Efficacy of Bt proteins and the effect of temperature on the development of spiny bollworm in South Africa

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Abstract

Genetically modified cotton expressing *Bacillus thuringiensis* (Bt) proteins has been cultivated in South Africa since 1998 for control of the bollworm complex. Spiny bollworms, *Earias biplaga* (Walker) (Lepidoptera: Noctuidae) *and Earias insulana* (Boisduval) (Lepidoptera: Noctuidae) belong to this complex. Exposure to Bt crops could contribute to resistance development to the insecticidal proteins expressed in these crops. The aim of this study was to determine the efficacy of Bt proteins for control of and the effect of temperature on development of *E. biplaga* in South Africa. There is currently no resistance of *E. biplaga* to Bollgard® and Bollgard II® cotton in South Africa. The use of Bt spray applications for control of *E. biplaga* on cotton was also evaluated, although it is currently not registered for control of this pest on cotton in South Africa. Half the dosage rate registered for bollworm control on cotton, was too low for effective control. The recommended dosage rate controlled the larvae as effective as Bollgard® and Bollgard II®, but 100% mortality was not achieved. Environmental factors such as UV light and rain may reduce the efficacy of Bt sprays. The final instar larvae might not be controlled by Bt sprays. Effective coverage with the spray application is essential for successful control. The effect of temperature on the development of *E. biplaga* was studied at four different temperature regimes, namely 18, 20, 25 and 30 ± 1°C. Development time for all life stages was inversely related to temperatures from 18 to 30 °C. The relationship between temperature and developmental rate of *E. biplaga* was linear between 18 and 30 °C and more rapid development was observed with increasing temperatures. The total development period was 68.9 to 22.5 days at 18 and 30 days, respectively. The thermal thresholds for *E. biplaga* were 15.2, 11.3, 12.8 and 12.2°C and the thermal constants were 34.3, 195.1, 156.45 and 369.6 °D, for the completion of the egg, larval, pupal and egg-to-adult stages, respectively.

Key words: *Bacillus thuringiensis,* Bt spray*, Earias biplaga***,** degree-days, development, resistance, temperature

Table of content

List of Figures

List of Tables

Chapter 1
Introduction and literature review

1. Introduction

The growing population and the need for increased food and fibre production put a lot of pressure on farmers (Smith & McDonald, 1998). Research provides new technology, formulation and improved cultivars which are made available to the public (Sunding & Zilberman, 2002). Development and adoption of technologies that will increase plant production and minimise economic losses caused by insect pests are essential. Chemical insecticides were mainly used to control insect pests before the introduction of biotechnologically engineered (biotech) crops.

1.1. Biotechnologically engineered crops

Insect resistant crops have been transformed to express different *cry* genes that originate from the soil bacterium *Bacillus thuringiensis* (Bt) (Höfte & Whiteley, 1989). The Cry protein is an endotoxin to different insect groups, including those of agricultural importance and acts as a self-producing insecticide (Crickmore *et al.,* 1998; Perlak *et al.*, 2001).

There was a steady growth and adoption of biotech crops in the world since its inception in 1996 (Figure 1.1) (James, 2013). In 2011, 160 million hectares of biotech crops was planted globally (James, 2011), and it increased to 175.2 million hectares in 2013 planted by 18 million farmers, in 27 countries (James, 2013). The global area of genetically modified cotton increased rapidly from 1996, with 800,000 hectares to 5.7 million hectares in 2003 (James, 2003). The aggregated global cotton lint production during 2012 was approximately 26 million tons (FOASTAT, 2015).

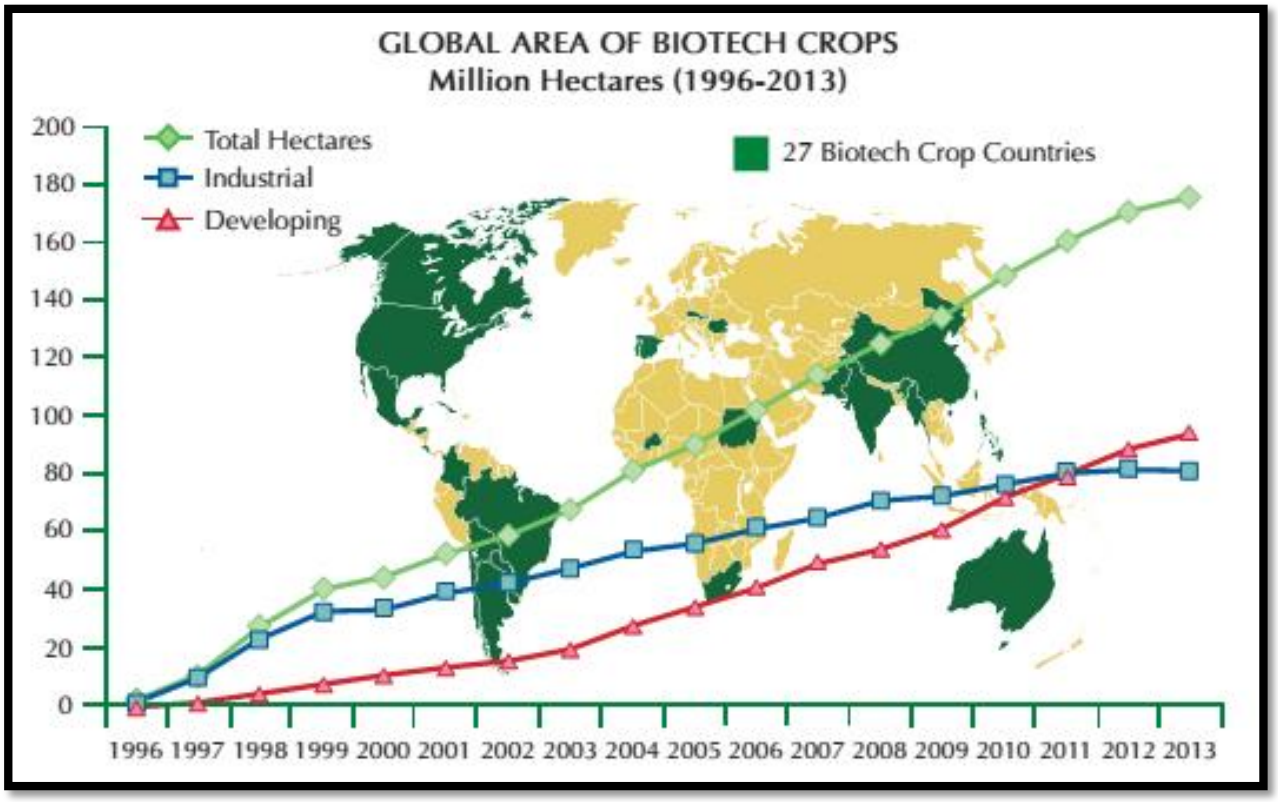

Figure 1.1 Global area of biotech planted crops (James, 2013).

The first Bt cotton in Australia was Ingard®, also known as Bollgard® elsewhere in the world, expressing the Cry1Ac protein (Carpenter *et al.*, 2002; Whitburn & Downes, 2009). During the 2004/05 growing season Ingard cotton in Australia were replaced by Bollgard II® which expressed the Cry2Ab and Cry1Ac proteins (Whitburn & Downes, 2009). Bollgard II® cotton production in Australia declined from 2005 to 2008 from 230,000 hectares to 61,000 hectares due to competition with other crops (Whitburn & Downs, 2009).

South Africa is one of the first countries in Africa where GM crops were approved (James, 2013), and include maize, cotton and soybean (Biosafety SA, 2013). Sixteen GM events were approved for general release, under which six were cotton cultivars produced by Monsanto as summarised in table 1.1 (Biosafety SA, 2013).

Table 1.1 GM cotton events approved for general release in South Africa (Biosafety SA, 2013).

GM event	Resistance	Cry proteins	Year approved
Bollgard ll x RR flex (MON15985 x MON88913)	Insect resistant (IR) and herbicide tolerant (HT)	Cry1Ac and Cry2Ab2	2007
MON88913 (RR Flex)	Herbicide tolerant (HT)	Absent	2007
Bollgard® RR	Insect resistant (IR) and herbicide tolerant (HT)	Cry1Ac	2005
Bollgard ll® 15985	Insect resistant (IR)	Cry1Ac and Cry 2Ab2	2003
RR line 1445	Herbicide tolerant (HT)	Absent	2000
Bollgard® line 531	Insect resistant (IR)	Cry1Ac	1997

The area planted to GM crops South Africa during the 2013 growing season was 2.9 million hectares of which 2.4 million was maize, 478000 hectares is herbicide tolerant soybeans and 8000 hectares of cotton (James, 2013).

1.2 History of cotton in South Africa

The first cotton seed in South Africa was planted in the Western Cape in 1960, about 38 years after Jan van Riebeeck arrived in the Cape (Cotton SA, 2012). It was officially declared an agricultural crop in 1939 according to the Co-operative Societies Act (Act 29 of 1929.

1.3 Bt- cotton

The first Bt cotton was planted in 1996 in Australia, and grown commercially in New South Wales and Queensland to control lepidopteran pests including bollworm and budworm using Bollgard® genetics (Pedigo, 2002; Wilson *et al*., 2013). Bt cotton is cultivated in South Africa since 1998 (Cotton SA, 2012). Of the total cotton production in 2013 in South Africa, 8000 ha genetically modified cotton planted, accounted for 95% of the country's cotton production of which 95% was stacked and the remaining

5% herbicide tolerant, the latter being used as refuge areas (James, 2013). Cotton production in South Africa has declined from 11000 hectares in 2012 to 8000 hectares in 2013 (James, 2013). Production of cotton lint in South Africa was 426 165 tonnes and cotton seed 729 965 tonnes in the 2013 production year (FAOSTAT, 2015). The decline in cotton production resulted from competition with soybean and maize (James, 2013).

1.4 Management strategy for Bt-Cotton

The possibility for insect pests to develop resistance to insect-tolerant crops that have the Bt-genes responsible for expressing the Cry proteins is possible due to the capability of insects to develop resistance to chemicals rather quick (McGaffery, 1998; Pray *et al*., 2002; Bennett-Nell *et al*., 2005; Alvi *et al*., 2012). Bollworms is one of the worst cotton pests in China and chemical insecticides with different modes of action are used for its control (Pray *et al.*, 2002). These include chlorinated hydrocarbons, organophosphates, pyrethroids and even mixtures of pyrethroids, organophosphates and other chemicals including DDT (cholorinated hydrocarbons), even though the use of DDT is illegal. Bollworms became resistant to many of these chemicals rather quick and soon farmers used more pesticides on their cotton than any other field crop in China (Huang *et al*., 2002). The use of more pesticides has environmental and human health risks, and the development of Bt cotton reduced pesticide use, increased yields and reduced labour in China (Pray *et al*., 2002).

To preserve the benefits of Bt technology it is necessary to incorporate Integrated Pest Management (IPM) practices and to develop and implement insect resistance management (IRM) in the cropping system (Bennett, 2007). According to Kumar *et al.* (2008), the four strategies that can contribute to a more sustainable deployment of the insect resistant genes in transgenic crops are:

i. Gene strategies: The combination of two or more strategies by deploying one or several genes.
ii. Expression strategies: Expressed effectively in the plant or in a tissue-specific manner.

iii. Dose strategies: Produce a high dose of the endotoxin. A high-dose Bt plant for one pest species is not necessarily a high dose against another target pest (Huang *et al*., 2011).
iv. Field strategies: It should be grown with refuge areas, to act as a mixture of genes or a rotation of genes, to help prevent resistance development.

The Genetically Modified Organism Act (15 of 1997) of the Republic of South Africa, prescribes that a refuge area should be planted as part of an IRM strategy where insect resistant crops are grown. The distance that the adult moth will travel before mating must be considered when deciding on a location for the refuge area, and it is therefore important to plant the refuge area next to the Bt plants in order for cross-mating to take place (Cohen *et al*., 2000).
Cross-mating may be successful when a susceptible population is established next to a resistant population, insects from the different populations can then mate and produce heterozygote offspring that in turn is susceptible to the high dose Bt plants (Cohen *et al*., 2000). According to Bennett (2007) the prescribed refuge options for Bollgard® cotton are the following:

i. For each 100 ha of Bollgard® product planted, a refuge area of non-Bt cotton must be 20 ha of cotton where insecticides may be sprayed on the non-transgenic cotton
ii. For each 100 ha of Bollgard® product planted a refuge area of 5 ha non-Bt cotton must be planted where insecticides may not be sprayed on the non-transgenic cotton. The refuge area may not be sprayed with any products that contain Bt because it is possible to increase the risk of pest to become resistant to Bt crops (Monsanto, 2014).

