midgut **(Burton *et al.*, 1999 ; Angst *et al.*, 2001).**

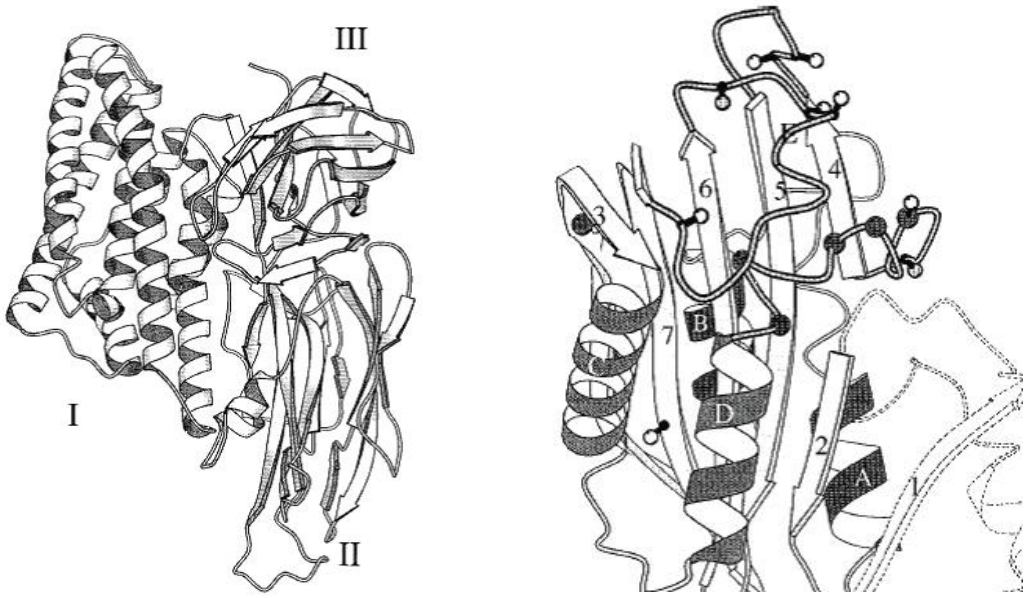
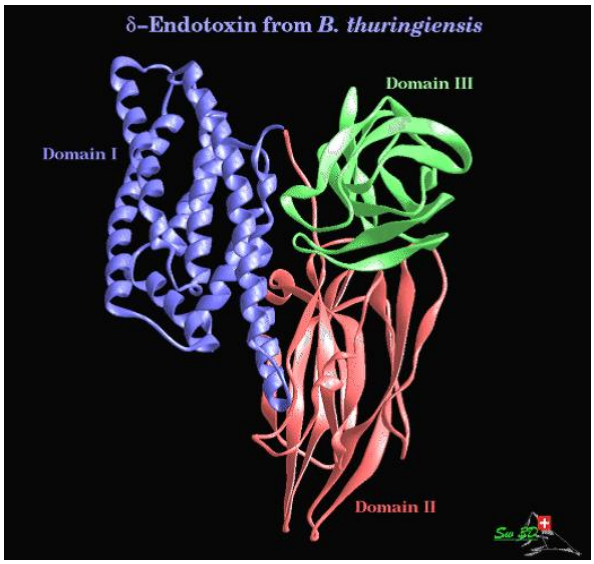


Figure 2.4 Structure of cry1Aa and cytB δ –endotoxin

Cyt2A contains a single domain in which two outer layers of α-helix wrap around a mixed β -sheet. Cyt1A is also thought to have a similar structure **(Schnepf *et al.*, 1998).** Figure 2.6 represents the structure of Cry1A and CytB δ-endotoxins.

A



B

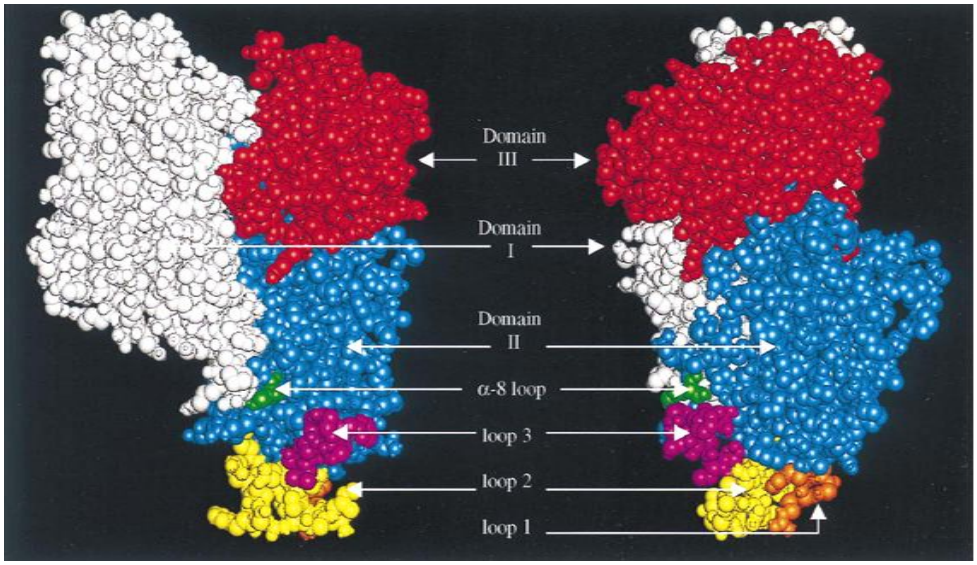


Figure 2.5 : (**A**): Crystal structure of the δ-endotoxin, or insecticidal crystal protein, of *Bacillus thuringiensis* illustrating the three protein domains **(Gutierrez, 2001).** (**B**) 3D structure through two planes indicate the position of the various domains **(Schnepf *et al.*, 1998).**

2.9.5 Action Mechanism of δ-Endotoxins

Crystals are formed as protoxins by Bt. To become active; a suspectible insect must ingest them. After being ingested, the crystals are solubilized in the alkaline environment in the insect midgut (pH>10). After solubilization, midgut proteases convert the protoxins into active toxins. The active toxin binds to specific receptors on the membranes of epithelial midgut cells; this interaction provides the insertion of the toxin into the lipid bilayer and formation of pores (0.5 to 1 nm). As a result, pore formation leads to gut paralysis. Finally, insect larvae stop feeding and die from lethal septicemia **(Aranson *et al*., 1986; Knowles and Ellar, 1987; Höfte and Whiteley, 1989; Lereclus *et al*., 1989; Adang, 1991; Gill *et al*., 1992).** Figure 2.9 illustrates the action mechanism of Cry proteins. The mode of action of Cyt toxins has not been fully determined. It has been suggested that these toxins could also be involved in colloid-osmotic lysis like Cry toxins but the formation of lesions in the cell membrane may be different **(Butko *et al*., 1996; Butko *et al*., 1997; Crickmore *et al*., 1995; Höfte and Whiteley, 1989).** All Cyt toxins react directly with phospholipids without the need for a membrane protein receptor **(Thomas and Ellar, 1983).** Serine proteases such as chymotrypsin, thermolysin, elestase are important in both solubilization and activation of protoxins **(Yang and Davies, 1971; Spiro-Kern, 1974; Borovsky, 1986; Dai and Gill, 1993).** Besides these digestive proteases, a novel DNase from an insect has been found to act synergistically with the crystal protein and to convert it to the active DNA-free toxin in the larval gut **(Clairmont *et al*., 1998; Milne and Kaplan, 1993).** Spores are known to synergize the insecticidal activity of crystals when tested against insects. This may be related to the invasion of haemocele through the ulcerated midgut, and the subsequent development of septicemia **(Li *et al*., 1987).**

The efficiency and potency of Cry toxins to control insects could be increased by the addition of enzyme chitinase in Bt preparations. The chitinase acts on the peritrophic membrane which is composed of a network of chitin and proteins **(Smirnoff, 1973).**

This enzyme hydrolyses the β -1,4 linkages in chitin so it may distrupt the peritrophic membrane by creating holes and facilitates the contact between δ-endotoxins and membrane receptors in the midgut epithelium **(Regev *et al*., 1996).** Some factors such as PH, enzymes, peritrophic membrane, enzyme detoxification, and antimicrobial

characteristics of gastric juice of insect gut make insects resistant to the toxin **(Davidson, 1992).**

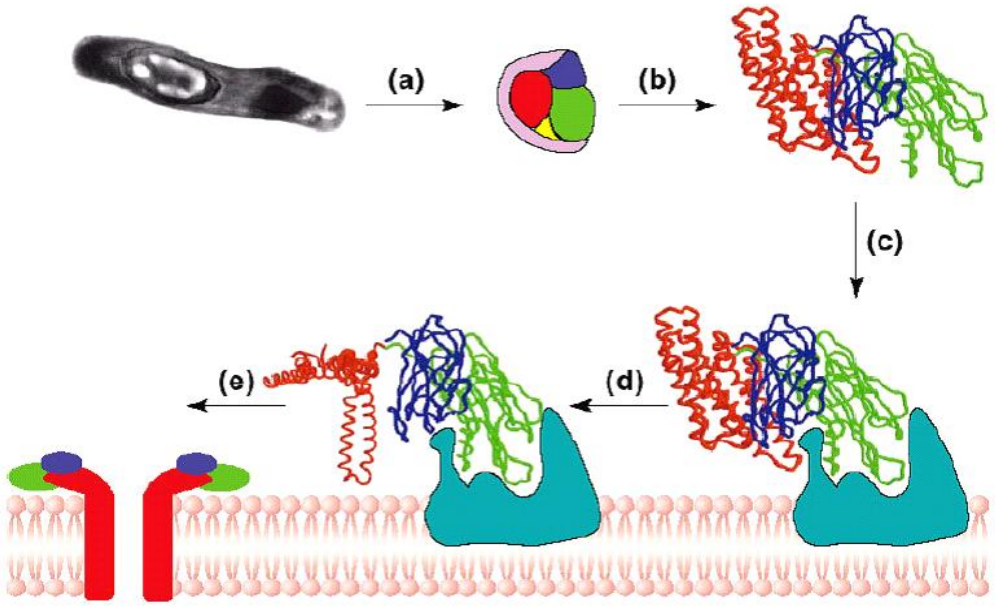


Figure 2.6 Action mechanism of cry δ-endotoxin (Source: de Maagd *et al.*, 2001)

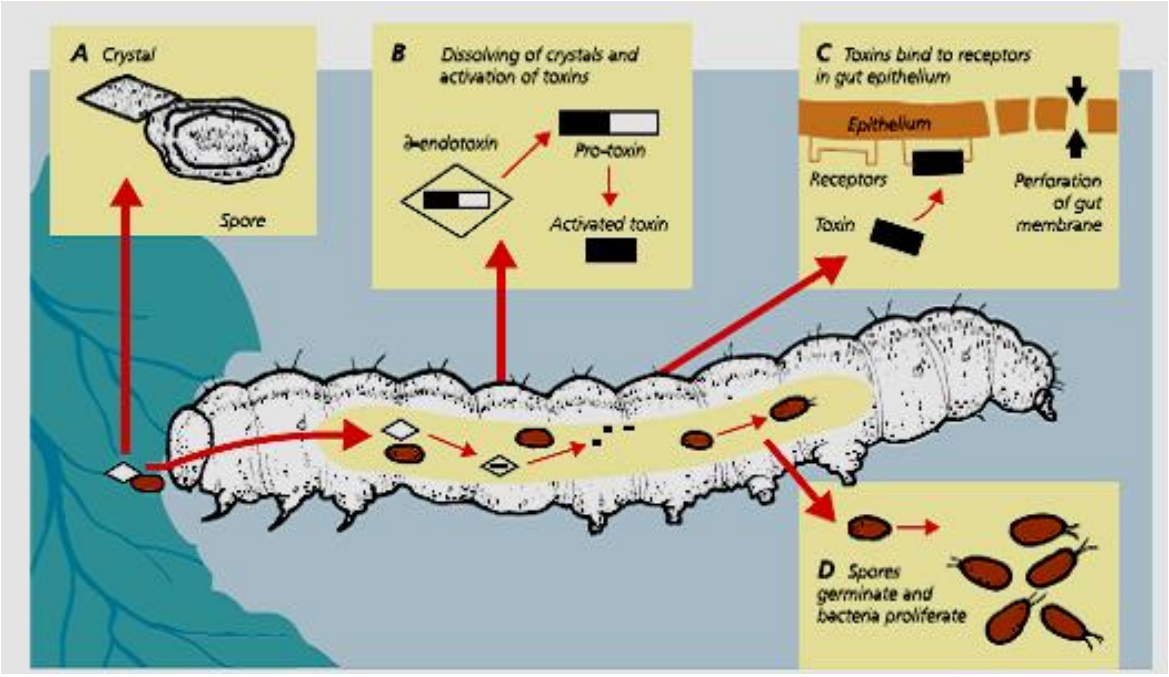


Figure 2.7 Mechanism of toxicity of bt

2.9.6 Insecticidal Spectrum of Bt δ-Endotoxins

More than 3000 insect species included in 16 orders have been found to be susceptible to different crystal proteins **(Lin and Xiong, 2004).** Insecticidal crystal proteins are toxic to insects within the orders *Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga* as well as non-insect organisms such as *nematodes, mites, protozoa, and plathelmintes* **(Feitelson, 1993).** The toxicity is high against the insects belonging to the first three orders.

Lepidopteran and *Coleopteran* insects are leaf-feeders with chewing mouthparts, whereas *Dipterans* feed by filtering water. These two different feeding behaviours provide the possible intake of Bt spores /crystals **(Borror *et al*., 1989).**

2.10 Other Pathogenic Features of *Bacillus thuringiensis*

B. thuringiensis produces various virulance factors other than δ-endotoxins. Vegetative insecticidal proteins (VIP) expressed and secreted during vegetative growth and sporulation, were described as toxic against *lepidopteran* insects **(Estruch *et al.*, 1996).** Beside VIP, a series of extracellular compounds synthesized and contribute virulence, such as δ-exotoxins, phospholipases, proteases, and chitinases **(Levinson,1990 ; Lövgren *et al.*, 1990 ; Zhang *et al.*, 1993 ; Sonngay and Panbangred, 1997).** Also، the spores themselves contribute to pathogenity, often synergizing the activity of the crystal proteins **(Johnson *et al.*, 1996).**

2.11 Previous Studies

In 2001 Giustolin *et al.*, found that *B. thuringiensis var. kurstaki* (Btk) can cause mortality in all *T. absoluta* instars and that the use of Btk has synergistic or additive effects when applied to tomato resistant genotypes.

In 2001 Giustolin *et al.*, expressed the mortality of *Tuta absoluta* (Meyrick) larvae that were fed on leaves of *Lycopersicon hirsutum f. glabratum* (PI 134417, insect resistant) and *Lycopersicon esculentum* (cultivar Santa Clara, susceptible) treated with *Bacillus thuringiensis var. kurstaki* (Btk) was evaluated. Feeding on untreated PI 134417 was detrimental to the survival of *T. absoluta* larvae. When Btk was applied to the two *Lycopersicon* plants, mortality occurred in all *T. absoluta* instars.

Application of Btk on tomato leaves had synergistic or additive effects with the resistant genotype on larval survival. This effect was dependent on the instar at which the larvae were fed Btk-treated leaves. Delayed Btk application may cause higher insect mortality if the insects become more susceptible to the pathogen after a longer period of feeding on the resistant crop.

In 2003 Theoduloz *et al.*, expressed a *B. thuringiensis* toxin in other *Bacillus* species that naturally colonize the phylloplane of tomato plants, showing that these plant-associated microorganisms could be useful as a delivery system of toxins from *B. thuringiensis,* which would allow a reduction in pesticide applications.

In 2005 Reyes *et al*., according to the biochemical and molecular characterizations, 10 native isolates were selected as promising for *Tuta absoluta* control and challenged against 2nd instar larvae. To carry out this procedure five methods were evaluated with three commercial products (based on bioinsecticides crystals and spores of *B. thuringiensis*: Dipel® Turilav® and Xentari® : 1) immersion of the leaves, 2) by spraying airbrush on tomato leaves, 3) Bottles with leaflets of tomato plants, 4) culture medium of tomato extract leaves and 5) plastic containers. The bioassays optimum methodology to try Bt on *Tuta absoluta* was method 1, with immersion of leaves into the evaluated product, (96% control survival and 100% mortality at a 2.5 g/L concentration of commercial product Dipel®. The strain native ZBUJTL39 and ZCUJTL11, showed better biological activity that the reference strain: Bt var *kurstaki* HD1. Strain native ZCUJTL11 presented a LC50 of 2.4 mg/ml ($P<0.05$). Results showed the enormous biodiversity of strain native that could be found in Colombian soil. Apparently the great variability in ecosystems and diversity in insects, both pests and beneficials, determines different coevolution ways to be followed for *Bacillus thuringiensis*. The methodology allows selecting Bt strains according to their potential biological activity, as a preliminary step to bioassays. The native bacillus strain identified with potential to control *Tuta absoluta* is promising for further research leadingt to develop a biopesticide or a genetically engineered tomato, resistant to *Tuta absoluta*.

In 2006 Niedmann and Meza-Basso, performed bioassay screens of native *B. thuringiensis* strains from Chile and found that two of them were even more toxic for *T. absoluta* than the strain isolated from the formulate Dipel® **.**

In 2006 Niedmann *et al.*, the insecticidal potential of native *Bacillus thuringiensis* (Bt) strains against *T. absoluta* was studied. Bt isolates were collected from soil samples of the VII Region of Chile, and characterized using different criteria: colony and parasporal inclusion morphologies, SDS-PAGE, western blotting analysis and bioassays against *T. absoluta* larvae. Two isolates displayed a relevant toxic activity against *T. absoluta* larvae and could constitute an alternative for controlling this pest. These strains proved to be more effective than the isolate obtained from the commercial Dipel® Bt formulation (*B. thuringiensis* var. *kurstaki*).

In 2010 Nannin *et al.*, in order to monitor the population trends of *T. absoluta* in greenhouse tomatoes and to evaluate the effectiveness of the control measures applied by growers, from February 2009 to July 2010 they surveyed several commercial crops grown in a major fresh market tomato production area. During the study period they recorded the number of adults caught by pheromone traps every week and assessed monthly the percentage of infested plants, the mean number of live larvae per plant, and the mortality of 2nd-4th-instar larvae. Finally, for each crop they noted the treatment schedules. The highest numbers of moths caught in traps were observed between April and June and in September-October. Similarly, the highest levels of tomato borer infestation were observed in spring and, to a lesser extent, in autumn. In fact, while in spring *T. absoluta* frequently reached maximum densities of 30-100 larvae/plant, during autumn infestation did not exceed 25 larvae/plant. This may probably explain the intensive application of insecticides recorded in spring. The products most commonly used by growers for pest management were spinosad, abamectin and azadirachtin, but *Bacillus thuringiensis*-based insecticides and indoxacarb were also applied. Evidence of enhanced biological control of the tomato borer by native natural enemies was observed in several crops at the end of the growing period.

In 2010 Cabrera, *et al.*, laboratory, semi field and field trials conducted at The Entomology Unit of IVIA have evidenced the high efficacy of active ingredient *B. thuringiensis* against *T. absoluta*. The pest impact was reduced to minimum levels without chemical treatments. Integration with other biological control methods such as the use of mirid predators should contribute to improve the fruit safety and quality.

In 2011 Gonza´lez-Cabrera *et al.*, the laboratory, greenhouse, and open-field experiments presented in this work are evidence that *B. thuringiensis* is highly efficient in controlling *T. absoluta*. First instar larvae were the most susceptible, while susceptibility was lower in second and third instar larvae. Their results have shown that the impact of *T. absoluta* can be greatly reduced by spraying only *B. thuringiensis*-based formulates, with no need for chemical insecticides. Furthermore, the integration of this technology with other biological control methods focused on *T. absoluta* eggs, such as the use of mirid predators or parasitoids, could reduce the number of *B. thuringiensis* treatments and the use of chemicals, with the consequent reduction of residues on fruits.

In 2011 Ladurner *et al.*, the field studies carried out in Southern Italy, the efficacy of two formulations of Btk strain EG2348, respectively a wettable powder (f.p. Lepinox Plus®) and a suspension concentrate (f.p. Rapax®), was investigated against *T. absoluta*. The suspension concentrate proved to be more effective than the wettable powder.

In 2011 Mollá *et al.*, *B. thuringiensis* formulations were sprayed weekly for two months, three months or throughout the growing cycle, and in all cases, one *N. tenuis* per plant was also released. Control plants were completely destroyed by the infestation levels reached by *T. absoluta*. In contrast, all treatments based on *B. thuringiensis* treatments and releases of *N. tenuis* reduced leaf damage by more than 97% when compared to the untreated control, with no significant differences among them. Furthermore, yield in the control plants was significantly reduced when compared with all Bt–*N. tenuis* treatments. Their results demonstrate that when *B. thuringiensis* treatments are applied immediately after the initial detection of *T. absoluta* on plants, they do not interfere with *N. tenuis* establishment in the crop because *T. absoluta* eggs are available. According to their data, treatments with *B.*

thuringiensis later in the growing season would no longer be necessary because mirids alone would control the pest.

In 2011 Gonza´lez-Cabrera *et al.*, expressed the laboratory, greenhouse, and open-field experiments presented in this work are evidence that *B .thuringiensis* is highly efficient in controlling *T. absoluta*. First instar larvae were the most susceptible, while susceptibility was lower in second and third instar larvae. their results have shown that the impact of *T. absoluta* can be greatly reduced by spraying only *B. thuringiensis*-based formulates, with no need for chemical insecticides.

In 2012 Sellami *et al.*, they screened a set of 212 *B. thuringiensis* strains to search the higher insecticidal activities. These strains had bipyramidal and cubic crystal morphologies and 30% of them showed PCR amplification of *vip3* internal region, from which five isolates (S1/4, S17, S122, S123 and S144) showed plasmid profile variability. These five strains contained the *cry1I*, *cry1Aa* and/or *cry1Ac*, *cry1Ab* and *cry2* genes, and S1/4 harbored in addition the *cry1C*, *vip1* and *vip2* genes. They produced from 25 to 46 µg δ-endotoxin/10^7 spores. Their δ-endotoxins displayed distinct lethal concentrations 50% against either *Spodoptera littoralis* or *Ephestia kuehniella* larvae with the lowest one for S1/4, which was also active against *Tuta absoluta*. Fortunately, the analysis of the culture supernatants revealed that S1/4 had the higher toxicity towards these *lepidopteron* but it didn't show any toxicity against the *Tribolium castaneum coleopteran* larvae; additionally, S1/4 displayed an antibacterial activity. S1/4 is a good candidate for agricultural pest control, as it is more efficient than the reference strain HD1.

In 2012 Aziz *et al.*, conducted experiment to evaluate the effect of bacteria *Bacillus thuringiensis* in characters of *Tuta absoluta*. Results showed that using bacteria *Bacillus thuringiensis* due to significant decrease in egg hatching percentage to 33.36% compared with control treatment which gave 86.74%, meanwhile, the bacteria was using due to increase on killing ratio of larval instars and make it reached to 65.92, 66.55, 64.98, 70.78% for 1st, 2nd, 3rd and 4th instars, respectively, compared with control treatment which have 0% of mortality ratio. Also, the using of *B .thuringiensis* bacteria was affected on mortality ratio of adults of *T.absoluta* and gave

78.75% compared with control treatment which did not appear any mortality ratio for insect adults.

In 2012 Aziz *et al.*, conducted a series of laboratory and field experiments to determine the effects of biological control agents such as *Bacillus thuringiensis* and *Beauveria bassiana* and chemical control elements such as Match, Proteus, King Bo, Evisect and Phytomax insecticides on some biological performance of *T.absoluta*. The compatibility between the biological and chemical control agents was studied, too. The results showed that:

The biological control agents used in laboratory study had significant effect in decreasing of egg mortality percentage of *T.absoluta*; the treatment with *B.thuringiensis* (3200 IU/L concentration) gave 66.37%, while the treatment with *B.bassiana* (50% concentration) gave 63.16%. The mortality percentage of 1st, 2nd, 3rd and 4th larval instars treated was 74.90, 46.36, 42.69 and 49.91% respectively, respectively.The bioagents used in this study were also had significant effect on adults killing percentage which gave 78.75 and 90.45% in both bacteria and fungus treatments, respectively.

Lucia Zappalà, Antonio *et al.*, several commercially available strains of the entomopathogenic bacteria *Bacillus thuringiensis* (Bt) were tested to evaluate their efficacy in controlling *Tuta absoluta,* in terms of larval mortality, reduction in larval feeding activity and subsequent damage. Tests were conducted on *T. absoluta* young larvae under laboratory and extended laboratory conditions, both after ingestion and topical application. The mortality rate, the number of infested leaflets and the amount of infested surface per each leaflet were recorded. The formulation containing the strain kurstaki SA12 resulted the most effective in controlling *T. absoluta* in terms of induced mortality as well as damage reduction, both in the topical and ingestion toxicity trial. Similar trend, although without significant differences, was recorded in the extended laboratory trial.

M.M. Sabbour the three microbial control agents *Bacillus thuringiensis var. kurstaki* (Bt), *Beauveria bassiana* (Bb) and *Metarhizium anisopliae* (Ma) were tested against tomato pinworm *Tuta absoluta (Lepidoptera: Gelechiidae)* under laboratory and greenhouse conditions. The results showed that under laboratory conditions, the LC50

values was 243.9 Ug/ml for Bt, 129.4 × 10^4 spores/ml for Bb, and 98.7 × 10^4 spores/ml for Ma. Under greenhouse conditions, the LC50 values corresponding to microbial control agents were 211Ug/ml (Bt), and 102 × 10^4 (Bb) and 100×10^4 (Ma) spores/ml.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Apparatus

The apparatus that were used are listed in Table 3.1 below.

Table 3.1 List of the apparatus used in this work

Apparatus	Manufactures
Vortex mixer	Labret's VX-1OO (USA)
Incubator	Memmert (Oxford)
Shaker	Memmert (Oxford)
Autoclave	Tuttnauer (USA)
Refrigerator	UGUR (Turkey)
Light microscope	Olympus cx21
Digital camera	Hp (China)
Safety cabinet	Walker(USA)
Hot plate	Scientific company (China)
PH meter	Scientific company (China)
Electronic balance	Scientific company (China)

3.1.2 Equipments

Inoculating needle
Aluminum paper
Cotton
Filter paper
Tissue paper
Parafilm
Plastic droppers
Plastic Petri plates
Plastic tubes
Sterile cotton
Labels

3.1.3 Stains

Table 3.2 List of the stains used in this work

Stain	Manufactures
Gram stain kit	Hi Media Company, India
Malachite green stain	Hi Media Company, India
Coomassie Blue stain	Hi Media Company, India

3.1.4 Reagents

Table 3.3 List of the reagents used in this work

Reagents	Manufactures
Sterile distilled water	Islamic University Lab
Hydrogen peroxide (3%)	Hi Media Company, India
Glycerol	Hi Media Company, India
Ethanol	Hi Media Company, India
Acetic acid	Hi Media Company, India
Oil Immersion	Hi Media Company, India
Potassium nitrate	Hi Media Company, India
Zinc powder	Hi Media Company, India
α- naphthylamine	Hi Media Company, India
Sulphanilamide	Hi Media Company, India
gram's iodine	Hi Media Company, India

3.1.5 Culture Media

Table 3.4 List of the culture media used in this work

Culture Media	Manufactures
Nutrient agar medium	Hi Media Company, India
Nutrient broth medium	Hi Media Company, India
Nutrient broth containing 50 % glycerol	Hi Media Company, India
Simmons citrate agar medium	Hi Media Company, India
Starch nutrient agar medium	Hi Media Company, India
Milk agar medium	Hi Media Company, India
Motility agar medium	Hi Media Company, India
Egg Yolk Nutrient Agar medium	Hi Media Company, India
7% Nacl Nutrient Agar medium	Hi Media Company, India
Peptone	Hi Media Company, India
Beef extract	Hi Media Company, India

3.1.6 *Bacillus thuringiensis* Commercial Products

Table 3.5 List of the commercial product used in this work

commercial product	Manufactures
(Agerin®) Bt k product	Biogro international – Egypt
(Back tosh ®) Bt i product	Bio dalia – technologies company Israel

3.1.7 Source of Tomato Leafminer, *Tuta absoluta*

Tomato leafminer, *Tuta absoluta* (larval stages and adult insect) were collected from greenhouses planted tomatoes infected with *Tuta absoluta* pests in the Gaza Strip .

3.1.8 Source of Bacterial Strains

Two bacterial strains of *B. thuringiensis* will be used in this study. The first one *B. thuringiensis var. kurstaki* will be isolated from the commercial product (Agerin ®) produced by company biogro international – Egypt as package powder container spores and crystals with concentration of 32000 IU \ mg

The second bacterial strain is *B. thuringiensis var. israelensis* will be isolated from the commercial product (Back tosh ®) product by bio dalia – technologies company Israel as a bottle container spores and crystals with concentration of 1200 AAU \mg.

3.2 Isolation of *Bacillus thuringiensis* Strains from Different Commercial Products

to isolate *B. thuringiensis* Strains *(Bt k , Bt I)* from different commercial products samples (Btk Agerin product , Bti Back tosh product) Approximately, 0.25 g of each product samples will be suspended in test tubes containing 10 ml nutrient broth. Next, suspensions will be vortexed vigorously and incubated overnight at 37 ℃ in the incubator. The samples will be plated on nutrient agar plates, which will be incubated overnight at 35 ℃. Finally, bacterial colonies will be separated by their colony morphology. The colonies, which showed *B. thuringiensis*-like colony morphology will be rough, white and spread out over the plate. These colonies will be subcultured on nutrient agar plates and incubated for 48h at 35 ℃ to check the presence of crystals protein beside bacterial spores by light microscopy. For this purpose crystal protein will be stained by Coomassie blue, and endospore will be stained by Malachite green.

3.3 Characterization

The characterization of the isolates from the commercial product was carried out according to bergay‘s manual as shown in Table 3.6.

3.3.1 Morphological Characteristics

3.3.1.1 Examination of Crystal Protein

The isolates were grown on Nutrient agar medium for 48 h at 37 ℃. The cells were suspended in drop of sterile distilled water on the microscope slide . The slide was

heat-fixed smears were prepared and stained with Coomassie blue stain (0.133% Coomassie blue stain in 50% acetic acid) rinsed with distilled water, dried, and observed with bright field microscopy using a 100 x oil immersion objective. The presence of parasporal bodies was immediate and strikingly evident by the presence of numerous dark blue staining objects.

3.3.1.2 Gram Stain

This staining procedure, developed in 1884 by the Danish physician Christian Gram, is the most important procedure in microbiology. It separates most bacteria into two groups: the gram-positive bacteria, which stain blue, and the gram-negative bacteria, which stain red. The Gram stain involves the following four-step procedure was carried out according to the procedures as follow:

1-The crystal violet dye stains all cells blue/purple.

2-The iodine solution (a mordant) is added to form a crystal violet–iodine complex; all cells continue to appear blue.

3-The organic solvent, such as acetone or ethanol, extracts the blue dye complex from the lipid-rich, thin-walled gram-negative bacteria to a greater degree than from the lipid-poor, thick-walled gram-positive bacteria. The gram-negative organisms appear colorless; the gram-positive bacteria remain blue.