A license agreement is signed with the company providing the seed that one of the above mentions choices for the planting of refuge will be followed (Bennett, 2007; Monsanto, 2012; Monsanto, 2014).

1.5 Resistance development to Bt cotton

The development of resistance in Bt cotton is of great concern. Cases of field resistance development against Bt cotton in insect pest populations have been reported in the following countries:

- United States - *Helicoverpa zea* (Lepidoptera: Noctuidae) to Bt cotton expressing Cry1Ac and Cry2Ab (Anikulmar *et al*., 2008; Tabashnik *et al*., 2009).
- Northern China - *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Bt cotton expressing Cry1Ac (Zhang *et al*., 2011; Tabashnik *et al*., 2012).
- Pakistan - *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Cry1Ac in the field of Bt cotton (Alvi *et al*., 2012).
- India and China - *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) developed field evolved resistance to Cry1Ac on cotton (Dhurua & Gujar, 2011; Alvi *et al*., 2012; Tabashnik *et al*., 2012; Wan *et al*., 2012).

Cases of field resistance of other lepidopteran pests to other Bt crops have also been reported from:

- South Africa - *Busseola fusca* (Lepidoptera: Noctuidae) to Bt maize expressing the Cry1Ab protein (Van Rensburg, 2007; Kruger *et al*., 2009).
- Puerto Rico - *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to Bt maize expressing the Cry1F protein (Tabashnik *et al.,* 2009).

It is important to evaluate the possibility of resistance development in insect pests regularly. It can then be noted in an early stage and the necessary research and control measures can be implemented (Tabashnik, 2008).

1.6 Cotton Pests

There are a number of insect pests that is of great concern to the cotton growing industry globally, and a constant concern for cotton growers (Malinga, 2010). A list of arthropod pests that attack cotton in South Africa is provided in Table 1.2.

Table 1.2 Cotton pests in South Africa (Bennett, 2015).

Order	Group of pests	Pest species common name	Scientific name
Lepidoptera	Bollworm	African bollworm Spiny bollworm Red bollworm Pink bollworm	*Helicoverpa armigera* (Hübner) (Noctuidae) *Earias biplaga* Walker (Noctuidae) *Diparopsis castanea* Hapson (Noctuidae) *Pectinophora gossypiella* (Saunders) (Gelechiidae)
Lepidoptera	Leaf caterpillars	Tomato semi-looper Cabbage semi-looper Cotton semi-looper Leaf worm Cotton leaf worm	*Chrysodeixis acuta* (Walker) (Noctuidae) *Trichoplusia orichalcea* (F.) (Noctuidae) *Anomis flava* (F.) (Noctuidae) *Xanthodes graellsi* Feisthamel (Noctuidae) *Spodoptera littoralis* (Boisduval) (Noctuidae)
Lepidoptera	Cutworms	Black cutworm Brown cutworm Common cutworm	*Agrotis ipsilon* (Hufnagel) (Noctuidae) *Agrotis longidentifera* (Hampson) (Noctuidae) *Agrotis segetum* (Denis & Schiffermüller) (Noctuidae)
Coleoptera	Leaf beetles	Black cotton beetle	*Syagrus rugifrons* Baly (Chrysomelidae)
Hemiptera	Cotton aphid	Cotton aphid	*Aphis gossypii* Glover (Aphididae)
Hemiptera	Tobacco whitefly	Whitefly	*Bemisia tabaci* (Gennadius) (Aleyrodidae)
Hemiptera	Leafhoppers	Cotton leafhopper Leafhopper	*Jacobiella facialis* (Jacoby) (Cicadellidae) *Jacobiasca libyca* (de Bergevin & Zanon) (Cicadellidae)
Hemiptera	Cotton stainers	Dusky cotton stainer Cotton stainer Cotton stainer Cotton stainer	*Oxycarenus hyalinipennis* (Costa) (Lygaeidae) *Dysdercus fasciatus* Signoret (Pyrrhocoridae) *Dysdercus nigrofasciatus* Stål (Pyrrhocoridae) *Dysdercus intermedius* Distant (Pyrrhocoridae)
Trombidiformes	Mites	Red spider mite Carmine cotton mite Dark red spider mite	*Tetranychus cinnabarinus* (Boisduval) (Tetranychidae) *Tetranychus lombardinii* Baker & Pritchard (Tetranychidae) *Tetranychus ludeni* Zacher (Tetranychidae)

Bollworm as a complex is responsible for the most insect damage to cotton in South Africa, with three different species namely the African bollworm (*H. armigera*), red bollworm (*D. castanea*) and spiny bollworm (*E. biplaga* and *E. insulana*) (Bennett, 2007; Malinga, 2010). For the purpose of this study, emphasis will be placed on one of the spiny bollworm species (*E. biplaga*).

1.7 Spiny bollworm

There are seven species of spiny bollworms but only two of these species occur in Africa namely *E. biplaga* and *E. insulana* (Bennett, 2015). These pests are common in the cotton growing regions, and the damage caused by spiny bollworm is often underestimated (Bennett, 2007). There are very few differences between the larvae of the two species. The larvae with an orange-brown appearance seem to be *E. biplaga* and the yellower-green larvae seem to be *E. insulana* (Pearson & Darling, 1958; Bennett, 2007). The colour pattern of the forewing of moths distinguishes the two species (Bennett, 2015). The colour of the forewings of *E. insulana* may vary from silvery green to straw yellow and the outer fringe has the same colour (Bennett, 2015). In *E. biplaga* the colour of the wings may vary from metallic green to gold and the outer fringe has a brownish colour (Bennett, 2015).

Characteristics of the juvenile life stages of *Earias* spp. as described by Hashmi (1994) are as follows:

- Eggs: The eggs have a spherical shape, and vary from light green to blue in colour and there is about 30 parallel longitudinal ridges on the surface. Every alternated ridge point upwards to form a crown like structure, the egg is about 0.5mm in diameter).
- Larvae: Neonate larva has a dark head and is brownish in colour. Tubercles become prominent about four days after emergence on the second and third thoracic, and first abdominal segment. It is about 1.3 mm in length.
- Final instar larvae: A fully grown larva is spindle shaped and there are spine like hairs or setae on each segment of the body. The name spotted bollworm relates to the two pairs fleshy tubercles on the abdominal segments and last two thoracic segments of the larvae. These tubercles may vary in shape and

size and one pair is lateral while the other pair is dorsal. Protuberances are found on the last three abdominal segments.

- Pupae: The pupa is protected by a closely woven silk cocoon which is light brown or white in colour and represents the shape of a boat. The pupa is purplish brown in colour and has a distinct medium carina on the thorax.
- Moths: The moths have a silvery creamy white abdomen and the hind wings are uniform and the same colour as the abdomen. The fore wings vary in colour from species to species and may include patterns. Copulation is affected by temperature and takes place between 01:00 and 07:00. Eggs are laid during the night and temperature influences the capacity of the females to lay eggs (Hashmi, 1994). One female can lay about 385 eggs (Vennilla *et al*., 2007). The eggs are laid on flowers, buds young shoot tips and cotton balls (Hashmi, 1994; Vennila *et al*., 2007; Bennett, 2015). According to Hashmi (1994) research from Pakistan showed that the neonate larvae hatch about three to four days after oviposition during the summer, but during winter it takes about seven to nine days for neonate larvae to hatch.

Spiny bollworms feed mostly on cotton plants in South Africa, but if there is no cotton available they feed on other wild host plant species like *Abutilon* (Malvaceae)*, Cienfuegosia* (Malvaceae)*, Sida* (Malvaceae) and *Hibiscus* (Malvaceae) (Green *et al*., 2003). Damage is done to the growth-points or internodes by larvae tunnelling into it and feeding on the soft growing tissue, the flower buds or green cotton bolls (Hashmi, 1994, Bennett, 2015). The larvae block the entrance with excreta (Bennett, 2015).
The pest status of spiny bollworms can be significant and yield losses of more than 10 % were reported in New Delhi, India (Venilla, 2007). It is speculated that if the growing season of cotton in South Africa is largely extended the pest status will probably increase (Bennett, 2015). At 600 m above sea-level, there is a greater chance *of Earias*- species investing crops, this might be because of the abundance of more host plants (Bennett, 2015). No literature is currently available in South Africa on the influence of temperature on the development of the *Earias* spp. which may also influence the pest status in cotton growing areas.

According to Shah *et al*. (2012) the life cycle of the *Earias vittella* was noted to be dependent on the temperature, and studies from Sindh Pakistan showed that development of *E. vittella* at a temperatures of 27°C was longer than the development at 35 °C. This data is important to understand the biology and survival of pests.

1.8 Control methods for spiny bollworm

Spiny bollworm numbers can be suppressed if good Integrated Pest Management (IPM) strategies are followed. It should include chemical- , biological- and cultural control as well as host plant resistance (Hashmi, 1994).
Cultural control:

Spiny bollworms can be affected by cultural practices such as sanitation and there are a few recommendations which may help to decrease the pest status. These include:

- Destruction of wild host plants. Host plants can provide shelter during the winter which may contribute to the following season's pest status (Hashmi, 1994).
- Stems should be cut under the soil surface, cotton plant residues should be removed after harvest and the field should be ploughed (Hashmi, 1994; Malinga, 2010).
- The planting dates should be planned in such a manner that the crop is in its most susceptible stages during periods when the pest is present at low numbers (Malinga, 2010).
- Late irrigation should be avoided (Hashmi, 1994).
- In cotton producing areas where okra is also cultivated, all okra should be eradicated before planting since okra is a very suitable host plant for the spiny bollworm (Hashmi, 1994).
- Crop rotation or intercropping can help reduce the pest status (Malinga, 2010).

Biological control:

Eilenberg *et al.* (2001) defined biological control as “the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be”. Natural enemies can reduce pest numbers, and can be of great advantage in cotton fields by postponing the use of chemicals (Malinga, 2010). In Pakistan there are 27 parasite species reported that can help control *Earias* species which include *Elasmus johnstoni* (Hymenoptera: Elasmidae), *Rogas testaceu* (Hymenoptera: Braconidae) and *Goryphus nursei* (Hymenoptera: Ichneumonidae) (Hashmi, 1994).

Chemical control:

The use of chemicals to control bollworms should be done before larvae tunnel into the fruiting bodies of the plant, thus during the first and second larval stage of their development (Hashmi, 1994; Malinga, 2010). A threshold level for chemical control of bollworm on the fruiting parts is a 5-10% infestation in Pakistan (Hashmi, 1994). In South Africa, an economic threshold level for spiny bollworms is two larvae per 24 plants (Malinga, 2010). Scouting is very important, and all insecticide applications should be based on exceeding of the threshold level.

Host plant resistance:

Host plant resistance should also be considered when choosing a new cultivar for production. Three different plant resistance concepts can be described.

- Antibiosis can be described as the resistance mechanism that involved characteristics of a plant that have a negative effect on the insect survival (Manglitz & Danielson, 1992). This can influence mortality in insect pests and may reduce their longevity and reproduction (Manglitz & Danielson, 1992; Teetes, 2009). It can be caused by chemicals produced by the plant, that for example can reduce growth in insect pests or this can be because of structures produced by the plant such as trachoma’s that prevent an insect to harm the plant (Chadwell *et al.,* 2005).
- Antixenosis or non-preference is a resistance mechanism that influences an insect’s behaviour (Manglitz & Danielson, 1992).

- Tolerance describes the plant's response to damage (Teetes, 2009) and represents how the plant grows, its reproduction and repairing ability to damage caused by insects or other herbivores (Manglitz & Danielson, 1992).

1.9 The effect of temperature on cotton

The growth and development of the cotton plant is highly dependent on temperature (Ritchie *et al.*, 2004). Growth ceased when the daily mean temperature is below the threshold of 15.5 °C but when the temperature rises above this critical threshold, growth can increase (Macaskill, 2010). The relationship between the growth rate and temperature are used to determine the timing for various developmental stages of the plant (Macaskill, 2010). The growth of cotton during the season can therefore be calculated by using the heat units of a crop over time (Ritchie *et al*., 2004).
Root development is also dependent on heat units, and if the temperature is below 10 °C during germination the growing root point can be destroy and the seedling will die or will never develop into a productive plant (Macaskill, 2010).