4-The red dye safranin stains the decolorized gram-negative cells red/pink; the gram-positive bacteria remain blue **(Tortora *et al.*,2010) .**

3.3.1.3 Endospore Stain

The spore stain is a differential stain used to detect the presence and location of spores in bacterial cells. Only a few genera produce spores. Among them are the genera *Bacillus* and *clostridium* **.**

3.3.1.3.1 Principle of the Endospore Stain

An endospore is a dormant form of the bacterium that allows it to survive poor environmental condition. Spores are resistant to heat and chemicals because of a tough outer covering made of the protein keratin. The keratin also resistant staining, so extreme measures must be taken to stain the spore. In the Schaeffer-Fulton method

1 – Cells and spores prior to staining are transparent.

2- After staining with malachite green, cells and spores are green. Heat is used to force the stain into spore, if present.

3- Decolorization with water removes stain from cells, but not spores.

4- Safranin is used a counterstain cells **(Michaael *et al.*,2005) .**

3.3.1.4 Motility Determination

Motility test medium is a semisolid medium designed to detect bacterial motility. Its agar concentration was reduced from the typical 1.5% to 0.4% just enough to maintain its form while allowing movement of motile bacteria. It is inoculated by stabbing with a straight transfer needle. Motility is detectable as diffuse growth radiating from the central stab line **(Benson, 2001).**

3.3.2 Biochemical Characterization of *Bacillus thuringiensis*

Table 3.6 Biochemical characterization of *Bacillus* species (Bergay's manual).

species	Motlity	Catalase production	Parasporal bodies	Lipid globules in protoplasm	Lecitnovitellin reaction	Citrate utilization	Anaerobic growth	V-P reation	PH in V-P medium 60	Growth at 50°C	Growth at 60°C	Growth in 7%Nacl	Acid from AS glucose	Acid +gas from AS glucose	Nitrate reduction	Casein hydrolysid	Starch hydrolysis	Propionate uilization
Morphologic group 1																		
B megaterium	V	+	-	+	-	+	-	-	V	-	-	+	+	-	V	+	+	n
B cereus	+	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	n
B cereus subsp mycoides	-	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	n
B anthracis	-	+	-	+	+	V	+	+	+	-	-	+	+	--	+	+	+	n
B thuringiensis	+	+	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	n
B licheniformis	+	+	-	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+
B subtilis	+	+	-	-	-	+	-	+	V	V	-	+	+	-	+	+	+	-
B pumilus	+	+	-	-	-	+	-	+	+	V	-	+	+	-	-	+	+	-
B firmus	v	+	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-
B coagulans	+	+	-		-	V	+	+	+	+	V	+	+	-	V	V	+	-
Morphologic group 2																		
B polymyxa	+	+	-	-	-	-	+	+	V	-	-	-	+	+	+	+	+	n
B macerans	+	+	-	-	-	V	+	-	+	+	-	-	+	+	+	-	+	n
B circulans	V	+	-	-	-	V	V	-	+	V	-	V	+	-	V	V	+	n
B stearothermophilus	+	V	-	-	-	-	-	-	+	+	-	-	+	-	-	V	+	n
B alvei	+	+	-	-	-	-	+	+	+	-	+	-	+	-	+	+	+	n
B laterosporus	+	+	-	-	+	-	+	-	+	-		-	+	-	V	+	-	n
B brevis	+	+	-		-	V	-	-	-	V	V	-	+	-	+	+	-	n
Morphologic group 3				-														
B sphaericus	+	+	-		-	V	-	-	-	-	-	V	+	-	-	V	-	n

The basic characteristics which have been used for the identification of selected *Bacillus species*

Key of abbreviations: V-P, Voges-Proskaure, AS ammonium salt; +,v, variable; n, test not applicable; (+), under colony which must be scraped off see positive reaction.

3.3.2.1 Catalase Test

Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide to water and oxygen. The presence of catalase is important in the prevention of toxic byproducts of oxygen metabolism that can kill the cell. Isolates were grown on nutrient agar at 37 ℃ overnight. Catalase activity was detected by adding a drop of 3% hydrogen peroxide solution onto an isolated colony. Immediate and vigorous bubbling indicated a strong catalase reaction whereas scant or no bubble formation indicated a negative test **(Michaael *et al.*, 2005).**

3.3.2 .2 Lecithinase Activity

Lecithinase activity was detected by spotting or streaking a very small amount of bacteria onto the center of the nutrient agar plate supplemented with egg yolk emulsion (100 ml/L). After incubation for up to 48 h at 37 ℃, lecithinase-producing isolates were determined by the formation of white precipitate around the colonies **(Michaael *et al.*, 2005) .**

3.3.2 .3 Amylase Activity

A plate of starch-nutrient agar plate was streaked once with the organism. After incubation for 24 h at 37 ℃, plates were flooded with 5-10 ml of Gram's iodine solution. Any clear area around the growth of the culture indicated the breakdown of starch by the organism due to its production of amylase. Unhydrolyzed starch formed a blue color with the iodine **(Michaael *et al.*, 2005).**

3.3.2 .4 Citrate Utilization Test

This test is used to study the ability of an organism to utilize citrate present in Simmon's media ($MgSO_4$-0.2g/l, $NH_4H_2PO_4$-1g/l, K_2HPO_4-1g/l, sodium citrate-2g/l, NaCl-5g/l, Bromothymol blue-0.08ml, agar-15g/l, distilled water-1liter) as a sole source of carbon for growth. The Simmon's media was prepared, sterilized and poured into the tubes. The media was allowed to cool and solidify in the form of slants. The slant was then stabbed with the isolated bacterial sample using a needle loop and incubated at 37°C for 36 hours **(Michaael *et al.*, 2005).**

3.3.2 .5 Nitrate Reduction Test

The enzyme nitrate reductase reduces nitrate to nitrite, ammonia, nitrous oxide, nitrogen, etc.This test is used to detect the production of nitrate reductase. The isolated test organism was inoculated in 5ml of nitrate broth (beef extract-3g/l, peptone-5g/l, NaCl-5g/l, potassium nitrate- 1g/l, distilled water-1 liter) and incubated at 37°C for 96 hours. Then we added 1ml of α- naphthylamine reagent and 1ml of sulphanilamide reagent and the results were immediately read **(Michaael *et al.*, 2005).**

3.3.2 .6 Growth on 7% NaCl Test

This test determines whether the microbe can grow on a nutrient medium where the concentration of sodium chloride (NaCl) is 7%. If the microbe can grow in the presence of 7% NaCl, the broth will become turbid (cloudy) after incubation. If an agar plating medium is used, growth is indicated by appearance of bacterial colonies following incubation. An inoculum from a pure culture is transferred aseptically to a sterile plate of 7% NaCl nutrient agar and streaked for isolation across its surface. The inoculated plate is incubated at 35-37 °C for 24 hours. A positive test is indicated by the presence of colonies on the streak marks placed on the agar surface **(Michaael *et al.*, 2005).**

3.4 Preparation of Bacterial *B. thuringiensis* Concentrations

For commercial product (Agerin ®) of *B. thuringiensis var. kurstaki* bacteria has been prepared in several different concentrations by dissolving (1 - 2 - 3- 4 - 5 g) in 1000 ml of water respectively .

As for the commercial product (Back tosh ®) of *B. thuringiensis var. israelensis* bacteria has also been prepared in several different concentrations by dissolving (6 - 8 - 10 - 12 -14 ml) in 1000 mL of water respectively.

3.5 Toxicity Test

The larvae were classified according to their metamorphic cycle in 1st, 2nd, 3rd and 4th instars and adults of the tomato leafminer, *Tuta absoluta*. The instars larvae and adults of the tomato leafminer, *Tuta absoluta*, which was reared in the laboratory for several generations away from any insecticide contamination, was used in toxicity

tests to evaluate the efficacy of the different commercial products of *B. thuringiensis* to determine their lethal concentrations.

3.6 Effect of Commercial Products of *B. thuringiensis* on the Different Larval Stages of *T.absoluta* in the Laboratory (Bioassay).

The infected tomato leaves were collected from greenhouse of tomato plants then the larval stages were classified according to their metamorphic cycle in 1st, 2nd, 3rd and 4th instars depending on the color and size of larvae.

We put ten larvae from each instar stage in one petri dish contains a leaf that had already dipped in diffrent concentrations of commercial product of *B. thringiensis* .The different concentrations of commercial products were bioassayed against 1st, 2nd, 3rd and 4th instars and adults of the tomato leafminer, *Tuta absoluta*, using the leaf dipping technique **(Makkar and EI Mandrawy, 1996)** under their preferable natural conditions. For testing the effectiveness of commercial products, on fresh tomato leaves were washed with tap water followed by rinsing in sterile water and then left to dry for 10 minutes. Plant leaves were dipped in commercial bacterial suspensions containing different concentrations. The tested plant leaves were left to dry for 10 min, then placed into Petri dishes. Ten larvae of each stage for each concentration were put in each Petri dish , then the Petri dish were placed at room condition held at *25*°C ± 2°C for 2-4-6 days. Three replicates were used for each commercial product, for control, plant leaves were treated with distilled water only.

3.7 Effect of Commercial Products of *B. thuringiensis* on the Adult Insect of *T.absoluta* in the Laboratory (Bioassay).

The adult insects of *T.absoluta* were brought and put in plastic jars so that each jar contains a leaves of tomato plant that dipped in specific concentration of commercial product of *B. thringiensis* and ten adult insects *T. absoluta* .

The different concentrations of commercial products were bioassayed against adult insects of the tomato leafminer, *Tuta absoluta*, using the leaf dipping technique **(Makkar and EI Mandrawy, 1996)** under their preferable natural conditions. For testing the effectiveness of commercial products, on fresh tomato leaves were washed with tap water followed by rinsing in sterile water and then left to dry for 10 minutes. Plant leaves were dipped in commercial bacterial suspensions containing different

concentrations. The tested plant leaves were left to dry for 10 mints, then placed into plastic jar. Ten adult insects *T.absoluta* for each concentration were put in each plastic jar. For feeding the adults, a piece of cotton wetted with sugar solution (10%) mixed with different concentrations of commercial products was used. The plastic jars, were placed at room condition held at *25*°C ± 2°C for 1-2-3 days. Three replicates were used for each commercial product. For control, plant leaves were treated with distilled water only and a piece of cotton wetted with sugar solution (10%) was used for feeding the adults.

3.8 Evaluation of Lethal Concentrations (LC) Values.

The tested different commercial products of *B. thringiensis* were used at 6 different concentrations to evaluate LC-values. The bioassay was done as previously mentioned in 3.6 then the percentage of mortality was recorded and corrected according to **Abbott's formula (1925).** Then, LC50 and LC90 values were determined.

3.8.1 Larvae Mortality and LC-Values Calculations

Assessment of larval mortalities was expressed as corrected percentage of total larvae of each treatment, as well as in the control, after 2 up to 6 days daily. Corrected mortality was calculated according to Abbott's formula **(Abbott, 1925),** as follows:

Corrected mortality (%) = (T -C) ⁄(10-C)x100 Where,

T = No. of dead larvae in treated replicates.

C = No. of dead larvae in control replicates.

LC50 = the lethal concentration that kills 50 % of larval individuals.

LC90 the lethal concentration that kills 90 % of larval individuals.

3.8.2 Adults Mortality and LC-Values Calculations

Assessment of adults mortalities was expressed as corrected percentage of total adults of each treatment, as well as in the control, after 1 up to 3 days daily. Corrected mortality was calculated according to Abbott's formula **(Abbott, 1925),** as follows:

Corrected mortality (%) = (T -C) ⁄(10-C)x100 Where,

T = No. of dead adult in treated replicates.

C = No. of dead adult in control replicates.

LC50 = the lethal concentration that kills 50 % of adult individuals.

LC90 the lethal concentration that kills 90 % of adult individuals.

3.9 Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 19. Statistical comparison of data was carried out using one-way analysis of variance (**ANOVA**). For all analyzed P value less than 0.05 was considered significant.

CHAPTER 4

RESULTS

4.1 Isolation of *Bacillus thuringiensis* Strains from Different Commercial Products

Two different bacterial strains were isolated from two different commercial products samples by the enrichment culture technique as follows:

1. B. thuringiensis var kurstaki from (Agerin®) product.

2. B. thuringiensis var. israelensis from (Back tosh ®) product.

Two isolates were bioassayed against the different larval stages and adults of tomato leafminer, *Tuta absoluta.*

B. thuringiensis var kurstaki ***B. thuringiensis var. israelensis***

Figures 4.1 *B. thuringiensis var kurstaki* and *israelensis* culture in petri dishes.

4.2 Morphological Characteristics

4.2.1 Examination of Crystal Protein

For testing the presence of the proteinaceous parasporal inclusion bodies, optical microscope was employed. So, after 72 hr incubation of culturing each of the previous isolate, previously prepared from single colonies, were examined by optical microscope by staining with Coomassie blue stain .The crystalline body of the *B. thuringiensis* strains could be detected dark blue.

4.2.1.1 B. thuringiensis var kurstaki

Strain produce parasporal crystal inclusions bodies are apparent; bipyramidal crystals. Bipyramidal shaped crystals are related to Cry 1 proteins that are toxic against *lepidopteran* species.

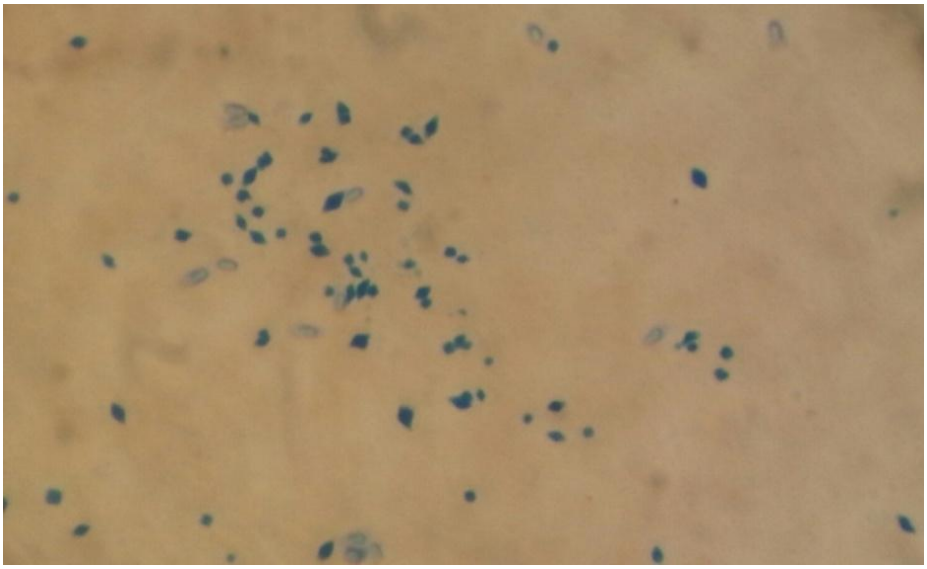


Figure 4.2 Crystal proteins of *B. thuringiensis var kurstaki*.

4.2.1.2 B. thuringiensis var. israelensis

Strain produce parasporal crystal inclusions bodies are apparent; spherical crystals. Spherical shaped crystals are related to Cry4 and Cry11 proteins are specifically toxic against *Dipterans* species.

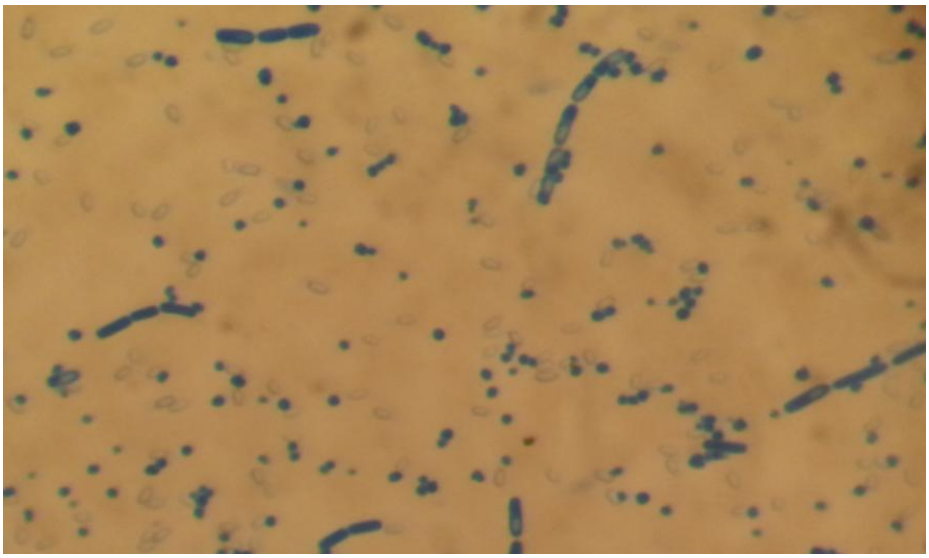


Figure 4.3 Crystal proteins of *B. thuringiensis var israelensis*.

4.2.2 Gram Stain

The two bacterial isolates are gram positive rod shaped as seen in Figures 4.4 and 4.5.

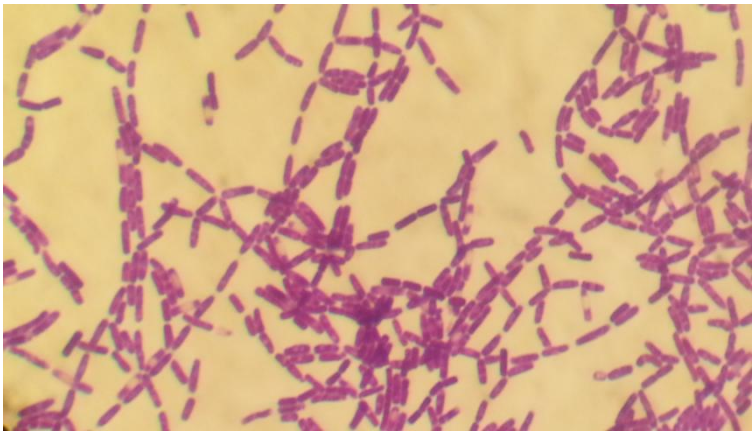


Figure 4.4 *B. thuringiensis var kurstaki* gram stain.

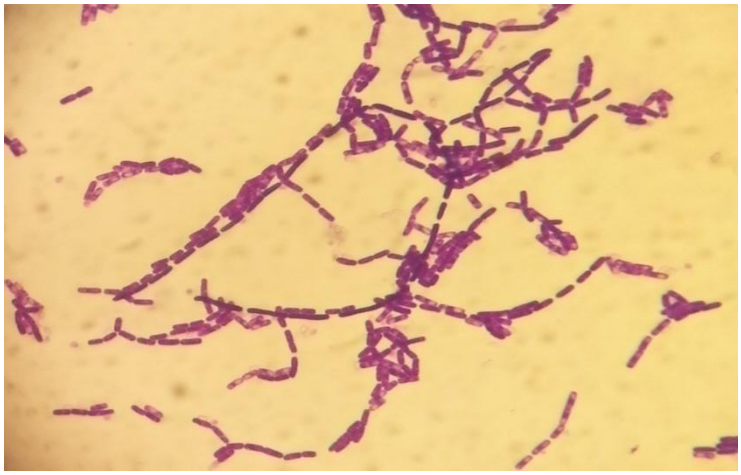


Figure 4.5 *B. thuringiensis var. israelensis* gram stain.

4.2.3 Endospore Stain

The two bacterial isolates that tested positive for endospores were seen to contain brightly stained green elliptical structures. Slides prepared at time intervals provided additional evidence that these were in fact endospores, where small green spores were clearly visible within pink vegetative tissue at early stages of spore development. The presence of the elliptical green spheres were present in two samples (Figure 4.6 and 4.7).

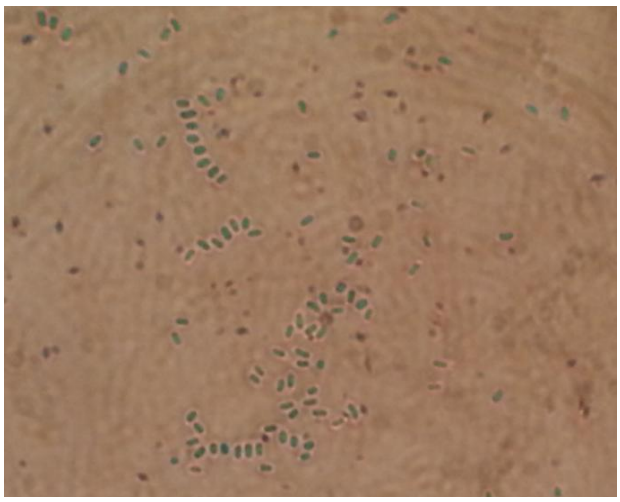


Figure 4.6 *B. thuringiensis var kurstaki* endospores stain.

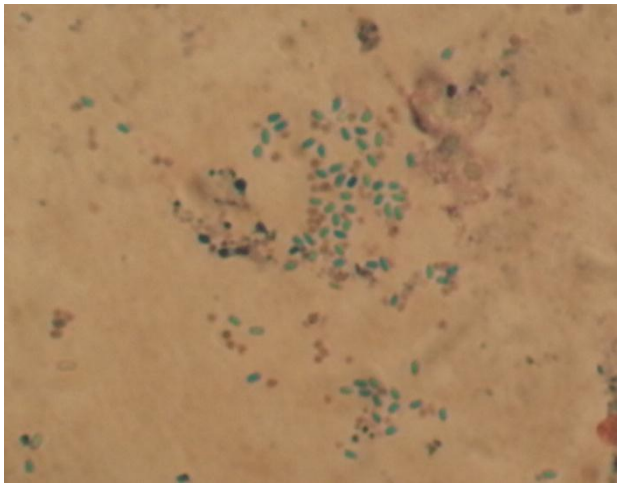


Figure 4.7 *B. thuringiensis var. israelensis* endospores stain.

4.2.4 Motility Determination

As shown in Figure 4.8 the motility test is positive for the two bacterial isolates there is growth going out away from the stab line.

Figure 4.8 Motility test of *B. thuringiensis var kurstaki* and *israelensis*.

4.3 Biochemical Characterization of *Bacillus thuringiensis*

4.3.1 Catalase Test

The two bacterial isolates exhibited catalase activity. The catalase slide test in which visible bubble production indicates a positive result (Figures 4.9).

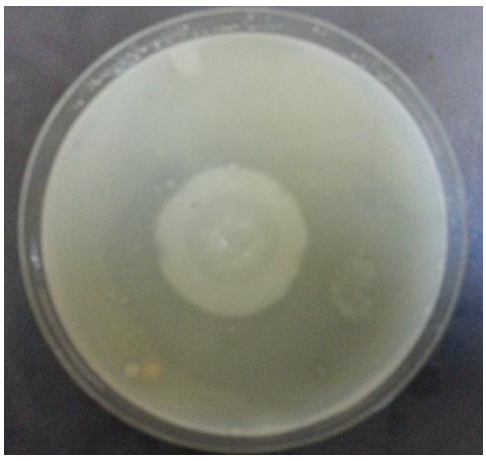
B. thuringiensis var kurstaki

B. thuringiensis var israelensis

Figure 4.9 Catalase test of *B. thuringiensis var kurstaki* and *israelensis.*

4.3.2 Lecithinase Activity

The two bacterial isolates exhibited lecithinase activity. Lecithinase - positive on egg yolk agar media determined by the formation of white precipitate around the colonies (Figures 4.10).



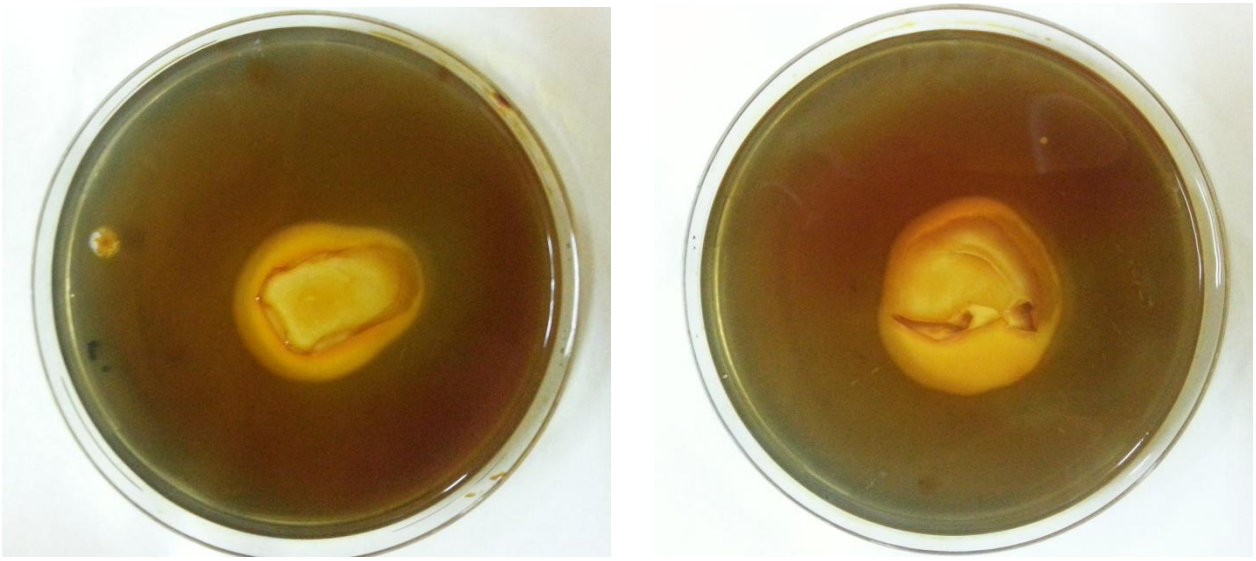
B. thuringiensis var kurstaki

B. thuringiensis var. israelensis

Figures 4.10 Lecithinase activity of *B. thuringiensis var kurstaki* and *israelensis*.

4.3.3 Amylase Activity

The two bacterial isolates exhibited amylase activity. Amylase-positive on starch agar media formed zones of clearance indicating amylase activity (Figures 4.11).

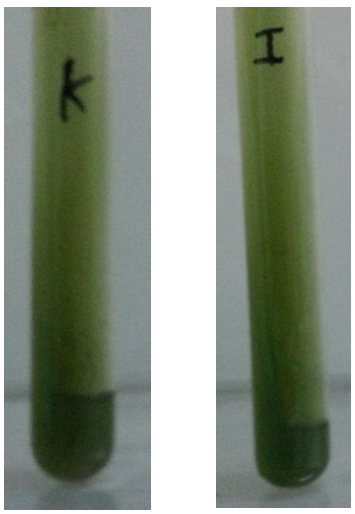


B. thuringiensis var kurstaki ***B. thuringiensis var. israelensis***

Figures 4.11 Amylase activity of *B. thuringiensis var kurstaki* and *israelensis*.

4.3.4 Citrate Utilization Test

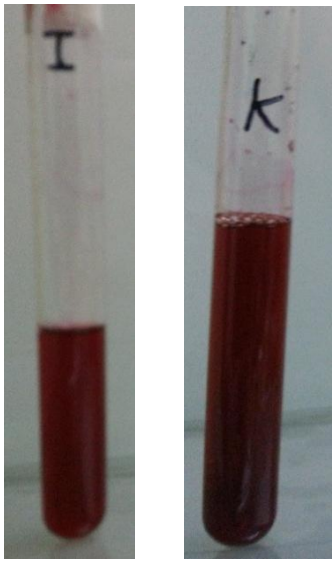
The two bacterial isolates were inoculated on simmon's citrate agar medium. Are negative in citrate utilization were detected by no change in PH medium to acidic which indicated by the no colour change of medium from green to blue (Figures 4.12).



Figures 4.12 Citrate test of *B. thuringiensis var kurstaki* and *israelensis*.

4.3.5 Nitrate Reduction Test

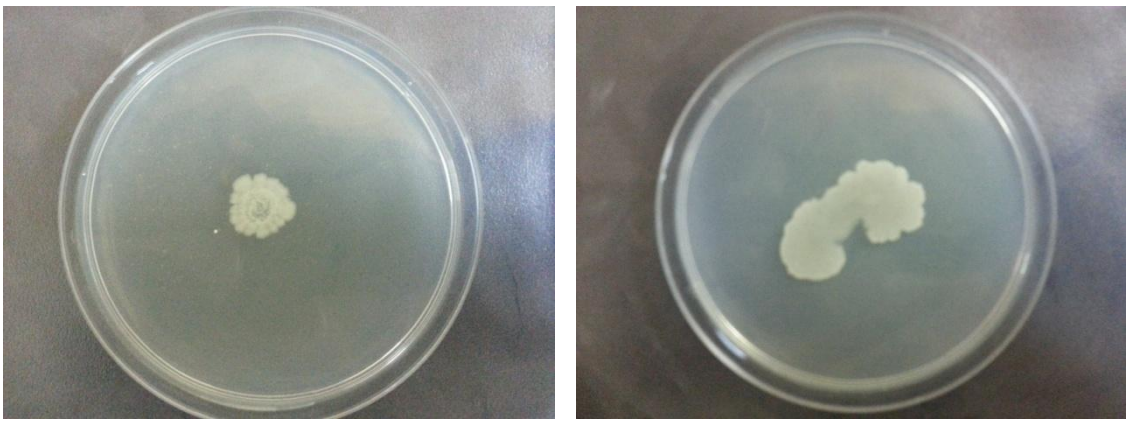
As shown in Figure 4.13 the nitrate reduction test was positive for both isolates by giving the pink colour which means nitrate reduced to nitrite (Figures 4.13).



Figures 4.13 Nitrate reduction test of *B. thuringiensis var kurstaki* and *israelensis*.

4.3.6 Growth on 7% NaCl Test

The two bacterial isolates were able to grow on nutrient agar containing 7% NaCl . As shown in Figure 4.14 the isolates were able to grow and spread on the medium containing 7% NaCl .



B. thuringiensis var kurstaki ***B. thuringiensis var. israelensis***

Figures 4.14 Growth on 7% NaCl test of *B. thuringiensis var kurstaki* and *israelensis*.

Table 4.1 Morphological characterisation of *B. thuringiensis kurstaki* and *B. thuringiensis israelensis*.

Morphological Characteristics	*B. thuringiensis kurstaki*	*B. thuringiensis israelensis*
Crystal protein	bipyramidal	spherical
Gram stain	Gram positive	Gram positive
Endospore stain	Endospore positive	Endospore positive
Motility	positive	positive

Table 4.2 Biochemical characterization of *B. thuringiensis kurstaki* and *B. thuringiensis israelensis*.

Biochemical test	*B. thuringiensis kurstaki*	*B. thuringiensis israelensis*
Catalase test	+	+
Lecithinase activity	+	+
Amylase activity	+	+
Citrate utilization test	-	-
Nitrate reduction test	+	+
Growth on 7% NaCl test	+	+

4.4 Effect of Commercial Product of *B. thuringiensis var.kurstaki* on the Different Larval Stages of *T. absoluta* (Bioassay).