1.10 Correlation of temperature between spiny bollworm and cotton

In order to manage pests in the field, the importance of temperature in the development of the pest and the plant should be understood (Shah *et al*., 2012; Kandil, 2013). Temperature is crucial when it comes to population dynamics and the fluctuation thereof under field conditions and season changings (Kandil, 2013). Whenever the conditions and temperature in the field and the growth and growth stage of the plant, in this case cotton, is favourable for the pest, the pest will have everything in its favour to develop and reproduce. Development and reproduction will be affected by unfavourable elements, and even one element can lead to a decline in the pest status (Ismail *et al*., 2005; Shah, 2012; Kandil, 2013).

1.11 Biopesticides

Biopesticides are biological products or organisms, which are produced from biological sources outside the field and can include viruses, bacteria, fungi, predators, parasites and pheromones (Gupta & Dikshit, 2010). More than 430 biopesticide active ingredients and 1320 active products were registered in 2014 (Flores-Lopez *et al.*, 2015).

1.12 *Bacillus thuringiensis* as a microbial pesticide

Although Bt sprays or Bt spray products were available for farming systems, it is more known in the organical farming industry since 1930 for the control of insect pests (De Maagd *et al*., 1999). Formulations of Bt are mostly used within the agricultural system, but can also be used in food storage facilities, soil and water environments and foliage. These products include spray concentrations, wettable powders, dusts, liquid concentrates, baits and time released rings (USDA, 2014). The use of bio-pesticides, which mostly consist of Bt sprays, is less than 1% of the crop protection market, although the forestry industry in Europe and North America has effectively replaced chemical control with Bt for control of defoliating larvae.

The crystal proteins are responsible for the control of pests and are usually inactive within hours or days (USDA, 2014). Bt is species specific and there are a lot of different strains that work on certain species but usually only target a certain pest or pest group, this is why non-target species and beneficial insects are usually not harmed during application (Copping & Menn, 2000; USDA, 2014; Flores-Lopez *et al*., 2015). Some of the popular Bt strains according to USDA (2014) that are used, are listed in table 1.3

Table 1.3 Popular Bt strains used currently (USDA, 2014).

***Bt* Strain**	**Effective against**
Bt kurstaki (*Btk*)	Lepidopterous insect pests, gypsy moth, cabbage looper
Bt aizawai (*Bta*)	Wax moth larvae in honeycombs
Bt israelensis (*Bti*)	Mosquitoes, blackflies, midges
Bt san diego	Certain beetle species, boll weevil

Dipel is the most used Bt *kurstaki* (HD-1 strain) product, and is used to control over 100 species of Lepidoptera globally. It is registered for the control of the African bollworm, diamondback moth, pine tree emperor moth, leafrollers, orange dog caterpillar, lawn caterpillar, lily borer and semi-looper in South Africa (Van Zyl, 2013). Sprays against the African bollworm can be applied on apples, pears, beans, citrus, Cruciferae, herbs, peas, tomatoes, ornamentals, flowers and lawns (Van Zyl, 2013). South Africa, but not against the other two species in the bollworm complex, namely the red - and spiny bollworms. Compared to chemical insecticides, biopesticide is an environmentally friendly option of pest control (Hynes & Boyetchko, 2005) and its efficacy for control of the bollworm complex should be determined.

DiPel contains five different bacterial protein toxins and spores which include: Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cp-ry2Ab and living spores thus the diversity of proteins and spores makes this insecticide suitable for the use in insect resistant management programmes (Valent Bioscience, 2014).

1.13 *Bacillus thuringiensis* mode of action

Ingestion of Bt toxins can cause the insect to stop feeding minutes after the gut cells are damage due to crystal solubilisation and therefore cease the initial damage to the plant (Valent Bioscience, 2014). The larvae may eventually starve to death. Those not killed rapidly by direct action of the toxin may die from bacterial infection over a longer period (Copping & Menn, 2000).

Bt has a series of effects on larvae that ingest the insecticide (Copping & Menn, 2000). The larvae ingest the crystal proteins from the Bt spray or plant; once in the insect, the crystal proteins are solubilised within the midgut and converted into a combination of

smaller toxins (Copping & Menn, 2000). The toxins bind to the midgut of the insect and interfere with the potassium ion dependent active amino acid support mechanism (Copping & Menn, 2000). Large cation-selective spores are formed that increase the water permeability of the cell membrane causing the cells to swell and rupture, which lead to the disintegration of the midgut lining (Copping & Menn, 2000). The spores enter the blood cavity through the gut opening, germinate rapidly and cause blood poisoning (Valent Bioscience, 2014). Different toxins bind to different receptors within the different insect species and with varying intensities. The live spores within these products enhances the activity.

There are four DiPel formulations available on the market according to Valent Bioscience (2014):

- DiPel DF – Dry flowable
- DiPel ES – Emulsifiable suspension.
- DiPel 10G – Corn grit
- DiPel SG – Sand granule

1.14 Application of biopesticides such as DiPel

Biopesticides should be sprayed when larvae are still young, during early instars and before crop damage occurs (Hynes & Boyetchko, 2005; Valent Bioscience, 2014). The whole plant should be covered during application and environmental factors such as wind, rain, dew and sunlight which can have an effect on the field performance should be taken into account (Hynes & Boyetchko, 2005). Application should be repeated in intervals of usually three to fourteen days to maintain control, depending on factors such as weather conditions and growth rate of the plant (Valent Bioscience, 2014).

1.2 Problem statement and substantiation

Cotton is damaged globally by a complex of bollworm species, with the most important *Heliothis* spp., *Helicoverpa* spp., *Diparopsis* spp., *Earias* spp. and *Pectinophora* spp. (Hill, 1983). Genetically modified (GM) Bt cotton is cultivated in South Africa since 1998 to control lepidopteran pests. It is well known that pests may develop resistance to the insecticidal Cry proteins expressed in Bt cotton, thereby compromising sustainable cotton production. No evaluation of resistance of the spiny bollworm *(E. biplaga*) has been done. At its inception, all commercial Bt crop cultivars were effective against their specific major target pest species (Huang *et al*., 2011).Continued and widespread use of Bt products can have a negative effect on sustainability of the products and strong selection pressure can enhance the development of resistance to Bt- based insecticides (Ibargutxi, 2008; Zhang *et al.*, 2011). Thus is it necessary to evaluate resistance development in insect pests regularly.

Evaluation of the development of the spiny bollworm at constant temperatures will contribute to knowledge on the estimated number of generations per cotton production season in the different production areas and also to the development of an effective Integrated Pest Management strategy for spiny bollworm on cotton in South Africa. Their status of resistance to Bt proteins expressed by plants and contained in Bt sprays in South Africa should therefore be investigated to contribute to sustainable management.

1.3 Objectives

The objectives of the study was to:

1. Evaluate the status of resistance of the spiny bollworm (*E. biplaga*) (Walker) to Bt cotton.
2. Determine the efficacy of *Bacillus thuringiensis* spray applications for control of the spiny bollworm (*E. biplaga*).
3. Determine the development of the spiny bollworm (*E*. *biplaga*) at constant temperatures.

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Chapter 2

Evaluation of the status of resistance of the spiny bollworm (*E. biplaga*) (Walker) to Bt cotton.

2.1 Abstract

Although the bollworm complex of cotton in South Africa is dominated by the African bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and the red bollworm *Diparopsis castanea* (Hamps) (Lepidoptera: Noctuidae) the spiny bollworms *Earias biplaga* (Walker) (Lepidoptera: Noctuidae) *and* (*Earias insulana* (Boisduval) (Lepidoptera: Noctuidae) also attack this crop. Genetically modified (GM) Bt cotton is cultivated in South Africa since 1998 to control these lepidopteran pests. It is well known that pests may develop resistance to insecticidal Cry proteins expressed in Bt cotton, thereby compromising sustainable cotton production. No evaluation of resistance of spiny bollworm (*E.biplaga*) has been done in South Africa. The aim of this study was therefore to screen *E. biplaga* for resistance to Bollgard® and Bollgard II® cotton to confirm that it is still susceptible and if not, to determine the levels of tolerance already developed by this species. Results indicated that there is no resistance to Bollgard® and Bollgard II® cotton in South Africa.

2.2 Introduction

2.2.1 Damage caused by bollworms

The bollworm complex of cotton in South Africa consists of the African bollworm (*Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)), red bollworm ((*Diparopsis castanea* (Hamps) (Lepidoptera: Noctuidae)) and the spiny bollworms (*Earias biplaga* (Walker) (Lepidoptera: Noctuidae)) *and* ((*Earias insulana* (Boisduval) (Lepidoptera: Noctuidae)) (Bennett, 2007). Larvae of these species tunnel into the growing tips, flowering buds and cotton bolls, resulting in damage and subsequent yield losses (Bennett, 2015).

Bollworms start to attack cotton plants from flowering until mature bolls are present (Van Hamburg & Guest, 1997). Shedding of cotton bolls occur when a plant is stressed, resulting in yield losses. Bollworms tunnelling into the cotton bolls, cause damage to the skin and lint, which in turn may cause secondary pests and pathogens to destroy the leftover lint (Ahmed *et al*., 2012; Bennett, 2015). There is a long flowering period followed by forming and maturation of bolls. It results in a long period of possible injuriousness by *E. bipalga*. This long period of vulnerability impedes control of this pest.

Spiny bollworms are regarded as major pests of cotton due to their wide distribution and the significant impact the larvae can have on boll development and yield (Bennett, 2015).

Genetically modified (GM) transgenic cotton plants that express Cry proteins (Bt cotton) had been cultivated in South Africa for control of these lepidopteran pests (Thirtle *et al.,* 2003).

Resistance to Bt-cotton may develop in pest populations if species are constantly exposed to GM cotton expressing only one Bt gene (Yang *et al*., 2013). Bollgard®, expressing the Cry1Ac protein, was first commercially produced in South Africa in 1998 and removed from the marked after the 2010 growing season (ICAC, 2007). Bollgard ll®, also registered for control of economically important lepidopteran pests on cotton, is a stacked variety and expresses two Bt proteins, Cry1Ac and Cry2Ab2 (Taverniers *et al*., 2008; Showalter *et al*., 2009).

2.2.2 Resistance development

Cases of resistance development to Cry1Ac protein expressed by Bollgard® cotton has been confirmed in Pakistan and northern China for *H. armigera* (Zhang *et al*., 2011; Alvi *et al*., 2012), in India and China for *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Dhurua & Gujar, 2011; Alvi *et al*., 2012; Wan *et al*., 2012;) and the United States for *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Luttrell *et al*., 2004; Anikulmar *et al*., 2008; Tabashnik *et al*., 2009; Tabashnik & Carrière, 2010).

2.2.3 Gene pyramiding and the high dose refuge strategy

Gene pyramiding implies introducing multiple genes, each with its own independent mechanism of action against the target pest (Taverniers *et al.,* 2008). This combination of genes may include multiple modes of actions such as insect control (Bt) or glyphosate tolerance (Taverniers *et al*., 2008; Showalter *et al*., 2009). Pyramiding with more than two genes also exists, for example the Bollgard II® in RoundupReadyFlex cotton which is a triple stack, containing Cry1Ac and Cry2Ab2 genes as well as the trait for glyphosate resistance (Taverniers *et al*., 2008; Showalter *et al*., 2009). The possibility for insect pests to develop resistance to insect-tolerant crops that express the insecticidal proteins is highly likely since insects have the capability to develop resistance to chemicals rather quickly (Bennett-Nell *et al*., 2005).

The high-dose / refuge strategy has been agreed to be the most practical approach to prolong the effectiveness of Bt crops (Cohen *et al*., 2000). It is essential to remember that a high-dose Bt plant for one pest species is not necessarily a high dose for another target pest (Huang *et al*., 2011). Refuges consist of areas planted with non-Bt plants (Cohen *et al*., 2000), adjacent to an area with Bt plants that express a high dose of the Bt toxin. The refuge strategy has two critical assumptions: that inheritance of resistance is recessive and that mating between the resistant and susceptible insects occur randomly (Liu *et al*., 1999). The hybrid first generation offspring produced by mating between susceptible and resistant adults are killed when they feed on Bt plants. If the mating is random, mating between the rare homozygous resistant adults that

emerged from Bt plants will more likely be with the homozygous susceptible adults that emerges from the non-Bt plants. Mating between these adults produce hybrid F1 progeny that cannot survive on Bt plants (Liu *et al*., 1999) (Fig. 2.1). The offspring that is not resistant to the Bt toxin will complete their life cycle if they are in the refuge area and will again produce offspring that is not resistant to Bt. Refuges should therefore be planted adjacent to the Bt cropping area so that cross-mating can take place between the resistant and susceptible individuals (Cohen *et al.,* 2000).