Table 4.3 Effect of commercial products of *B. thuringiensis var.kurstaki* on the first larval stage (L1) of an insect *T.absoluta.*

Concentration (g/l)	1			2			3			4			5			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	2	4	7	3	6	9	4	7	10	4	9	10	4	9	10	0	0	0
2	2	5	9	5	7	9	3	6	10	4	9	10	4	8	10	0	0	0
3	3	5	8	4	8	9	5	8	10	4	8	10	5	8	10	0	0	0
Mean	2.3	4.6	8	4	7	9	4	7	10	4	8.6	10	4.3	8.3	10	0	0	0
Mean cumulative	4.96			6.6			7			7.5			7.5			0		
% mortality	49%			66%			70%			75%			75%			0		
S.D	2.55			2.29			2.69			2.74			2.55			0		
ANOVA test	F test = 1.508									p-value = 0.218								

LC-values for first larval stage after 6 days: LC50 < 1 (g/l) LC90 = 2 (g/l)

Table (4.3) shows that the p-value (Sig.) is greater than the level of significance $\alpha = 0.05$, then there is insignificant difference in concentration to the first larval stage (L1) due to *B. thringiensis var.kurstaki*. We conclude that the *B. thuringiensis var.kurstaki* has no different effect for the five concentration in effectiveness on first larval stage (L1) of an insect *T. absoluta*. This means that the lowest concentration of the product acts as the highest concentration in effectiveness against the first larval stage (L1).

Table 4.4 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the first larval stage of *T. absoluta.*

Concentration (g/l)	Mean cumulative Corrected % Mortality	± S.D
1	4.96	2.55
2	6.6	2.29
3	7	2.69
4	7.5	2.74
5	7.5	2.55

Table 4.4 and Figure 4.15 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var.kurstaki* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.16 shows the test before and after applying the commercial product.

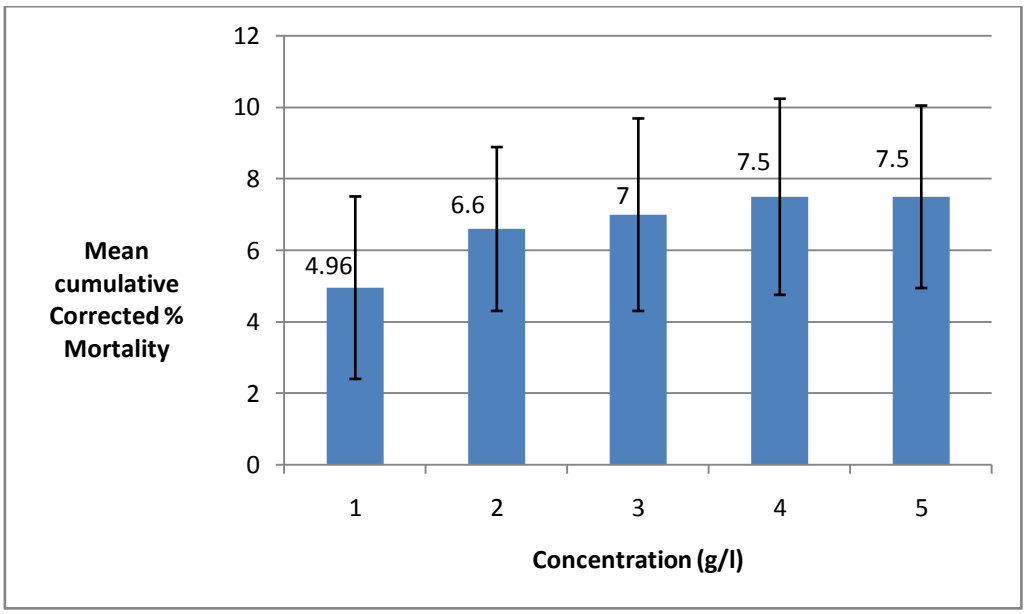


Figure 4.15 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the first larval stage of *T. absoluta*.

(a) **(b)**

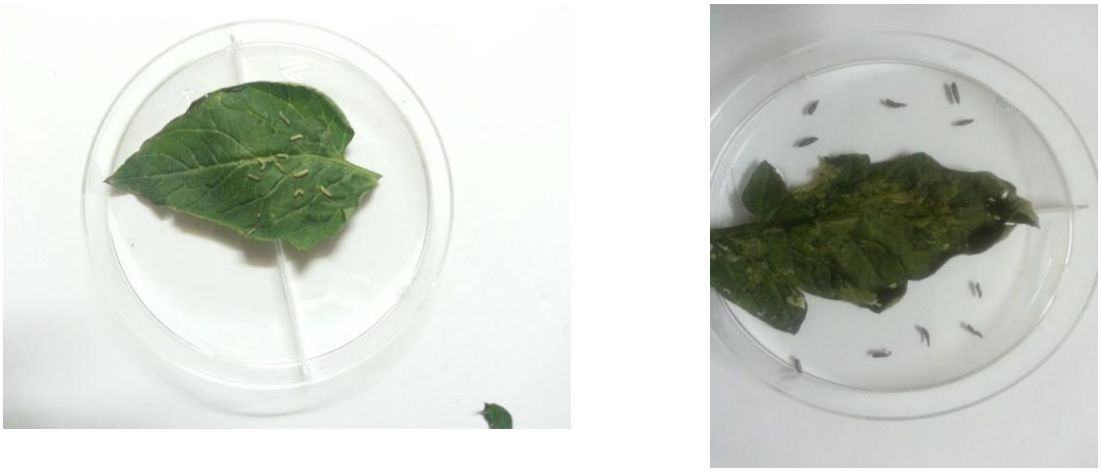


Figure 4.16 Effect of commercial product of *B. thuringiensis var.kurstaki* on the first larval stage (L1) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.5 Effect of commercial products of *B. thuringiensis var.kurstaki* on the second larval stage (L2) of an insect *T. absoluta.*

Concentration (g/l)	1			2			3			4			5			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	1	5	7	2	7	9	3	6	9	3	7	10	4	7	10	0	0	0
2	2	5	7	3	6	8	4	7	9	5	7	10	4	9	10	0	0	0
3	2	4	7	3	7	8	3	7	9	5	8	10	4	8	10	0	0	0
Mean	1.6	4.6	7	1.6	6.6	8.3	3.3	6.6	9	4.3	7.3	10	4	8	10	0	0	0
Mean cumulative	4.4			5.8			6.3			7.2			7.3			0		
% mortality	44%			58%			63%			72%			73%			0		
S.D	2.35			2.57			2.50			2.54			2.69			0		
ANOVA test	F test = 1.934									p-value = 0.123								

LC-values for second larval stage after 6 days: LC50 < 1 (g/l) LC90 = 3 (g/l)

Table (4.5) shows that the p-value (Sig.) is greater than the level of significance $\alpha = 0.05$, then there is insignificant difference in concentration to the second larval stage (L2) due to *B. thuringiensis var.kurstaki*. We conclude that the *B. thuringiensis var.kurstaki* has no different effect for the five concentration in effectiveness on second larval stage (L2) of an insect *T. absoluta*. This means that the lowest concentration of the product acts as the highest concentration in effectiveness against the second larval stage (L2).

Table 4.6 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the second larval stage of *T. absoluta.*

Concentration (g/l)	Mean cumulative Corrected % Mortality	± S.D
1	4.4	2.35
2	5.8	2.57
3	6.3	2.50
4	7.2	2.54
5	7.3	2.69

Table 4.6 and Figure 4.17 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var.kurstaki* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.18 shows the test before and after applying the commercial product.

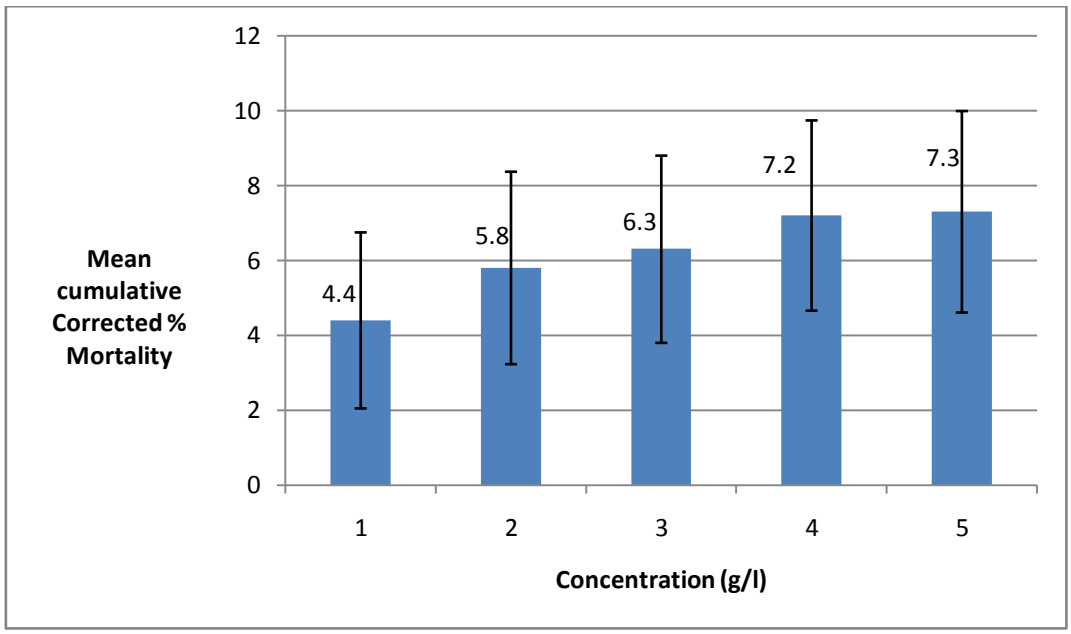


Figure 4.17 Commercial product concentration of *B .thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the second larval stage of *T. absoluta.*

(a) **(b)**

Figure 4.18 Effect of commercial products of *B. thuringiensis var.kurstaki* on the second larval stage (L2) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.7 Effect of commercial products of *B. thuringiensis var.kurstaki* on the third larval stage (L3) of an insect *T. absoluta.*

Concentration (g/l)	1			2			3			4			5			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	1	3	6	4	6	8	4	7	9	4	6	10	5	8	10	0	0	0
2	0	4	6	3	6	8	3	7	9	4	7	10	3	7	10	0	0	0
3	1	3	6	3	5	8	3	6	9	4	6	9	4	7	10	0	0	0
Mean	.6	3.3	6	3.3	5.6	8	3.3	6.6	9	4	6.3	9.6	4	7.3	10	0	0	0
Mean cumulative	3.3			5.6			6.3			6.6			7.1			0		
% mortality	33%			56%			63%			66%			71%			0		
S.D	2.35			2.06			2.50			2.50			2.67			0		
ANOVA test	F test = 3.393									p-value = 0.018								

LC-values for third larval stage after 6 days: LC50 < 1 (g/l) LC90 = 3 (g/l)

Table (4.7) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the third larval stage (L3) due to *B. thuringiensis var.kurstaki*. We conclude that the *B. thuringiensis var.kurstaki* has different effect in the five concentration on third larval stage (L3) of an insect *T. absoluta*.

Table 4.8 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the third larval stage of *T. absoluta.*

Concentration (g/l)	Mean cumulative Corrected % Mortality	± S.D
1	3.3	2.35
2	5.6	2.06
3	6.3	2.50
4	6.6	2.50
5	7.1	2.67

Table 4.8 and Figure 4.19 illustrated the relation between the different concentration of the commercial product of *B. thringiensis var.kurstaki* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.18 shows the test before and after applying the commercial product.

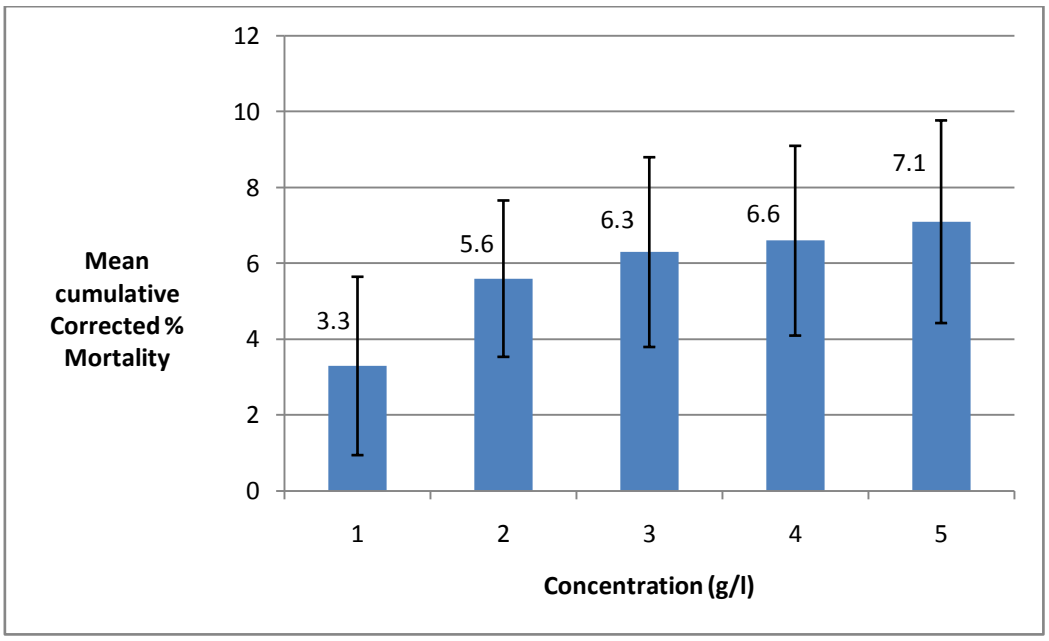


Figure 4.19 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the third larval stage of *T. absoluta*.

(a) **(b)**

Figure 4.20 Effect of commercial products of *B. thuringiensis var.kurstaki* on the third larval stage (L3) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.9 Effect of commercial products of *B. thuringiensis var.kurstaki* on the forth larval stage (L4) of an insect *T. absoluta.*

Concentration (g/l)	1			2			3			4			5			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	0	2	5	2	5	7	3	5	8	4	7	10	5	7	10	0	0	0
2	1	3	6	3	5	6	3	6	9	4	7	10	4	7	10	0	0	0
3	0	2	5	3	5	7	3	5	9	3	6	9	4	7	10	0	0	0
Mean	.6	2.3	5.3	2.6	5	6.6	3	5.3	8.6	3.6	6.6	9.6	4.3	7	10	0	0	0
Mean cumulative	2.7			4.7			5.6			6.6			7.1			0		
% mortality	27%			47%			56%			66%			71%			0		
S.D	2.24			1.79			2.50			2.65			2.47			0		
ANOVA test	F test = 5.087									p-value = 0.002								

LC-values for forth larval stage after 6 days: LC50 ≈ 1 (g/l) LC90 ≈ 4 (g/l)

Table (4.9) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the forth larval stage (L4) due to *B. thuringiensis var.kurstaki*. We conclude that the *B. thuringiensis var.kurstaki* has different effect in the five concentration on forth larval stage (L4) of an insect *T. absoluta.*

Table 4.10 Commercial product concentration of *B.thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the forth larval stage of *T.absoluta*.

Concentration (g/l)	Mean cumulative Corrected % Mortality	± S.D
1	2.7	2.24
2	4.7	1.79
3	5.6	2.50
4	6.6	2.65
5	7.1	2.47

Table 4.10 and Figure 4.21 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var.kurstaki* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.22 shows the test before and after applying the commercial product.

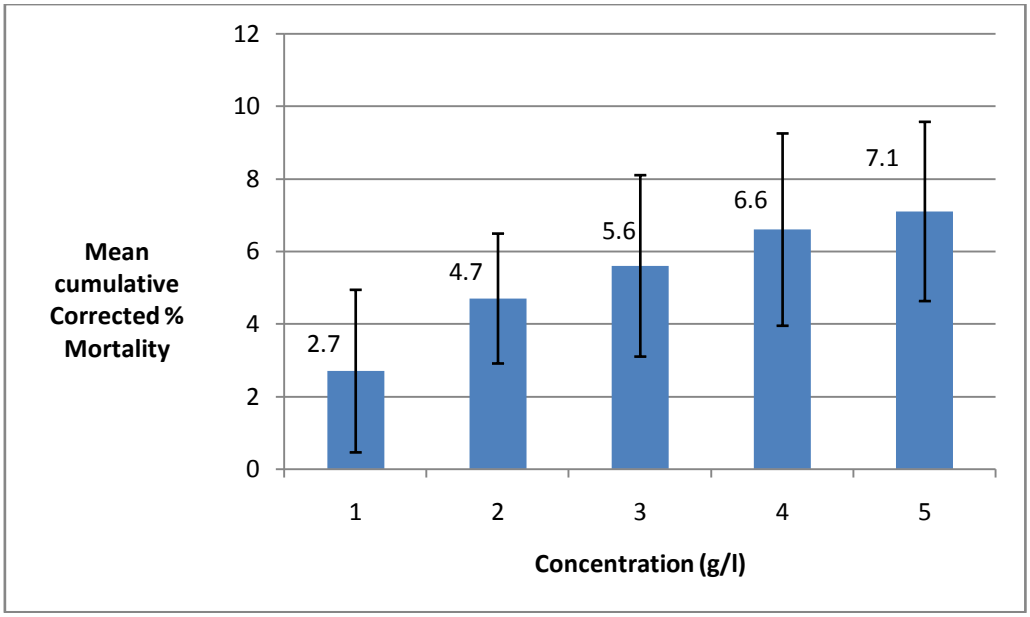


Figure 4.21 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the forth larval stage of *T .absoluta*.

(a) **(b)**

Figure 4.22 Effect of commercial product of *B. thuringiensis var.kurstaki* on the forth larval stage (L4) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

4.5 Effect of Commercial Products of *B. thuringiensis var.kurstaki* on the Adult Insect of *T. absoluta* in the Laboratory (Bioassay).

Table 4.11 Effect of commercial products of *B. thuringiensis var.kurstaki* on the adult of an insect *T. absoluta*.

Concentration (g/l)	1			2			3			4			5			control		
days / Replicate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	1	3	5	1	3	5	2	3	6	2	4	6	4	6	9	0	0	0
2	1	2	4	2	4	6	2	4	6	3	5	7	4	6	8	0	0	0
3	2	3	4	2	4	6	2	4	6	2	4	7	4	7	10	0	0	0
Mean	1.3	2.6	4.3	1.6	3.6	5.6	2	3.6	6	2.3	4.3	6.6	4	6.3	9	0	0	0
Mean cumulative	2.7			3.6			3.8			4.4			6.4			0		
% mortality	27%			36%			38%			44%			64%			0		
S.D	1.39			1.80			1.76			1.94			2.24			0		
ANOVA test	F test = 4.925									p-value = 0.003								

LC-values for adult stage after 3 days: LC50 ≈ 2 (g/l) LC90 = 5 (g/l)

Table (4.11) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the adult due to *B. thuringiensis var.kurstaki*. We conclude that the *B. thuringiensis var.kurstaki* has different effect in the five concentration on adult of an insect *T. absoluta*.

Table 4.12 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the adults of *T. absoluta*.

Concentration (g/l)	Mean cumulative Corrected % Mortality	± S.D
1	2.7	1.39
2	3.6	1.80
3	3.8	1.76
4	4.4	1.94
5	6.4	2.24

Table 4.12 and Figure 4.23 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var.kurstaki* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.24 shows the test before and after applying the commercial product.

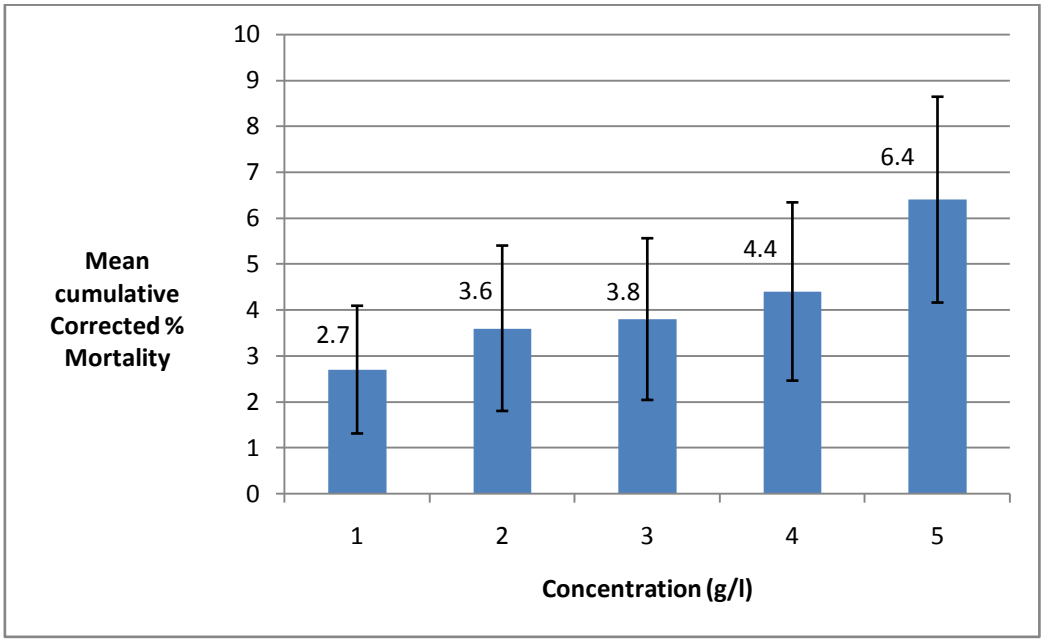


Figure 4.23 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the adult of *T. absoluta*.

(a) **(b)**

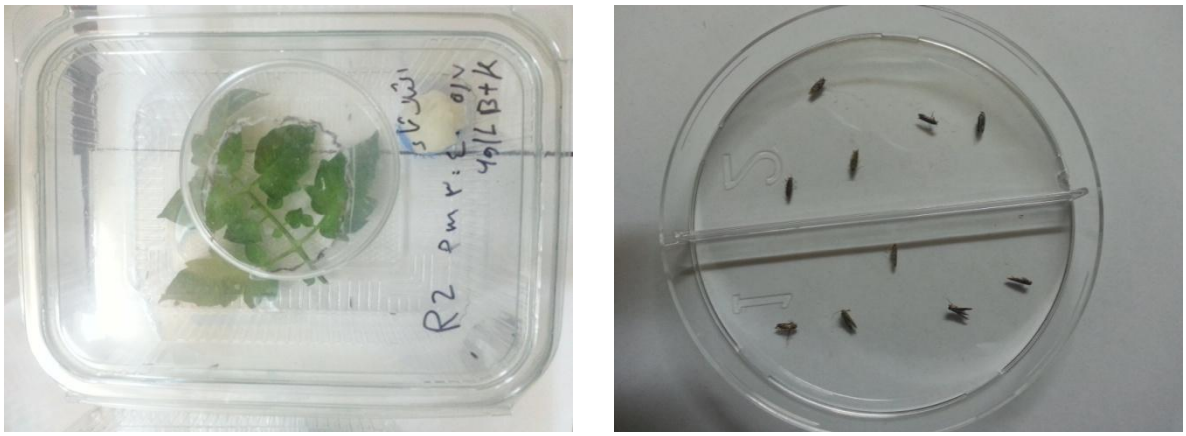


Figure 4.24 Effect of commercial product of *B. thuringiensis var.kurstaki* on the adult of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.13 Commercial product concentration of *B .thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the all stages larvae and adult of *T. absoluta*.

Concen / larval age	1	2	3	4	5	control	p-value	LC50 (g/l)	LC90 (g/l)
L1	4.96	6.6	7	7.5	7.5	0	0.218	≈ 1	2
L2	4.4	5.8	6.3	7.2	7.3	0	0.123	≈ 1	3
L3	3.3	5.6	6.3	6.6	7.1	0	0.018	≈ 1	3
L4	2.7	4.7	5.6	6.6	7.1	0	0.002	≈ 1	≈4
Adult	2.7	3.6	3.8	4.4	6.4	0	0.003	≈ 2	5

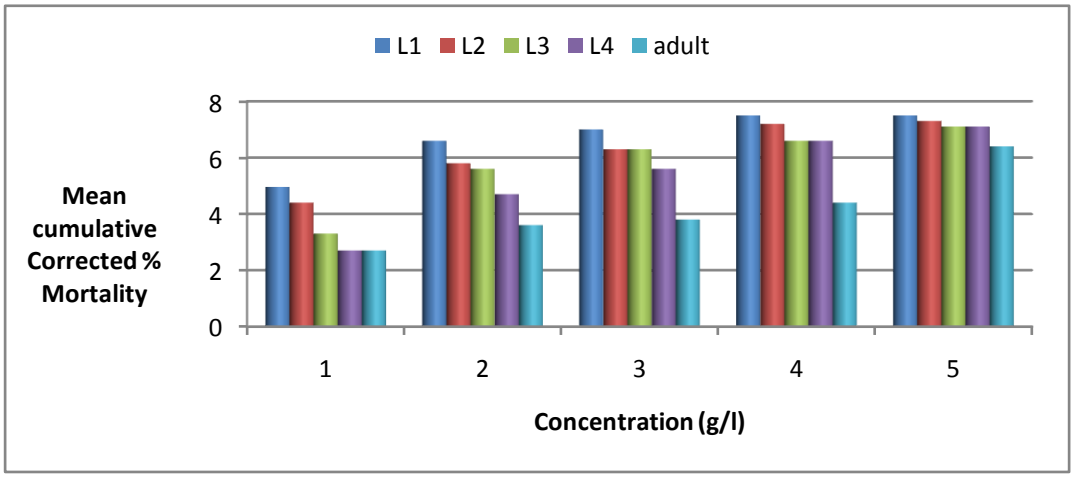


Figure 4.25 Commercial product concentration of *B. thuringiensis var.kurstaki* (g/l) and its relationship with mean cumulative corrected % mortality on the all stages larvae and adult of *T. absoluta*

Table 4.13 and Figure 4.25 Summarize the effect of different concentration of the commercial product of *B. thuringiensis var.kurstaki* on the different larval stages and the adult insect of *T. absoluta*. The results show significantly difference in using different concentration of the preperates on L3 , L4 and the adult insect.

4.6 Effect of Commercial Products of *B. thuringiensis var. israelensis* on the Different Larval Stages of *T. absoluta* (Bioassay).

Table 4.14 Effect of commercial products of *B. thuringiensis var. israelensis* on the first larval stage (L1) of an insect *T. absoluta*

Concentration (ml/l)	6			8			10			12			14			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	1	2	5	2	4	6	3	5	7	4	6	9	4	7	10	0	0	0
2	2	3	6	3	5	7	3	6	7	4	7	9	5	8	10	0	0	0
3	1	2	5	2	4	6	4	6	8	5	7	9	4	7	10	0	0	0
Mean	1.3	2.3	5.3	2.3	4.3	6.3	3.3	5.6	7.6	4.3	6.6	9	4.3	7.3	10	0	0	0
Mean cumulative	2.96			4.3			5.5			6.6			7.2			0		
% mortality	29%			43%			55%			66%			72%			0		
S.D	1.87			1.80			1.81			2.06			2.49			0		
ANOVA test	F test = 6.486									p-value = 0.000								

LC-values for first larval stage after 6 days: LC50 ≈ 6 (ml/l) LC90 = 12 (ml/l)

Table (4.14) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the first larval stage (L1) due to *B. thuringiensis var. israelensis*. We conclude that the *B. thuringiensis var. israelensis* has different effect in the five concentration on first larval stage (L1) of an insect *T. absoluta*.

Table 4.15 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the first larval stage of *T. absoluta*

Concentration (ml/l)	Mean cumulative Corrected % Mortality	± S.D
6	2.96	1.87
8	4.3	1.80
10	5.5	1.81
12	6.6	2.06
14	7.2	2.49

Table 4.15 and Figure 4.26 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var. israelensis* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.27 shows the test before and after applying the commercial product.

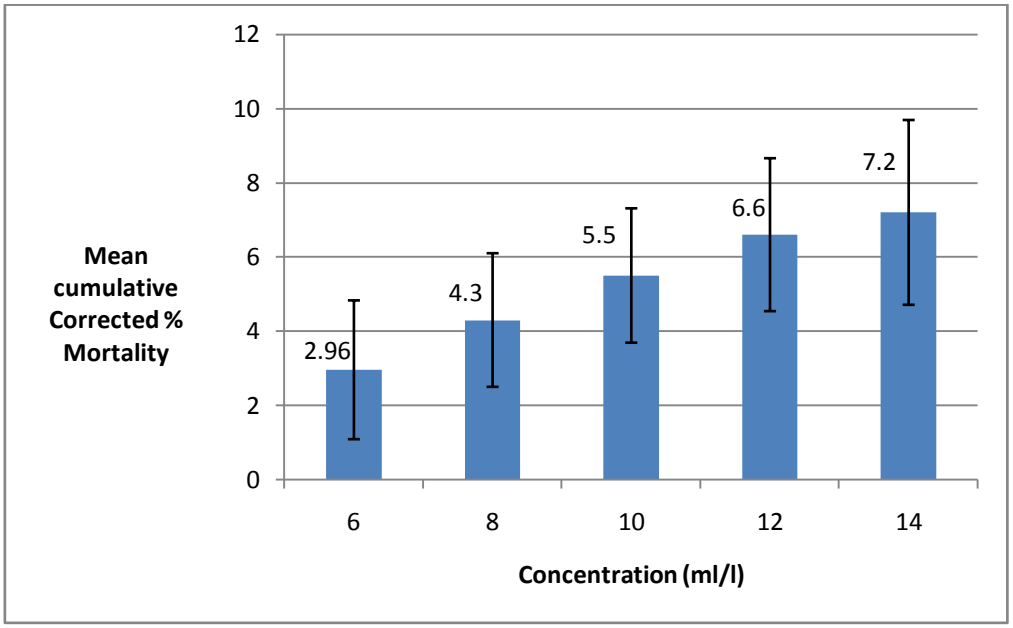


Figure 4.26 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the first larval stage of *T. absoluta*

(a) **(b)**

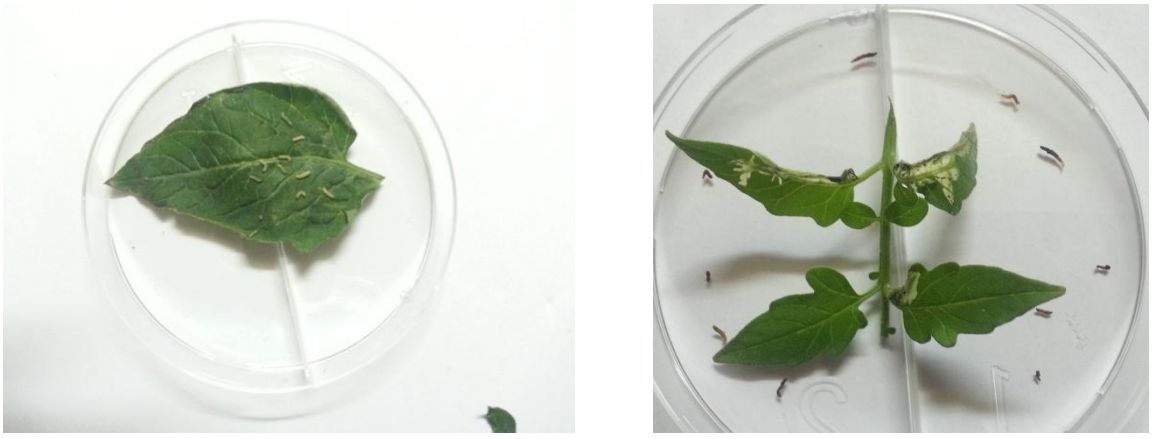


Figure 4.27 Effect of commercial products of *B. thuringiensis var. israelensis* on the first larval stage (L1) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.16 Effect of commercial products of *B. thuringiensis var. israelensis* on the second larval stage (L2) of an insect *T. absoluta*

Concentration (ml/l)	6			8			10			12			14			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	1	2	4	2	4	5	3	5	7	4	6	8	4	7	9	0	0	0
2	0	1	4	1	3	4	3	6	8	3	6	8	4	6	8	0	0	0
3	1	2	5	2	4	6	3	5	7	4	7	9	4	7	10	0	0	0
Mean	.6	1.6	4.3	1.6	3.6	5	3	5.3	7.3	3.6	6.3	8.3	4	6.6	9	0	0	0
Mean cumulative	2.16			3.4			5.2			6.06			6.5			0		
% mortality	21%			34%			52%			60%			65%			0		
S.D	1.72			1.59			1.92			2.09			2.24			0		
ANOVA test	F test = 8.138									p-value = 0.000								

LC-values for second larval stage after 6 days : LC50 = 8 (ml/l) LC90 = 14 (ml/l)

Table (4.16) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the second larval stage (L2) due to *B. thuringiensis var. israelensis*. We conclude that the *B. thuringiensis var. israelensis* has different effect in the five concentration on second larval stage (L2) of an insect *T. absoluta*.