This will not prevent resistance development but is set in place to delay the process (Van den Berg *et al*., 2013). Field evolved resistance is defined as a genetically based decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field (Tabashnik, 1994).

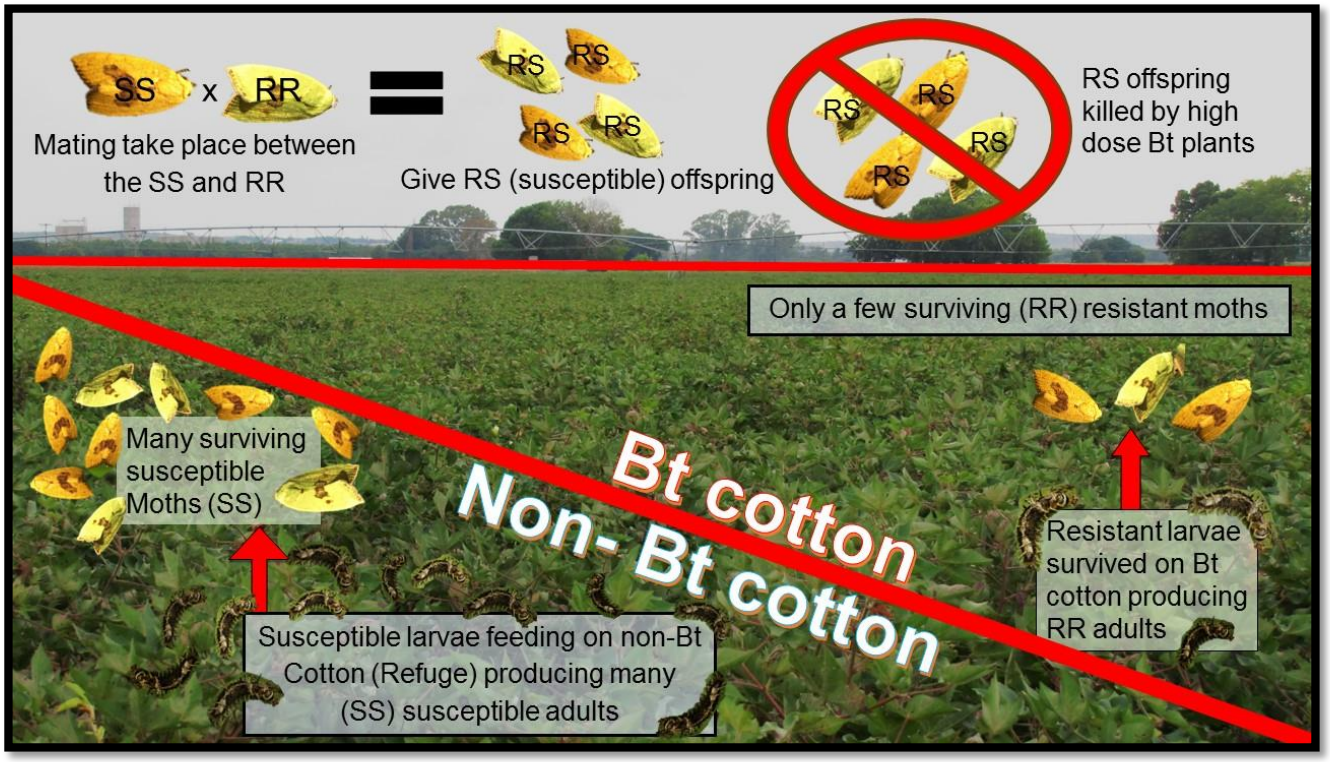

Figure 2.1 Cross mating between susceptible and resistant insect pests that fed on the refuge and Bt crops respectively.

It is thus necessary to have a high dose of the Cry toxin in the Bt cultivar that will kill almost all the RS insects (Cohen *et al*., 2000).The high-dose strategy, applied in South Africa, requires that Bt plants express a high concentration of Bt proteins to ensure that heterozygous insects that have one major resistance allele are killed (Andow, 2008; Huang *et al*., 2011). According to Tabashnik *et al*. (2008) and Tabashnik *et al.* (2009) the refuge strategy contributed to the delay of resistance development in insect pests, other than in the case of the pink bollworm in India where farmers neglected the compliance to refuge strategy requirements (Stone, 2004; Bagla, 2010).

To preserve the technology and Bt cotton that have been developed, research is needed to monitor pest populations on a continuous basis for possible resistance development to Bt proteins in order to prolong the benefits incurred from cultivating Bt cotton.

2.2.4 Objectives

Screening of *E. biplaga* for resistance to Bt cotton has never been done in South Africa. It is important that monitoring of Bt crops is done to evaluate changes occurring in the field, and to regularly test larvae in the laboratory to evaluate the level of resistance. The aim of the current study was therefore to determine the response of *E. biplaga* larvae from two different populations to Bt cotton in South Africa.

2.3 Materials and methods

2.3.1 Spiny bollworm stock colonies

Spiny bollworm larvae were collected from a cotton field at Potchefstroom (S26°68’668”, E27°15’801”) and moths were collected with light traps (Fig. 2.2) at Rustenburg (S25°72’401”, E27°28’944”), South Africa during the 2013/14 growing season.

Larvae were reared in cotton bolls in plastic containers (100 ml) covered with steel mesh in an insect rearing room at 26 ±1 °C, 70 ± 10 % RH and a 14L: 10D photoperiod

until pupation. Once the moths emerged, sugar water was provided as a food source and the moths were kept in plastic containers (40 x 15 x 20 cm), covered with an aerated plastic lid. Cotton plant material was used as stimulus for egg production and cotton wool as oviposition substrate.

Cotton wool with the eggs was removed from the containers and transferred to small plastic containers (52 mm high and 30 mm in diameter) with steel mesh infused lids. These plastic containers were kept in a glass desiccator (150 mm) in which RH was maintained at 70 ± 10 % using a potassium hydroxide solution according to the method of Solomon (1951). The desiccators were kept in the rearing room at 26° ±1°C and 14L:10D.

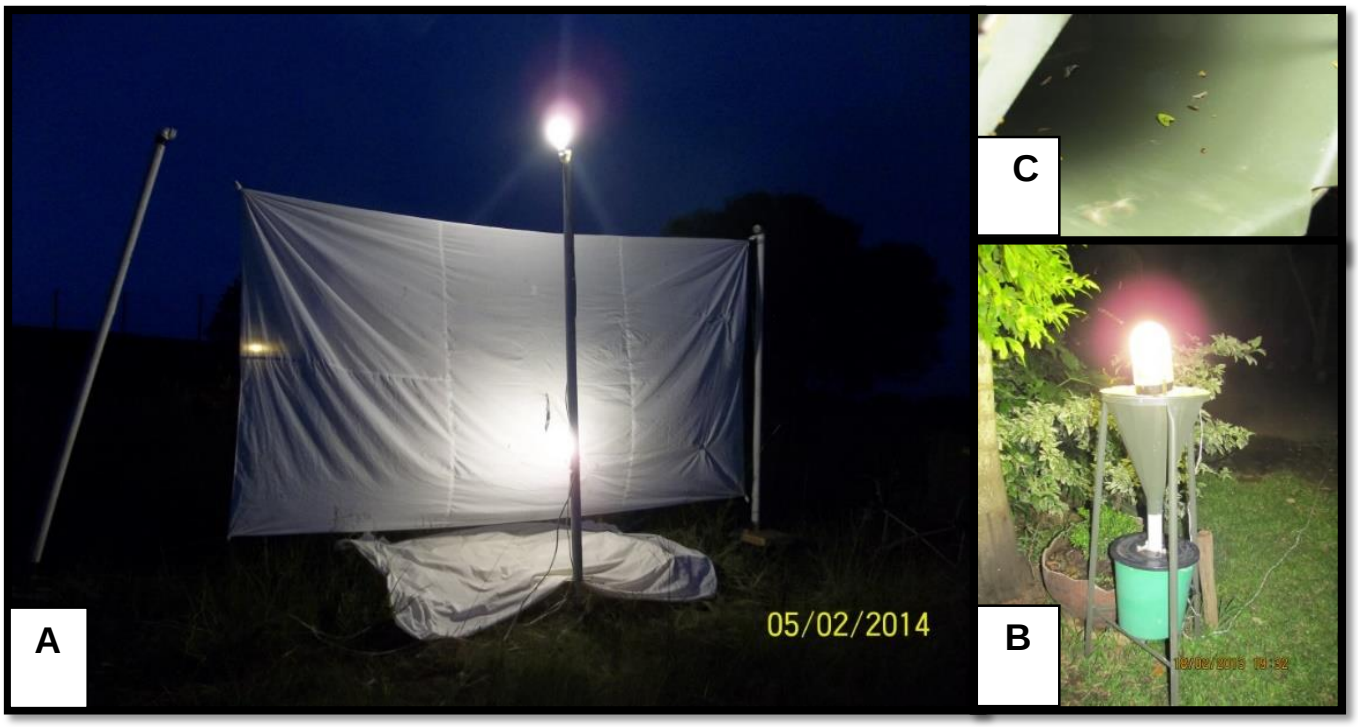

Figure 2.2 Light traps used to collect spiny bollworm moths.

2.3.2 Feeding study (Rustenburg and Potchefstroom populations)

2.3.2.1 Susceptibility bioassays

Bioassays to evaluate for possible resistance development to Bollgard® and Bollgard II® by two spiny bollworm populations (Rustenburg and Potchefstroom populations) were conducted using squares, cotton boll slices and cotton bolls (Fig. 2.3). These three different types of plant tissue were used to determine if larval mortality would be differentially affected by feeding on the softer inner parts of the cotton bolls that include cotton lint and immature seeds, or on cotton bolls with the outer capsule still in place. The experiments with squares and cotton boll slices were repeated twice and the bioassay using cotton bolls, was repeated three times with each population. Each bioassay consisted of three cotton treatments, namely Bollgard®, expressing the Cry1Ac protein, Bollgard ll®, a stacked variety expressing both the Cry1Ac and Cry2Ab2 proteins and the control treatment, a non-GM cultivar, Delta Opal.

Experiments using the Potchefstroom population consisted of 3 treatments and 6 replicates for cotton squares and cotton boll slices. Experiments on cotton bolls had 20 replicates. Experiments using the Rustenburg population also consisted of 3 treatments and 6 replicates for cotton squares and cotton boll slices. One of the experiments on cotton bolls for the Rustenburg population had 10 replicates and the other one, 5 replicates per treatments. Five neonate larvae were transferred to individual cotton squares, cotton boll slices or cotton bolls of the respective treatments for all experiments.

If there was any surviving larvae present after the 4th day, they were separated and kept individually. This was done to prevent any cannibalism and competition. Squares and cotton bolls were placed individually in plastic containers (55 mm high x 50 mm in diameter) closed with steel mesh infused lids while cotton boll slices were kept in Petri dishes. The containers and Petri dishes were kept in an incubator at 26±1 ºC with a 14L: 10D photoperiod.

Mortality was recorded and food was replaced on day 4, 7, and 10 after inoculation of larvae. The number of dead larvae was recorded at each time that fresh food was provided and expressed as a percentage of the initial number of larvae. The

experiments were terminated when 100% mortality was recorded on the Bt cotton cultivars.