Table 4.17 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the second larval stage of *T. absoluta*

Concentration (ml/l)	Mean cumulative Corrected % Mortality	± S.D
6	2.16	1.72
8	3.4	1.59
10	5.2	1.92
12	6.06	2.09
14	6.5	2.24

Table 4.17 and Figure 4.28 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var. israelensis* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.29 shows the test before and after applying the commercial product.

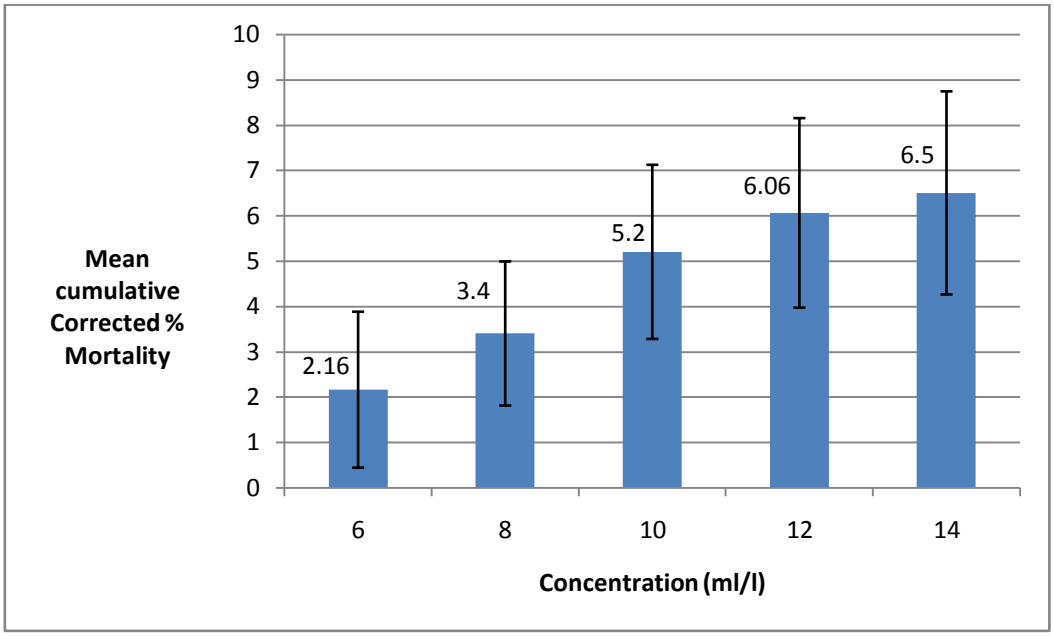


Figure 4.28 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the second larval stage of *T. absoluta*

(a) **(b)**

Figure 4.29 Effect of commercial products of *B. thuringiensis var. israelensis* on the second larval stage (L2) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.18 Effect of commercial products of *B.thuringiensis var. israelensis* on the third larval stage (L3) of an insect *T. absoluta*

Concentration (ml/l)	6			8			10			12			14			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	0	2	5	2	4	6	2	5	6	3	5	7	4	6	8	0	0	0
2	1	1	4	1	3	5	3	4	7	4	6	8	3	5	7	0	0	0
3	0	1	4	1	3	5	3	5	6	3	5	7	4	6	8	0	0	0
Mean	.3	1.3	4.3	1.3	3.3	5.3	2.6	4.6	6.6	3.3	5.3	7.3	3.6	5.6	7.6	0	0	0
Mean cumulative	1.96			3.3			4.6			5.3			5.6			0		
% mortality	19%			33%			46%			53%			56%			0		
S.D	1.87			1.80			1.67			1.80			1.80			0		
ANOVA test	F test = 6.423									p-value = 0.000								

LC-values for third larval stage after 6 days: LC50 ≈ 8 (ml/l) LC90 > 14 (ml/l)

Table (4.18) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the third larval stage (L3) due to *B. thuringiensis var. israelensis*. We conclude that the *B. thuringiensis var. israelensis* has different effect in the five concentration on third larval stage (L3) of an insect *T. absoluta*.

Table 4.19 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the third larval stage of *T. absoluta*

Concentration (ml/l)	Mean cumulative Corrected % Mortality	± S.D
6	1.96	1.87
8	3.3	1.80
10	4.6	1.67
12	5.3	1.80
14	5.6	1.80

Table 4.19 and Figure 4.30 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var. israelensis* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.31 shows the test before and after applying the commercial product.

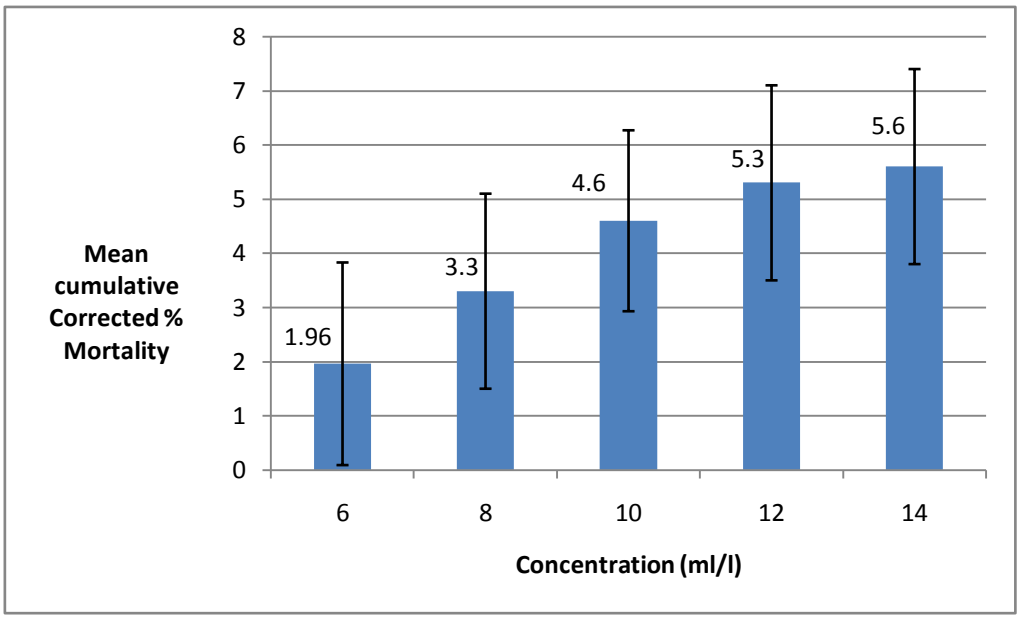


Figure 4.30 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the third larval stage of *T. absoluta*

(a) **(b)**

Figure 4.31 Effect of commercial products of *B. thuringiensis var. israelensis* on the third larval stage (L3) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.20 Effect of commercial products of *B. thuringiensis var. israelensis* on the forth larval stage (L4) of an insect *T. absoluta*

Concentration (ml/l)	6			8			10			12			14			control		
days / Replicate	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
1	1	2	5	1	3	5	2	5	7	3	5	7	3	5	7	0	0	0
2	0	1	4	2	3	5	2	4	6	3	5	7	3	5	7	0	0	0
3	0	1	3	1	4	6	3	5	7	4	6	8	4	6	8	0	0	0
Mean	.3	1.3	4	1.3	3.3	5.3	2.3	4.6	6.6	3.3	5.3	7.3	3.3	5.3	7.3	0	0	0
Mean cumulative	1.8			3.3			4.6			5.3			5.3			0		
% mortality	18%			33%			46%			53%			53%			0		
S.D	1.76			1.80			1.94			1.80			1.80			0		
ANOVA test	F test = 5.900									p-value = 0.001								

LC-values for forth larval stage after 6 days: LC50 ≈ 8 (ml/l) LC90 > 14 (ml/l)

Table (4.20) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the forth larval stage (L4) due to *B. thuringiensis var. israelensis*. We conclude that the *B. thuringiensis var. israelensis* has different effect in the five concentration on forth larval stage (L4) of an insect *T. absoluta*.

Table 4.21 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the forth larval stage of *T. absoluta*

Concentration (ml/l)	Mean cumulative Corrected % Mortality	± S.D
6	1.8	1.76
8	3.3	1.80
10	4.6	1.94
12	5.3	1.80
14	5.3	1.80

Table 4.21 and Figure 4.32 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var. israelensis* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.33 shows the test before and after applying the commercial product.

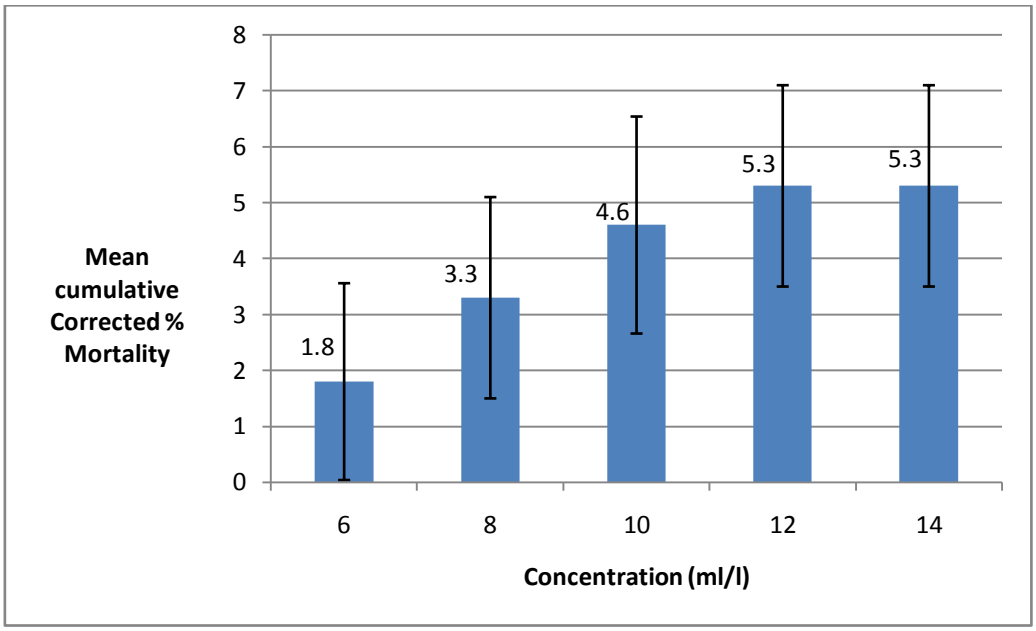


Figure 4.32 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the forth larval stage of *T. absoluta*

(a) **(b)**

Figure 4.33 Effect of commercial product of *B. thuringiensis var. israelensis* in the loss of the forth larval stage (L4) of an insect *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

4.7 Effect of Commercial Products of *B .thuringiensis var. israelensis* on the Adult Insect of *T. absoluta* in the Laboratory (Bioassay).

Table 4.22 Effect of commercial products of *B. thuringiensis var. israelensis* on the adult of an insect *T. absoluta*

Concentration (ml/l)	6			8			10			12			14			control		
days / Replicate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	1	1	2	1	2	2	1	2	2	1	2	3	2	3	4	0	0	0
2	0	1	2	1	1	2	1	2	3	2	3	3	1	2	3	0	0	0
3	0	0	1	0	1	1	1	2	2	1	2	3	2	3	4	0	0	0
Mean	.3	.6	1.6	.6	1.3	1.6	1	2	2.3	1.3	2.3	3	1.6	2.6	3.6	0	0	0
Mean cumulative	.8			1.1			1.7			2.2			2.6			0		
% mortality	8%			11%			17%			22%			26%			0		
S.D	0.78			0.67			0.67			0.83			1.00			0		
ANOVA test	F test = 7.339									p-value = 0.000								

LC-values for adult stage after 3 days: LC50 > 14 (ml/l) LC90 > 14 (ml/l)

Table (4.22) shows that the p-value (Sig.) is smaller than the level of significance α = 0.05, then there is significant difference in concentration to the adult due to *B. thuringiensis var. israelensis*. We conclude that the *B .thuringiensis var. israelensis* has different effect in the five concentration on adult of an insect *T. absoluta*.

Table 4.23 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the adults of *T. absoluta*

Concentration (ml/l)	Mean cumulative Corrected % Mortality	± S.D
6	.8	.78
8	1.1	.67
10	1.7	.67
12	2.2	.83
14	2.6	1.00

Table 4.23 and Figure 4.34 illustrated the relation between the different concentration of the commercial product of *B. thuringiensis var. israelensis* and the mean cumulative corrected % mortality with their corresponding stander deviations. Figure 4.35 shows the test before and after applying the commercial product.

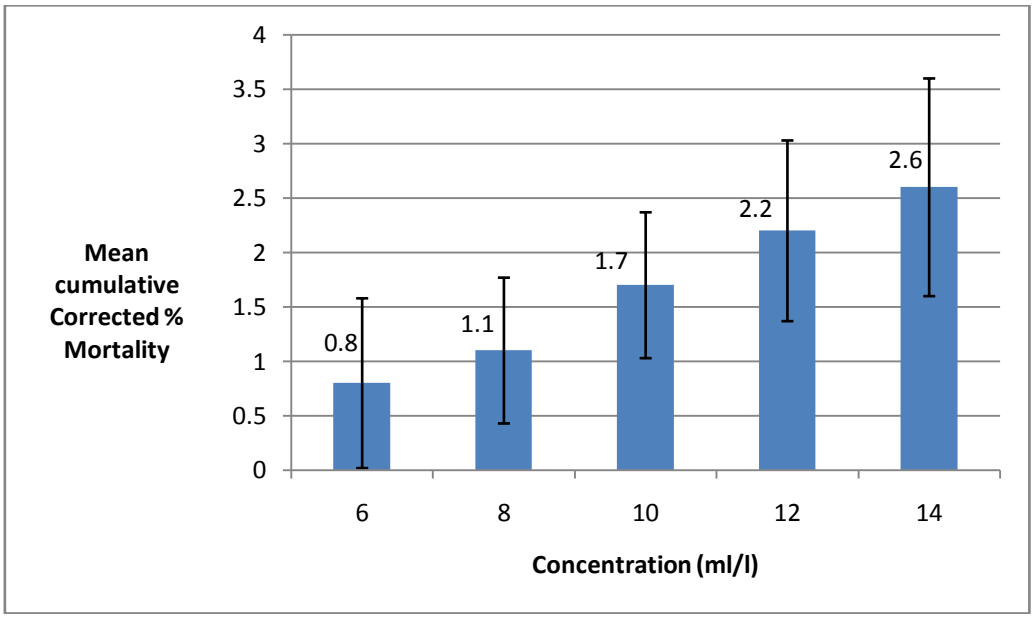


Figure 4.34 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the adult of *T. absoluta*

(a) **(b)**

Figure 4.35 Effect of commercial product of *B. thuringiensis var. israelensis* on the adult of *T. absoluta* (a) Before applying the preparates (b) At the end of the experimental period.