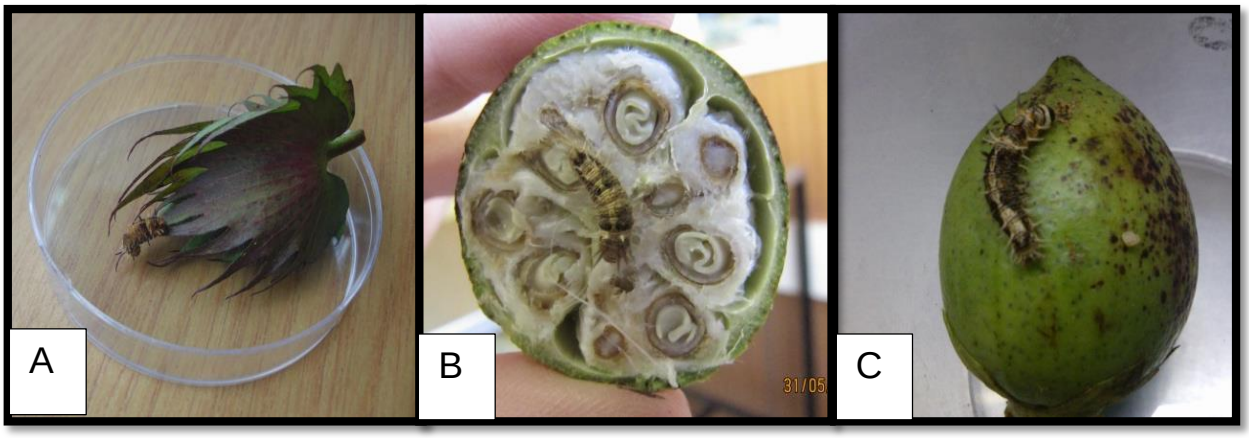

Figure 2.3 Squares (A), cotton boll slices (B) and cotton bolls (C) were used in bioassays to determine efficacy of Bollgard® and Bollgard ll® against spiny bollworm.

2.3 Data analysis

Two by two tables were used to evaluate the association between exposure to Bt toxin and mortality of spiny bollworm larvae. The odds ratios (OR) were calculated for each experiment 4, 7 and 10 days after inoculation. The OR represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure. Each Bt treatment was therefore compared to the control 4, 7 and 10 days after inoculation. Asterisks denote a significant difference with Bonferroni post hoc test adjusted to $P<0.0001$. The Pearson Chi-square was calculated to indicate whether there was a significant difference in mortality of *E. biplaga* between the cultivars evaluated 4, 7 or 10 days after feeding. The data was analysed using STATISTICA version 11 (StatSoft, Inc., 2015).

2.4 Results

2.4.1 Susceptibility bioassays

2.4.1.1 Cotton squares

Mortality of larvae 4, 7 and 10 days after inoculation on Bollgard® and Bollgard II® cotton squares differed significantly from mortality of larvae feeding on squares of the non-Bt cultivar for both the Rustenburg (Table 2.1) and Potchefstroom populations (Table 2.2) in experiments 1 and 2. Mortality increased over time and no larvae from the Rustenburg and Potchefstroom populations survived 10 days after initial feeding on Bollgard® and Bollgard II®. Most neonate *E. biplaga* larvae from the Rustenburg population died within 4 days of initial feeding on both Bollgard® and Bollgard II® cotton (Table 2.1). The odds for larvae that fed on both Bt cultivars not to survive 10 days after initial feeding, were similar when compared to the conventional cultivar (Table 2.1). Compared to the conventional cultivar, larvae from the Potchefstroom population feeding on Bollgard II® are more likely to die 7 days after initial feeding than those that fed on Bollgard® in both experiments (Table 2.2). However, 10 days after initial feeding, the likelihood of larvae to die on both Bollgard® and Bollgard II®, compared to non-Bt cotton squares, was similar in experiments 1 and 2 (Table 2.2).

2.4.1.2. Cotton slices

Mortality of larvae 4, 7 and 10 days after inoculation on Bollgard® and Bollgard II® cotton boll slices differed significantly from mortality of larvae feeding on the non-Bt cultivar for both the Rustenburg (Table 2.3) and Potchefstroom populations (Table 2.4) in experiment 1 and 2. Mortality increased over time and the mortality rate was already high 4 days after initiation of feeding. No larvae survived on either Bollgard® or Bollgard II® cotton boll slices 7 days after feeding commenced. Most neonate *E. biplaga* larvae from the Rustenburg population died within 4 days of initial feeding on both Bollgard® and Bollgard II® cotton. (Table 2.3). The odds for larvae that fed on both Bt cultivars not to survive 10 days after initial feeding, were similar when compared to the non-Bt cultivar (Table 2.3). Compared to the non-Bt cultivar, larvae from the Potchefstroom population feeding on Bollgard II® are more likely to die 7 days after initial feeding than those that fed on Bollgard®. (Table 2.2). However, 10 days

after initial feeding, the likelihood of larvae to die on both Bollgard® and Bollgard ll®, compared to conventional cotton squares, was similar in experiments 1 and 2 (Table 2.2).

2.4.1.3. Cotton bolls

Results for *E. biplaga* larval mortality after feeding on Bollgard® and Bollgard ll® cotton bolls were similar to mortality after feeding on squares and cotton boll slices. Mortality of larvae 4, 7 and 10 days after inoculation on Bollgard® and Bollgard ll® cotton bolls differed significantly from mortality of larvae feeding on bolls from the non-Bt cultivar for both the Rustenburg (Table 2.5) and Potchefstroom populations (Table 2.6) in experiments 1 and 2. Mortality increased over time and the mortality rate was already high 4 days after initial feeding for both populations. All larvae (except for experiment 1) of the Rustenburg population died within 4 days of feeding on Bollgard II® bolls and within 10 days on Bollgard®. More larvae from the Potchefstroom population (Table 2.6) survived a prolonged time on Bollgard® and Bolgard II® when compared to the Rustenburg population (Table 2.5), but mortality was also high from day 4 onwards. The odds for larvae from both populations that fed on cotton bolls of both Bt cultivars not to survive 10 days after initial feeding, were, however similar when compared to the non-Bt cultivar in all experiments (Table 2.3).

2.5 Discussion

Bt protein in Bt cotton is produced throughout the growing season but studies showed that the levels may fluctuate during the season (Fitt, 1998). The concentration of Bt protein in a plant decreases during the final stages of plant development (Kranthi *et al*., 2005). The Bt expression levels in flowers is the lowest followed by the young cotton bolls and the squares, with the highest expression in the leaves (Kranthi *et al*., 2005). The latter authors also reported a decline in Bt protein production of Cry1Ac within the plant during the season to such an extent that the Bt expression levels were less than the critical level of 1.9 µg/g 10 days after sowing. Although concentrations of Bt toxins in the different plant parts were not determined in this study it was still

sufficient to kill neonate *E. biplaga* larvae. Spiny bollworm larvae took longer to die on Bollgard I® compared to Bollgard II®, but all larvae died within 10 days of initial feeding on cotton squares, boll slices as well as bolls of both cultivars.

Earias biplaga larvae were 100% controlled on squares, cotton boll slices and cotton bolls of both cultivars 10 days after initial feeding, confirming no resistance development to either of these cultivars. Longer survival of larvae on Bollgard® cotton compared to Bollgard II® may be ascribed to the latter having two Bt toxins and Bollgard® containing Cry1Ac only.

It is crucial to cultivate Bt crops according to rules and regulations, because this contributes to prevention of resistance development against these pests. There are many advantages of Bt crops which include less pesticide usage. According to Ferry *et al*. (2006) there was a 21 000 ton reduction in pesticide usage in the USA in 2003, and Indian farmers had a 70% reduction in insecticides applied to cotton fields which was a 30$ saving per hectare for insecticide expenses and a yield increases of 80-89% occurred. The use of insecticides generally also reduced the numbers of natural enemies. During 1999 the use of Bt crops in China reduced poisoning symptoms, which was a very big health risk during pesticide application, with 17.3% (Huang *et al.,* 2002). The use of Bt cotton increased the yield and reduced the insecticide applications on Bt cotton by up to 7 applications in South Africa (James, 2002). The farmers do not need to spray insecticides as often as they used to, also reducing labour costs. It is important to evaluate resistance development so that small and commercial farmers can benefit from using a more environmental friendly product such as Bollgard II®. It is essential to evaluate tolerance levels and resistance development of insect pest to Bt crops in the field in order to preserve this technology with all its advantages.

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Table 2.1 Effect of Bollgard® and Bollgard II® on mortality of *Erias biplaga* larvae on cotton squares (Rustenburg population).

Experiment 1				
Feeding period	Treatment	% Mortality	X^2	Odds ratio
4 Days	Control	23.3		
	Bollgard®	76.7***	17.1	10.8
	Bollgard II®.	90.0***	27.2	29.6
7 Days	Control	36.7		
	Bollgard®	90.0***	18.4	15.5
	Bollgard II®.	96.7***	24.3	103.4
10 Days	Control	36.7		
	Bollgard®	100.0***	27.8	103.4
	Bollgard II®.	100.0***	27.8	103.4
Experiment 2				
4 Days	Control	26.0		
	Bollgard®	84.0***	34.0	14.9
	Bollgard II®.	96.0***	51.5	68.3
7 Days	Control	34.0		
	Bollgard®	94.0***	39.1	30.4
	Bollgard II®.	98.0***	45.6	45.6
10 Days	Control	40.0		
	Bollgard®	100.0***	42.9	150.3
	Bollgard II®.	100.0***	42.9	150.3

Each Bt treatment was compared to the control within an experiment per feeding time. Asterisks *** denote a significant difference: Bonferroni adjusted P<0.001.

Table 2.2 Effects of Bt1 (Bollgard®) and Bt2 (Bollgard ll®) on mortality of *Earias biplaga* larvae on cotton squares (Potchefstroom population).

Experiment 1				
Feeding period	Treatment	% Mortality	X^2	Odds ratio
4 Days	Control	26.7		
	Bollgard®	54.3***	4.4	3.1
	Bollgard ll®.	90.0***	24.8	24.8
7 Days	Control	26.7		
	Bollgard®	76.7***	15.0	9.0
	Bollgard ll®.	96.7***	31.1	79.8
10 Days	Control	26.7		
	Bollgard®	100***	34.7	161.5
	Bollgard ll®.	100***	34.7	161.5
Experiment 2				
4 Days	Control	20.0		
	Bollgard®	70.0***	15.9	9.3
	Bollgard ll®.	93.3***	32.9	56.0
7 Days	Control	33.3		
	Bollgard®	90***	20.4	18.0
	Bollgard ll®.	100***	30.0	119.1
10 Days	Control	33.3		
	Bollgard®	100***	30.0	119.1
	Bollgard ll®.	100***	30.0	119.1

Each Bt treatment was compared to the control within an experiment per feeding time. Asterisks *** denote a significant difference: Bonferroni adjusted P<0.001.

Table 2.3 Effect of Bollgard® and Bollgard II® on mortality of *Erias biplaga* larvae on cotton boll slices (Rustenburg population).

Experiment 1				
Feeding period	Treatment	% Mortality	X^2	Odds ratio
4 Days	Control	18.0		
	Bollgard®	88.0***	49.2	33.4
	Bollgard II®.	98.0***	65.7	223.2
7 Days	Control	24.0		
	Bollgard®	96.0***	54.0	76.0
	Bollgard II®.	100.0***	61.3	311.1
10 Days	Control	36.7		
	Bollgard®	100.0***	53.9	231.3
	Bollgard II®.	100.0***	53.9	231.3
Experiment 2				
4 Days	Control	13.3		
	Bollgard®	90.0***	35.3	58.5
	Bollgard II®.	100.0***	45.9	359.2
7 Days	Control	20.0		
	Bollgard®	100.0***	40.0	230.0
	Bollgard II®.	100.0***	40.0	230.0
10 Days	Control	20.0		
	Bollgard®	100.0***	40.0	230.0
	Bollgard II®.	100.0***	40.0	230.0

Each Bt treatment was compared to the control within an experiment per feeding time. Asterisks *** denote a significant difference: Bonferroni adjusted P<0.001.

Table 2.4 Effects of Bt1 (Bollgard®) and Bt2 (Bollgard II®) on mortality of *Erias biplaga* larvae on cotton boll slices (Potchefstroom population).

Experiment 1				
Feeding period	Treatment	% Mortality	X^2	Odds ratio
4 Days	Control	20.0		
	Bollgard®	90.0***	29.7	36.0
	Bollgard II®.	93.3***	32.9	56.0
7 Days	Control	30.0		
	Bollgard®	96.7***	28.7	67.7
	Bollgard II®.	96.7***	28.7	67.7
10 Days	Control	30.0		
	Bollgard®	100***	32.3	138.1
	Bollgard II®.	100***	32.3	138.1
Experiment 2				
4 Days	Control	20.0		
	Bollgard®	90.0***	29.7	36.0
	Bollgard II®.	93.3***	32.9	56.0
7 Days	Control	26.7		
	Bollgard®	96.7***	31.1	79.8
	Bollgard II®.	100***	34.7	161.5
10 Days	Control	26.7		
	Bollgard®	100***	34.7	161.5
	Bollgard II®.	100***	34.7	161.5

Each Bt treatment was compared to the control per feeding period. Asterisks denote a significant difference in mortality: Bonferroni adjusted $P<0.0001$.