Table 4.24 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the all larval stages and adult of *T .absoluta*

Concen / larval age	6	8	10	12	14	control	p-value	LC50 (ml/l)	LC90 (ml/l)
L1	2.96	4.3	5.5	6.6	7.2	0	0.000	≈ 6	12
L2	2.16	3.4	5.2	6.06	6.5	0	0.000	8	14
L3	1.96	3.3	4.6	5.3	5.6	0	0.000	≈ 8	> 14
L4	1.8	3.3	4.6	5.3	5.3	0	0.001	≈ 8	> 14
adult	.8	1.1	1.7	2.2	2.6	0	0.000	> 14	> 14

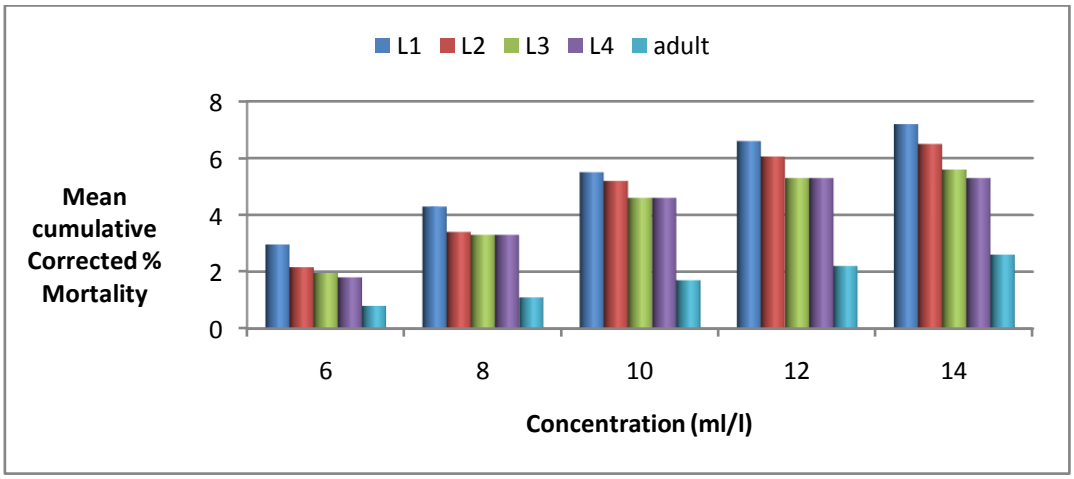


Figure 4.36 Commercial product concentration of *B. thuringiensis var. israelensis* (ml/l) and its relationship with mean cumulative corrected % mortality on the all stages larvae and adult of *T. absoluta*

Table 4.24 and Figure 4.36 summarize the effect of different concentration of the commercial product of *B. thuringiensis var. israelensis* on the different larval stages and the adult insect of *T. absoluta*. The results show significantly difference in using different concentration of the preparates on all larval stages and the adult insect.

CHAPTER 5

DISCUSSION

Insect pests that cause plant diseases have been the subject of intensive study and control measures. The use of biological insecticides is one effective way of coping with insect pests. There are predictions of an annual increase of biological pesticides production of 10-15%, in comparison with the increase in chemical pesticides production of 1-2%. Of the total production of insecticides, entomopathogenic bacteria (mostly *B. thuringiensis*) amount to 90% (**Powell and Jutsum**, **1993**). *B. thuringiensis* has been commercially used in the biological control of insect pests for the last four decades. Strains of Bt can produce toxic compounds of various chemical structures and properties. Most studies mentioned that δ-endotoxins acts selectively against the larvae of some target insects (**Stepanova *et al.*, 1996)**

The impact of *T. absoluta* can be greatly reduced by spraying only *B. thuringiensis* based formulates, with no need for chemical insecticides**.**

5.1 Characterization

On the basis of the morphological, and biochemical characteristics present in Table 3.6 and the "Bergey's Manual of Systematic Bacteriology". The bacterial strains was identified and confirmed as a member of the genus *Bacillus* and *B. thuringiensis* species *var*.(*kurstaki* (Btk) and *israelensis* (Bti)) .

5.1.1Morphological Characterization

B. thuringiensis var kurstaki produce parasporal crystal inclusions are apparent; bipyramidal crystals Figure 4.2. Based on literature, bipyramidal crystals, related to Cry 1 proteins **(Aronson *et al.*, 1976).** Crystal morphology of *B. thuringiensis* can provide valuable information on target insect spectra **(Maeda *et al.*, 2000).** For example, bipyramidal shaped crystals are related to Cry 1 proteins that are toxic against *Lepidopteran* species.

B. thuringiensis var. israelensis produce parasporal crystal inclusions are apparent; spherical crystals Figure 4.3. Based on literature, spherical crystals related to spherical (Cry4A and Cry4B), proteins **(Aronson *et al.*, 1976).** Crystal morphology of *B.*

thuringiensis can provide valuable information on target insect spectra **(Maeda *et al.*, 2000).** For example, spherical shaped crystals are related to Cry4 and Cry11 proteins are specifically toxic against to *Dipterans* species.

The two bacterial strains are gram positive rod shaped, endospore positive, contain brightly stained green elliptical structures, and motile positive, because there was growth going out away from the stab line (Figures 4.4 - 4.8 and Table 4.1). It was done according to the basis of the morphological, and biochemical characteristics shown in Table 3.6 and the "Bergey's Manual of Systematic Bacteriology". **(Murrang*, et al.*,1986) .**

5.1.2 Biochemical Characterization of *Bacillus thuringiensis*

The two bacterial strains exhibited catalase activity. The catalase slide test in which visible bubble production indicates a positive result, exhibited lecithinase activity, lecithinase -positive on egg yolk agar media determined by the formation of white precipitate around the colonies, exhibited amylase activity, amylase-positive on starch agar media formed zones of clearance indicating amylase activity, negative citrate utilization were detected by the no change in pH of the medium to acidic which indicated by the no colour change of medium from green to blue, exhibited nitrate reduction was reduced through appearance of a pink color indicated positive nitrite formation, and able growth and spread on nutrient agar containing 7% NaCl (Figures 4.9 - 4.14 and Table 4.2) .This was carried out according to the basis of the morphological, and biochemical characteristics shown in Table 3.6 and the "Bergey's Manual of Systematic Bacteriology" **(Murrang, *et al.*,1986) .**

5.2 Effect of Commercial Products of *B. thuringiensis* on the Different Larval Stages and Adults of *T. absoluta* (Bioassay).

Bioassays of the commercial biocide in laboratory showed high significant efficacy in reducing the damage caused by different larval instars (1st, 2nd, 3rd and 4th) of *T. absoluta* at different concentrations compared to non- treated once as a control.

First and second instars larvae recorded the highest mortality, while mortality was lower in third and forth instars larvae which show a pattern of immune-called

maturation immunity. Several *T. absoluta* instars were found to be susceptible to *B. thuringiensis*, to a different extent **(Giustolin *et al.*, 2001).** Also for the later instars larvae, low mortality was probably due to increase the maturation immunity of the larvae. Mortality progressively increased as the concentration of Agerin increased. On the other hand, the early instars suffered from higher mortality compared to the late instars. The potential of *B. thuringiensis* subsp *kurstaki* as commercial biocide in controlling pests of economic importance is well known as a key part of Integrated Pest Management Programs **(Roh *et al.*, 2007).**

These results are in agreement with those obtained by Cabello *et al.*, (2009) who reported that the effect of *B. thuringiensis* subsp *kurstaki* on all larval instars have exhibited satisfactory efficacy against *T. absoluta* larval infestations.

Mortality of the infected larvae may be due to the undigestion of the ingested food, or due to paralysis and/or the physiological disturbance due to the toxicity of the haemolymph **(Lotfy, 1988).**

In general the percent mortality increased with incubation period and maximum mortality was recorded at 6 days after feeding (Table 4.14, 4.16, 4.18, 4.20, 4.22) .

In the present investigation it was observed that the percent mortality was very low after 2 days of feeding. This might be due to the fact that *B. thuringiensis* being stomach poison, when Bt enters in to the midgut of insect, it gets dissolved in the alkaline pH, releasing δ-endotoxins **(Schnepf *et al.*, 1998)**.

Due to high toxicity of chemical pesticide to human beings, animals and beneficial insects, the use of chemical pesticides is being replaced by environment friendly biopesticides. The crystal proteins of *B. thuringiensis*, as bio-control agent, have been extensively studied worldwide. Under the present investigation, commercial product of *B. thringiensis var.kurstaki* were highly insecticidal on the different larval stages of *T. absoluta*. These results are in agreement with those obtained in literature (Youssef *et al.,* 2013). Commercial Product of *B. thuringiensis var.kurstaki* will be useful to use in integrated pest management for sustainable agriculture.

Higgins *et al.*, (1989) stated that the bacteria *B. thuringiensis* produces phosphatidylinositol containing enzyme phospholipase that broken down

phospholipid molecules existing in the basement membrane and goblet, vertical membrane cells in stomach tissue to stop its work.

5.2.1 Effect of Commercial Product of *B. thuringiensis var.kurstaki* on Different Larval Stages and Adults of *T. absoluta* (Bioassay).

As shown in Table 4.3, 4.5, 4.7, 4.9 and 4.11 and Figure 4.15, 4.17, 4.19, 4.21 and 4.23 the mean cumulative corrected % mortality of *T. absoluta* larvae and adults were recorded after 2,4, and 6 days. The highest mortality percentages were found for *B. thuringiensis var. kurstaki* bacteria with a gradual significant increase over the inspection period with different larval instars.

Data presented in Table 4.3 and Figure 4.15 indicated that increasing (Agerin®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of first larval stage from minimum 49 % at 1g\l to maximum of 75 % at 5 g\l compared with the untreated larvae after six days of exposure which agree with the previous studies **(Aziz *et al.*, 2012).** In most cases, first instar larvae were the most susceptible to *B. thuringiensis* which agree with the literature (**Ali and Young, 1996; Rausell *et al*., 2000).**

Data presented in Table 4.5 and Figure 4.17 indicated that increasing (Agerin®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of second larval stage from minimum 44 % at 1g\l to maximum of 73 % at 5 g\l compared by untreated larvae after six days of exposure which agree with the previous studies **(Aziz *et al.*, 2012).**

Data presented in Table 4.7 and Figure 4.19 indicated that increasing (Agerin®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of third larval stage from minimum 33 % at 1g\l to maximum of 71 % at 5 g\l compared by untreated larvae after six days of exposure which agree with the previous studies **(Aziz *et al.*, 2012).**

Data presented in Table 4.9 and Figure 4.21 indicated that increasing (Agerin®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of forth larval stage from minimum 27 % at 1g\l to maximum

of 71 % at 5 g\l compared by untreated larvae after six days of exposure which agree with the previous studies **(Aziz *et al.*, 2012).**

Data presented in Table 4.11 and Figure 4.23 indicated that increasing (Agerin®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of adults from minimum 27 % at 1g\l to maximum of 64 % at 5 g\l compared by untreated adults after three days of exposure which agree with the previous studies **(Aziz *et al.*, 2012).**

5.2.2 Effect of Commercial Products of *B. thuringiensis var. israelensis* on Different Larval Stages and Adult of *T. absoluta* (Bioassay).

Data presented in Table 4.14 and Figure 4.26 indicated that increasing (Back tosh ®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of first larval stage from minimum 29 % at 6 ml\l to maximum of 72 % at 14 ml\l compared by untreated larvae after six days of exposure .

Data presented in Table 4.16 and Figure 4.28 indicated that increasing (Back tosh ®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of second larval stage from minimum 21 % at 6 ml\l to maximum of 65 % at 14 ml\l compared by untreated larvae after six days of exposure.

Data presented in Table 4.18 and Figure 4.30 indicated that increasing (Back tosh ®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of third larval stage from minimum 19 % at 6 ml\l to maximum of 56 % at 14 ml\l compared by untreated larvae after six days of exposure .

Data presented in Table 4.20 and Figure 4.32 indicated that increasing (Back tosh ®) product concentration and duration of exposure increased the mean cumulative corrected % mortality of forth larval stage from minimum 18 % at 6 ml\l to maximum of 53 % at 14 ml\l compared by untreated larvae after six days of exposure.

Data presented in Table 4.22 and Figure 4.34 indicated that increasing (Back tosh ®) product concentration and duration of exposure increased the mean cumulative

corrected % mortality of adults from minimum 8 % at 6 ml\l to maximum of 26 % at 14 ml\l compared by untreated adults after three days of exposure.

B. thuringiensis var. israelensis from commercial product (Back tosh ®) lower effective than *B. thuringiensis var.kurstaki* from commercial product (Agerin®) in mortality rate of larval stages and adult insect *T.absoluta* due to the high specificity of *B. thuringiensis var kurstaki* against *Lepidopteran* insects.While *Bacillus thuringiensis* var. *israelensis* most effective against *Diptera* insects such as mosquitoes and blackflies **(Haider *et al*., 1986).**

CHAPTER 6

CONCLUSION AND RECOMMENDATION

CONCLUSION

1- B. thuringiensis, one of the most widely bio-control agents, is a gram positive soil bacterium that produces cellular inclusions during sporulation, which are specifically toxic to certain insects as *T. absoluta.*

2- Crystal morphology of *B. thuringiensis* can provide valuable information on target insect spectra. Accordingly, *B. thuringiensis var kurstaki* produced bipyramidal shaped crystals proteins that are toxic against *Lepidopteran* species and *B. thuringiensis var. israelensis* produced spherical shaped crystals proteins are specifically toxic against *Dipteran* species.

3- The results obtained from our experiments revealed that *B. thuringiensis* effectively controls *T. absoluta.* Accordingly, it is possible to design control programs based on this bacterium that will successfully manage this pest.

4-It is concluded that the commercial product (Agerin®) that contains *B. thuringiensis var kurstaki* more effective against four larval stages and adult insect *T.absoluta* compared with commercial product (Back tosh®) that contains *B. thuringiensis var.israelensis* and this was due to the highly specialized *B. thuringiensis var kurstaki* against *Lepidopteran* insects.

5-Results revealed that the size of the inoculums is very critical which is reflected from the different effect by using different concentrations.

6- The existence of a positive relationship between duration of exposure of the commercial product bio-pesticide and the effectiveness against the insect.

7- There is a difference in the susceptibility to commercial product bio-pesticide shown by each larval stage. First and second instars larvae were the most susceptible to bio-pesticide, while susceptibility was lower in third and forth instars larvae and adult which show a pattern of immune-called maturation immunity.

8-The results support the applicability of this commercial product (Agerin®) in controlling *T. absoluta* insect programs. Additional research work will be needed to conduct studies on the role of bioinsecticides of *B. thuringiensis* and other biological control agents in the control of *T. absoluta* insect under greenhouse and filed conditions.

RECOMMENDATIONS

1- It is recommended to use the safe commercial product (Agerin®) in biological control of the pest *T. absoluta* larvae and adult rather than chemical insecticides.

2- We strongly recommend Ministry of Agriculture and farmers to use this safe commercial product (Agerin®) in biological control of *T. absoluta* insect rather than chemical insecticides to protect tomato crops.

3- It is recommended to carry out training courses for the farmers to use this safe commercial product (Agerin®) in biological control of *T. absoluta* insect in Gaza Strip.

4- We also, recommend carrying out additional research work on the role of bioinsecticides of *B. thuringiensis* in the control of *T. absoluta* insect under greenhouse and filed conditions and other harmful insects.

CHAPTER 7

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