Table 2.5 Effect of Bollgard® and Bollgard II® on mortality of *Erias biplaga* larvae on cotton bolls (Rustenburg population).

Experiment 1				
Feeding period	Treatment	% Mortality	X^2	Odds ratio
4 Days	Control	20.0		
	Bollgard®	72.0***	13.6	10.3
	Bollgard II®.	98.0***	29.6	96.0
7 Days	Control	20.0		
	Bollgard®	100.0***	33.3	190.1
	Bollgard II®.	100.0***	33.3	190.1
10 Days	Control	20.0		
	Bollgard®	100.0***	33.3	190.1
	Bollgard II®.	100.0***	33.3	190.1
Experiment 2				
4 Days	Control	26.7		
	Bollgard®	80.0***	17.1	11.0
	Bollgard II®.	100.0***	34.7	161.5
7 Days	Control	33.3		
	Bollgard®	93.3***	23.3	28.0
	Bollgard II®.	100.0***	30.0	119.1
10 Days	Control	33.3		
	Bollgard®	100.0***	30.0	119.1
	Bollgard II®.	100.0***	30.0	119.1
Experiment 3				
4 Days	Control	12.0		
	Bollgard®	84.0***	51.9	38.5
	Bollgard II®.	96.0***	71.0	176.0
7 Days	Control	22.0		
	Bollgard®	94.0***	53.2	55.5
	Bollgard II®.	100.0***	63.9	346.9
10 Days	Control	22.0		
	Bollgard®	100.0***	63.9	346.9
	Bollgard II®.	100.0***	63.9	346.9

Each Bt treatment was compared to the control within an experiment per feeding time. Asterisks *** denote a significant difference: Bonferroni adjusted $P<0.001$.

Table 2.6 Effects of Bt1 (Bollgard®) and Bt2 (Bollgard ll®) on mortality of *Erias biplaga* larvae on cotton bolls (Potchefstroom population).

Experiment 1				
Feeding period	Treatment	% Mortality	X^2	Odds ratio
4 Days	Control	13.3		
	Bollgard®	56.7***	12.4	8.5
	Bollgard ll®.	96.7***	42.1	188.5
7 Days	Control	16.7		
	Bollgard®	93.3***	35.6	70.0
	Bollgard ll®.	100***	42.9	282.8
10 Days	Control	16.7		
	Bollgard®	100**	42.9	282.8
	Bollgard ll®.	100***	42.9	282.8
Experiment 2				
4 Days	Control	10		
	Bollgard®	66.7***	20.4	18.0
	Bollgard ll®.	93.3***	41.7	126.0
7 Days	Control	13.3		
	Bollgard®	93.3***	38.6	91.0
	Bollgard ll®.	96.7***	42.1	188.5
10 Days	Control	13.3		
	Bollgard®	100***	45.9	359.2
	Bollgard ll®.	100***	45.9	359.2
Experiment 3				
4 Days	Control	19		
	Bollgard®	88.9***	98.7	34.5
	Bollgard ll®.	97.0***	124.9	137.
7 Days	Control	22.0		
	Bollgard®	90***	106.4	55.0
	Bollgard ll®.	100***	124.1	351.0
10 Days	Control	26.0		
	Bollgard®	100***	117.5	565.1
	Bollgard ll®.	100***	117.5	565.1

Each Bt treatment was compared to the control within an experiment per feeding time. Asterisks *** denote a significant difference: Bonferroni adjusted P<0.001

Chapter 3

Efficacy of *Bacillus thuringiensis* spray applications for control of the Spiny bollworm.

3.1 Abstract

One of the three species of the bollworm complex in South Africa is known as the spiny bollworm (*Earias* spp.). There are seven *Earias* spp. known worldwide but only two of these occur in South Africa namely *E. biblaga* and *E. insulana*. Bt cotton is cultivated in South Africa since 1998 for control of the bollworm complex but Bt spray is currently not registered for control of *E. biplaga* on cotton in South Africa. The aim of this study was to determine the efficacy of *Bacillus thuringiensis* spray applications for the control of *E. biplaga* on cotton in South Africa. Half the dosage rate registered for bollworm control on cotton, was too low to control *E. biplaga* larvae effectively. The recommended dosage rate controlled the larvae as effective as Bollgard® and Bollgard II®, but 100% mortality was not achieved as with the latter two treatments. Bt sprays break down if exposed to UV light and rain can reduce the efficacy, final instar larvae will not be controlled by Bt sprays and effective coverage with the spray application is essential for successful control. Bt is, however, ever-present in Bt transgenic plants, is continuously expressed and correct timing of an insecticide application is therefore not needed. It will therefore be the preferred control method while there is not resistance to the Cry proteins contained in Bt cotton.

3.2 Introduction

3.2.1 *Bacillus thuringiensis* mode of action

Bacillus thuringiensis (Bt) was first discovered in Japan after it was responsible for killing silkworms in 1901 (Ishiwata, 1901) and another strain in Germany, killing grain moth larvae in stored grain (Berliner, 1915). It was named after the city Thuringen where it was found and thus the name *Bacillus thuringiensis* (Knowels, 1994).

Over 150 insecticidal crystal (Cry) proteins in *Bacillus thuringiensis* and *Bacillus cereus* species had been discovered (Schnepf *et al*., 1998). *Bacillus thuringiensis* (Bt) is a rod-shaped bacterium that belongs to the Bacillaceae family (Knowles, 1994). Research have been done on the insecticidal properties of Bt during 1950 and isolation of the proteins responsible for mortality was done which led to the first commercial production of Bt as a biological pesticide (Yamamoto, 2001).

Bt is a spore-forming gram-positive bacterium (Knowles, 1994; Bravo *et al*., 2007; Yul *et al*., 2007). The formation of intracellular crystals make Bt different from other bacilli (Yamamoto, 2001). Crystalline parasporal inclusions are formed consisting of δ-endotoxins (Knowles, 1994). The delta-endotoxin is formed at sporulation and contains the insecticidal proteins (Cry proteins) (Bravo *et al*., 2007).

Larvae ingest Bt crystal inclusions, the crystal proteins are solubilised and the protoxins are then proteolictic processed to the active form which binds with high affinity to the midgut receptors followed by irreversible insertion of the toxin into the membrane (Whalon & Wingerd, 2003). The Cry toxins aggregate to form spores in the membrane that leads to osmotic lysis. This damage to the midgut causes either starvation or septicaemia (Whalon & Wingerd, 2003) and once the gut is paralyzed the insect stop feeding and can die within one to three days (Hilder & Boulter, 1999). The larva then acts as food source for the vegetative bacterial growth (Knowles, 1994).

3.2.2 *Bacillus thuringiensis* as a sprayable insecticide

Cry proteins are the only active components of Bt-based microbial insecticides, which have been used as foliar sprays in agriculture and forests for several decades (Reed *et al*., 2001). Partly because of their selectivity and short half-life, Bt Cry proteins (as well as cell bodies and spores) are generally considered to have fewer adverse impacts on the environment than many broad-spectrum and persistent chemical insecticides (Schnepf *et al*., 1998). Dipel® is the most used Bt kurstaki (HD-1 strain) product and is applied for control of more than 100 species of Lepidoptera globally (Navon, 1993). Bt sprays have never occupied a large share of the insecticide market, and are largely used by organic farmers, gardeners and in forestry (De Maagd *et al*., 1999).

Dipel® was registered in South Africa for control of various lepidopterans as a biopesticide. DiPel DF® is registered for control of the African bollworm on apples, pears, beans, citrus, Cuciferae, herbs, peas, tomatoes, ornamentals, flowers and lawns, for the diamondback moth on Cuciferae, the pine tree emperor moth on afforestation, ornamentals, flowers and lawns, for leafrollers on avocadoes, citrus and grapes, for the orange dog caterpillar on citrus, for lawn caterpillar and the lily borer on ornamental, flowers and lawns, and for the semi-looper on beans, potatoes, tomatoes, ornamentals, flowers and lawns in South Africa (Van Zyl, 2013). Dipel DF® contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab (Valent Biosciences, 2002).

There are a few advantages of using Bt sprays to control insect pests. Bt sprays are considered to be more environmental friendly, with less negative impact on the environment (Schnepf *et al*., 1998). Bt spray is a better alternative to chemical pesticides and can be used to control pests in organic farming systems, forestry, storage facilities, and at home, because of their high specificity (Schnepf *et al*., 1998; Roh *et al*., 2006). Many Cry proteins have been classified and named and are highly toxic to different orders of insect pests (Yamamoto, 2001).

Thirteen crystal proteins or Cry genes have been divided into four major groups that have structural similarities and similarities in the insecticidal spectra of the encoded proteins (Höfte & Whiteley, 1989). These groups are:

- Cry1 which is Lepidoptera-specific,
- Cry2 which is Lepidoptera and Diptera specific,
- Cry3 which is Coleoptera specific and
- Cry4 which is Diptera specific. Two groups,
- Cry5 and Cry6 had been added for the control of nematodes (Feitelson, 1992).

Bacillus thuringiensis sprays can contain more than one protein belonging to different groups and the product can therefore be used on more than one pest species in contrast to Bollgard II® cotton that only express Cry 1Ac and Cry 2Ab (Whitehouse *et al*., 2005). Field activity of DiPel is less than seven days, which makes it safer to use than other pesticides (BCPC, 2009). It is possible to use DiPel® with a number of insecticides, fungicides and acaricides but the use of DiPel® together with alkaline products will not be effective (BCPC, 2009).

Bt sprays can be applied to plants as most other insecticides. It is usually found in a dry flowable, emulsifiable suspension, corn grit or sand granules form. It contains Bt spores and usually a few Cry proteins. Dipel® contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab, making it effective for control of a variety of target pests (Valent Bioscience, 2014). Bt plants is genetically engineered to produce the cry proteins that control the pest (Dutton *et al*., 2005). The gene producing the cry proteins are encoded into the genetic composition of the plant (Dutton *et al*., 2005). Bt is ever-present in the plant, are continuously expressed, and the farmer does not have to be specific on timing of insecticide application (Navon, 2000). The cry proteins are produced during the complete life span of the plant but may become less potent as the plant age (Whitehouse *et al*., 2005). Bt plants usually express only one or two cry proteins, for instance Bt cotton express Cry1Ac and Cry2Ab (Whitehouse *et al*., 2005). The target pests that will be controlled by Bt plants are more limited due to the limited number Cry proteins narrowing control to specific insect orders (Lemaux, 2009).

3.2.3 Resistance to Bt sprays

Resistance to the Cry1Ac protein in Bt sprays had been documented for field populations of diamondback moth (*Plutella xylostella* (L.)) (Lepidoptera: Plutellidae) in Hawaii (Tabashnik, 1990). Resistance to Cry1Ac and a high frequency of resistance to Cry2Ab in greenhouse populations of the cabbage looper (Hübner) (*Trichoplusia ni*) (Lepidoptera: Noctuidae) in British Columbia was also documented (Kain *et al*., 2015).

According to Ferré & Van Rie (2002) cases of resistance against DiPel® *kurstaki, HD-1* was reported for the Indian meal moth *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) almond moth, *Cadra cautella* (Walker) (Lepidoptera: Pyralidae) and the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Huang *et al*., 1999). *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) was reported to have high levels of resistance to Cry1Ac and cross resistance to Cry1Ab and Cry1Fa (Ferré & Van Rie, 2002). *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) showed resistance to Cry1Ca and cross-resistance to Cry1Ab, Cry2Aa and Cry9Ca (Ferré & Van Rie, 2002). *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) is resistant to Cry1Ac and have high levels of cross-resistance to Cry1Aa and Cry1Ab (Ferré & Van Rie, 2002). According to Atsumi *et al*. (2012), the silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae) also developed resistance to Cry1Ab. Resistance to Cry1Ac has also been reported for *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Zhang *et al*., 2009) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Ali *et al*., 2006).

Bt spray is currently not registered for control of *E. biplaga* on cotton in South Africa. The aim of this study was to determine the efficacy of *Bacillus thuringiensis* spray applications for the control of *E. biplaga* on cotton in South Africa.

3.3 Materials and methods

3.3.1 Susceptibility bioassay

Two *E. biplaga* populations were evaluated, namely a Rustenburg and Potchefstroom population. Larvae from these localities were collected and reared under laboratory conditions as described in chapter 2 (see 2.2.1). Bolls were removed from plants and secured in an upright position on a polystyrene board by means of a wooden skewer inserted into the petiole of each boll (Fig. 3.1). Inserting the skewer into the petiole rather than the cotton boll was to prevent a possible experimental error that the larvae may enter the cotton boll without ingesting plant material sprayed with the respective treatments. The calyx leaves were also left intact.

The susceptibility bioassay consisted of the following treatments: Bollgard® and Bollgard II® cotton bolls, bolls of a non-Bt cotton cultivar, Delta Opal sprayed with four concentrations Dipel®. These concentrations were 0.5, 1.0, 2.0 and 5.0 times the recommended dosage rate for African bollworm, *H. armigera* on cotton (500g/ha/100L water), deltamethrin (25g/L EC) applied at the recommended dosage rate for *H. armigera* control on cotton of 6.25 g a.i./ha (positive control), and water as an untreated (negative) control. Sprayfilm (sticker) was added to each spray treatment at a rate of 5ml/10L water. For ease of reading, the Dipel® concentrations will be referred to as 0.5, 1.0, 2.0 and 5.0 times "the recommended dosage rate" from this point onwards. The 2.0 times rate was not applied to the Potchefstroom population. The spray treatments were applied as full cover sprays to cotton balls. The sprayed cotton bolls were allowed to dry for 30 min and placed separately in round plastic containers of 100ml. One neonate larva was transferred to each cotton boll in the containers which were closed with aerated lids and kept in an incubator at 26±1 °C and a 14L:10D photoperiod. The bioassay was repeated 3 times for each population, two of the bioassays consisted of 6 replicates with 5 individuals per replicate each and the third bioassay consisted of 10 replicates with 5 individuals per replicate. This was the case for both populations. Survival of larvae was assessed at 4, 7 and10 days after application.

Figure 3.1 Skewers were inserted into the petiole, to prevent damage to the boll.

Figure 3.2 Cotton bolls on skewers for application of spray treatments.

3.3.2 Statistical analysis

Efficacy of control was calculated according to Abbott's formula (Abbott, 1925). The corrected percentage efficacy (Abbott, 1925) of each treatment was compared by means of one way ANOVA, followed by Tukey's HSD test to determine significant differences between treatments. Mortality over time was compared by means of repeated measures ANOVA, followed by Tukey's HSD test. ANOVA's were done with STATISTICA version 11 (StatSoft, Inc., 2012).

3.4 Susceptibility bioassays

3.4.1 Potchefstroom population

Mortality of neonate *E. biplaga* larvae occurred within 4 days after exposure to the respective Bt treatments and deltamethrin. Deltamethrin caused 100% mortality within 4 days, when the first evaluation was done. All Bt treatments except half the dosage rate of Bt spray controlled the larvae equally well, 10 days after treatment in experiment 1. In experiment 2, half the dosage rate was less effective in controlling *E. biplaga* larvae than Bollgard® and Bollgard II® 10 days after treatment. In experiment 3, the recommended dosage rate took 10 days to be as effective in controlling *E. biplaga* as 5 times the dosage rate, Bollgard®, Bollgard II® and deltamethrin. Half the dosage rate proofed to be too low for effective control of *E. biplaga* larvae. Bollgard® and Bollgard II® provided 100 % mortality 10 days after exposure to the respective Bt treatments in all three experiments.

Mean corrected percentage mortality for the Potchefstroom population increased over time in all three experiments (Figure 3.3). In all three experiments, significantly more larvae died 7 days after exposure to all treatments compared to 4 days after treatment. Mortality did, however, increased significantly from day 7 to 10 in experiment 1 and 3. Although mortality increased slightly from day 7 to 10 in experiment 2, mortality was not significantly different between day 7 and day 10 and corrected percentage mortality 7 days after treatment were therefore similar to that 10 days after treatment.

3.4.2 Rustenburg population

For experiment 1, the mean percentage mortality caused by the 0.5, 1.0 and 2 times dosage rates applied did not differ significantly from each other, 7 days after application (Table 3.4). However, the 0.5, 1 and 2 times dosage rate controlled first instar *E. biplaga* 7 days after application also similarly, with no significant difference in their respective mean corrected percentage mortality. Ten days after application, all treatments, except half the dosage rate with a lower mortality rate, were similarly effective in controlling *E. biplaga* larvae (Table 3.4). Bollgard® and Bollgard II® had a 100% mortality as well as experiment where deltamethrin was applied to the cotton.

In experiment 2, the corrected percentage mortality of all treatments applied, except half the dosage rate, was similar 7 days after treatment. Mortality of larvae in half the dosage rate treatment was lower compared to the Bollgard II® and deltamethrin treatments which controlled larvae 100% within 7 days, and compared to Bollgard® and Bollgard II® and deltamethrin 10 days after treatment. In experiment 3 for the Rustenburg population, percentage corrected efficacy of control achieved by half the dosage rate, was significantly poorer than the 100% control of both Bollgard® and Bollgard II® and deltamethrin treatments as well as the control by 5 times the recommended dosage rate ten days after treatment (Table 3.4).

Deltamethrin was 100% effective in controlling *E. biplaga* larvae within 4 days of application in all experiments for both the Potchefstroom and Rustenburg populations (Table 3.1 and 3.2).

The combined corrected percentage efficacy of control for all treatments applied, increased significantly from 4 to 7 days after treatment, but was similar 7 and 10 days after treatment (Fig. 3.5).

3.5 Discussion

Earias species tunnel into the flowering parts, cotton squares and cotton bolls causing little damage to the outside surface area, but substantial damage feeding inside the cotton boll (Abro *et al*., 2004). When insecticides with a stomach mode action are applied to cotton plants, only the sprayed plant material from the surface area where larvae feed on to enter the boll will be ingested.

Earias biplaga larvae in all experiments from both the Potchefstroom and Rustenburg populations evaluated were 100% controlled by Bollgard® expressing Cry1Ac and Bollgard II® expressing both Cry1Ac and Cry2Ab2. Therefore, to date there is no resistance development by *E. biplaga* to Bt cotton in South Africa. The recommended dosage rate of DiPel® for African bollworm control on cotton also proved to be effective in controlling *E. biplaga* larvae although 100% control was not achieved within 10 days of treatment application.

Bt sprays such as DiPel® as well as Bollgard® and Bolgard II® has to be ingested to have any effect on the larvae (Andow, 2008). Deltamethrin is a non-systemic pyrethroid with a strong contact and stomach mode of action (Tomlin, 2009) registered in South Africa for control of the African bollworm, red bollworm and spiny bollworm on cotton (Van Zyl, 2013). The high mortality in deltamethrin treatments can be ascribed to the contact mode of action which provide for an immediate knock down effect. This also explains complete control achieved within 4 days of application. A contact insecticide as well as the DiPel® spray application will, however, be ineffective once the larvae entered the bolls. The exposure time of larvae to contact with insecticides is very short. It is from the time the neonates hatch until they penetrate the cotton buds or bolls, or when the larvae move to another fruiting point (Tunstall, 1970). After the larvae have penetrated the cotton boll, the entrance is blocked with excreta (Bennett, 2015). This also prevents spray application of insecticides to enter the boll. Systemic insecticides or insecticides produced by the plant that can be ingested by the larvae such as Bollgard® and Bollgard II® is therefore a better control option (Casida & Quistad, 1998; Kumar, 2008).

Although the chances of survival on conventional cotton sprayed with Bt is better than on Bt cotton expressing Cry1Ac and Cry2Ab2 proteins, Bt sprays can still provide protection for crops where pests are foliage feeders (Roush, 1994). Bt as a biopesticide have some disadvantages. Weather conditions such as UV exposure to Bt sprays, heat and rain can also influence the efficacy of Bt sprays and can lead to reduced control (Whalon & Wingerd, 2003). The amount of Bt insecticide ingested and concentration may not be enough for mortality to occur and this might lead to survival or even possible resistance development. According to Valent Bioscience (2014) a lethal dose has to be ingested before feeding stops. This can also contribute to resistance development in Bt crops. It is effective against the immature stages of the insect pest that feed on the exposed surface of the plant, thus if the coverage is inadequate or if larvae are in the final instar before pupation, control might be unsatisfactory (Kumar, 2008). Another disadvantage is that the biopesticide needs to be applied repeatedly during the immature stages of larval development, before larvae tunnel into the cotton boll or square (Kumar, 2008). Improved control of *E. biplaga* was achieved with Bollgard® and Bollgard II® cotton compared to Bt-sprays and will therefore be the better control option.

Resistance is the ability of an individual to survive from egg to adult on a Bt plant and to produce viable offspring (Andow, 2008). Insect resistant management (IRM) should contribute to the delay or preventing the occurrence of control failures by means of preventing or delaying the evolution of resistance (Andow, 2008). Although a high percentage efficacy of control of *E. biplaga* larvae was achieved with Bt spray applications, control with Bollgard® and Bollgard II® was more effective in this study. It can serve as a base line for future studies to determine possible resistance development to Bt cotton in South Africa.

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Table 3.1 Corrected percentage mortality of first instar *Earias biplaga* larvae 4, 7 and 10 days after application of insecticides to cotton bolls for Potchefstroom.

Experiment 1			
Treatment	#Corrected % mortality 4 d.a.t	Corrected % mortality 7 d.a.t	Corrected % mortality 10 d.a.t
0.5 X dosage rate	15.7a	23.4a	43.8a
1.0 X dosage rate	57.9b	73.2b	88.0b
5 X dosage rate	61.7b	84.7bc	96.0b
Bollgardl®	56.5b	84.7bc	100.0b
BollgardII®	96.2c	100.0c	100.0b
Deltamethrin	100.0c	100.0c	100.0b
	$F_{5,30} = 23.05$	$F_{5,30} = 25.55$	$F_{5,30} = 15.72$
	$P < 0.000001$	$P < 0.000001$	$P < 0.00001$
Experiment 2			
Treatment	#Corrected % mortality 4 d.a.t	Corrected % mortality 7 d.a.t	Corrected % mortality 10 d.a.t
0.5 X dosage rate	29.2a	54.5a	59.1a
1.0 X dosage rate	83.3bc	81.8bc	86.4ab
5 X dosage rate	87.5bc	95.5bc	95.5ab
Bollgardl®	58.3ab	81.8bc	100.0b
BollgardII®	95.8bc	100.0c	100.0b
Deltamethrin	100.0c	100.0c	100.0b
	$F_{5,30} = 8.2$	$F_{5,30} = 2.7$	$F_{5,30} = 2.9$
	$P < 0.0001$	$P < 0.000001$	$P < 0.000001$
Experiment 3			
Treatment	#Corrected % mortality 4 d.a.t	Corrected % mortality 7 d.a.t	Corrected % mortality 10 d.a.t
0.5 X dosage rate	53.8a	60.53a	65.8a
1.0 X dosage rate	61.5a	71.1ab	84.2ab
5 X dosage rate	87.2b	86.8bc	92.1b
Bollgardl®	66.7ab	89.5bc	100.0b
BollgardII®	92.3c	100.0c	100.0b
Deltamethrin	100.0c	100.0c	100.0b
	$F_{5,54} = 9.3$	$F_{5,54} = 7.5$	$F_{5,54} = 7.0$
	$P < 0.00001$	$P < 0.00001$	$P < 0.0001$

Means within a column for each experiment, followed by the same letter do not differ significantly at P = 0.05 (Tukey's HSD). #Corrected % efficacy to account for mortality in the control treatment according to the Abbott formula (Abbott, 1925). Days after treatment = d.a.t.

Table 3.2 Corrected percentage mortality of first instar *Earias biplaga* larvae 4, 7 and 10 days after application of insecticides to cotton bolls for Rustenburg.

Experiment 1			
Treatment	#Corrected % mortality 4 d.a.t	Corrected % mortality 7 d.a.t	Corrected % mortality 10 d.a.t
0.5 X dosage rate	41.7a	50.0a	44.4a
1.0 X dosage rate	62.5ab	77.3ab	77.8ab
2 X dosage rate	62.5ab	77.3ab	83.3b
5 X dosage rate	70.8b	90.9b	94.4b
Bollgardl®	45.8a	86.4b	100.0b
BollgardII®	91.7b	100.0b	100.0b
Deltamethrin	100.0b	100.0b	100.0b
	$F_{6,35} = 6.3$	$F_{6,35} = 7.5$	$F_{6,35} = 7.0$
	$P < 0.001$	$P < 0.0001$	$P < 0.0001$
Experiment 2			
Treatment	#Corrected % mortality 4 d.a.t	Corrected % mortality 7 d.a.t	Corrected % mortality 10 d.a.t
0.5 X dosage rate	60.0a	72.7a	70.0a
1.0 X dosage rate	80.0ab	86.4ab	85.0ab
2 X dosage rate	80.0ab	86.4ab	85.0ab
5 X dosage rate	84.0ab	90.9ab	90.0ab
Bollgardl®	88.0ab	95.5ab	100.0b
BollgardII®	100.0b	100.0b	100.0b
Deltamethrin	100.0b	100.0b	100.0b
	$F_{6,35} = 4.4$	$F_{6,35} = 2.8$	$F_{6,35} = 3.5$
	$P < 0.01$	$P < 0.1$	$P < 0.01$
Experiment 3			
Treatment	#Corrected % mortality 4 d.a.t	Corrected % mortality 7 d.a.t	Corrected % mortality 10 d.a.t
0.5 X dosage rate	69.2a	75.0a	72.7a
1.0 X dosage rate	76.9ab	77.8a	78.8ab
2 X dosage rate	76.9ab	86.1ab	84.8ab
5 X dosage rate	89.7abc	94.4ab	97.0b
Bollgardl®	84.6abc	100.0b	100.0b
BollgardII®	97.4bc	100.0b	100.0b
Deltamethrin	100.0c	100.0b	100.0b
	$F_{6,63} = 4.6$	$F_{6,63} = 5.2$	$F_{6,63} = 5.0$
	$P < 0.001$	$P < 0.001$	$P < 0.001$

Means within a column followed by the same letter do not differ significantly at P = 0.05 (Tukey's HSD). #Corrected % efficacy to account for mortality in control treatment according to the Abbott formula (Abbott, 1925). Days after treatment = d.a.t

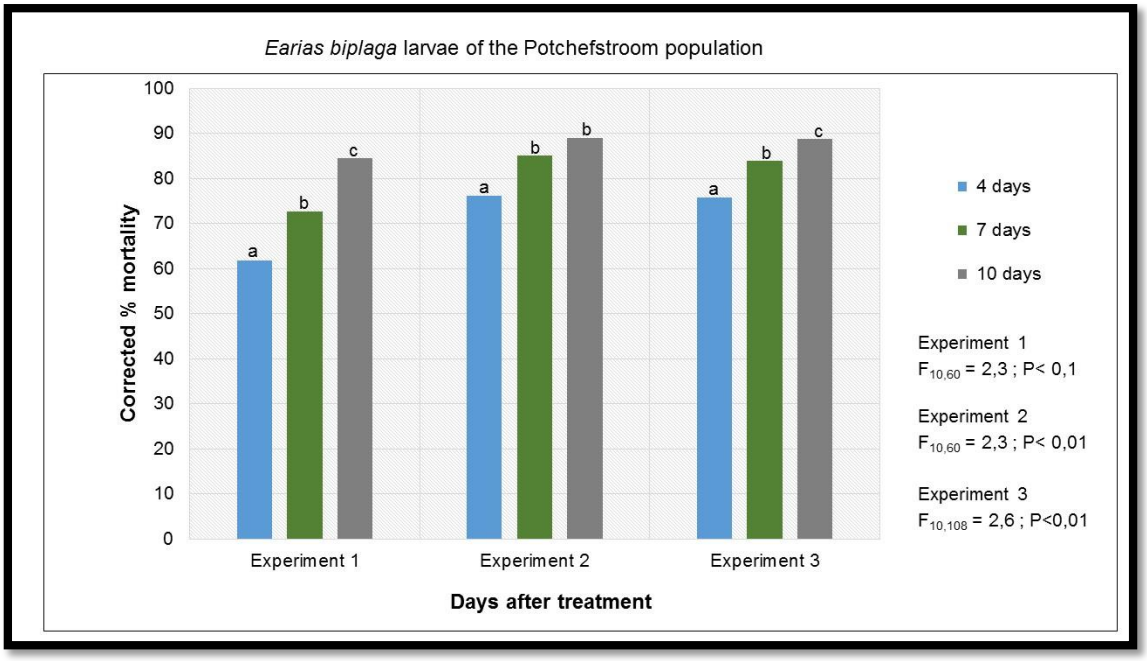

Figure 3.3 Corrected percentage mortality for all three experiments of the Potchefstroom population over time.

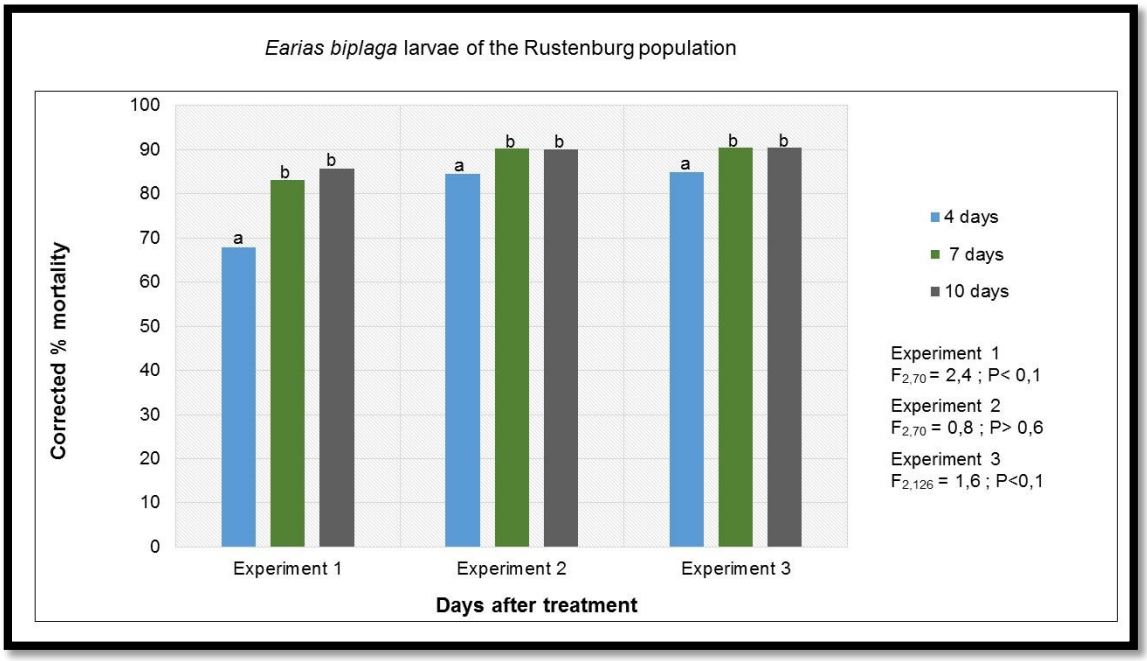

Figure 3.4 Corrected percentage mortality for all three experiments of the Rustenburg population over time.

Chapter 4

Development of the Spiny bollworm at constant temperatures

4.1 Abstract

Earias biplaga Walker (Noctuidae) is known to attack cotton in South Africa. The effect of temperature on the development of *E. biplaga* was studied at four different temperature regimes, namely 18, 20, 25 and 30 ± 1°C. Development time for all life stages was inversely related to temperatures from 18 to 30 °C. The relationship between temperature and developmental rate of *E. biplaga* was linear between 18 and 30 °C and more rapid development was observed with increasing temperatures. The total development period was 68.9 to 22.5 days at 18 and 30 days, respectively. The thermal thresholds for *E. biplaga* were 15.2, 11.3, 12.8 and 12.2°C and the thermal constants were 34.3, 195.1, 156.5 and 369.6 °D, for the completion of the egg, larval, pupal and egg-to-adult stages, respectively.

4.2 Introduction

4.2.1 The effect of temperature on insect development

Insects are the most successful animals on earth and the estimated ratio of insects to humans is 200 million to 1 (Pedigo & Rice, 1989). Environmental factors such as temperature effect poikilothermic animals, including insects (Taylor, 1981; Ju *et al*., 2011). Temperature is a key factor for insects to hatch, develop and reproduce (Bale *et al*., 2002). The survival rate and duration of the life cycle are affected by temperature (Danks, 2006), but is also affected by voltinism, population density, size, genetic composition, extent of host plant exploitation and geographical distribution (Bale *et al*., 2002; Sharma, 2010). Development occurs within a specific temperature range and is best performed at an optimum temperature. Development rate decreases as the temperature decreases or deviates from the optimum (Begon *et al*., 2006). Some insects have the ability to undergo diapause and although this is usually during the winter months it can also occur in the summer or both depending on the regional climatic patterns (Danks, 2006). Whenever the temperature is unfavourable it can induce insect diapause or extra moulds in larval stages, preventing premature development and prolongs the development time of an insect to become an adult (Danks, 2006). This enable survival of insects during cold and unfavourable weather conditions (Danks, 2006).

Temperature in nature is fluctuating and affects the development time of diverse insects differently (Bale *et al*., 2002). Changes in temperature at the polar regions have a greater effect on insects than in the tropical zones (Convey & Block, 1996). Global warming and climate change will affect insect host plant interactions and their synchrony, invasion rates of insect and the possible introduction of different host plants that can cause challenges in plant protection programs (Sharma, 2010).

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4.2.2 Other factors that might influence development of insects

Factors other than temperature that also affects development time of insects include topography, abundance of food and photoperiod (Danks, 2006). Abundance of high quality food may produce larger adults (Wissinger *et al.,* 2004). Bigger larvae of *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) from larger eggs perform better during food deficiency (Torres-Villa & Rodriquez-Molina, 2002). Limited resources, such as food, may influence some species, resulting in a reduction in size, but it may still maintain the development time, while others may have a longer growth rate but are normal in size (Danks, 2006). According to Khaldey (1977) can the photoperiod also determines whether a species will enter diapause or not and it may also have an effect on the duration of diapause, and if an additional instar might occur. The effect of photoperiod on larvae may also be different in the various life stages, for example the opposite effect may occur in first than in later instars (Khaldey, 1997).

4.2.3 *Earias* spp. (spiny bollworm) as pests of crops

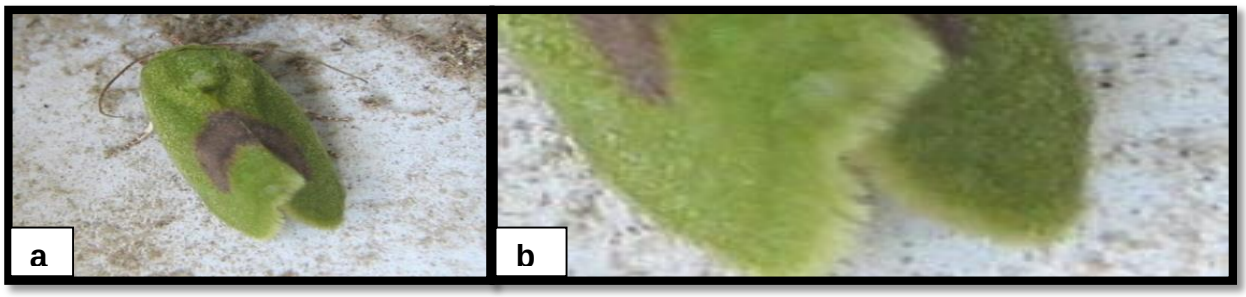
Earias insulana (Boisduval) (Lepidoptera: Noctuidae) is one of the most common pests that attack okra *Abelmos esculentaus* (Malvales: Malvaceae) in Egypt (Kandil, 2013), cotton *Gossypium hirsutum* (L) (Malvales: Malvaceae) in Pakistan (Shah *et al*., 2012), South Africa (Bennett, 2015) and India (Venilla, 2007) as well as other Malvaceae plant families (Kandil, 2013; Venilla, 2007). *Earias* species were responsible for more than 10% of damage on cotton in India during the 2005 growing season (Venilla, 2007).

4.2.4 Identification of *Earias* spp.

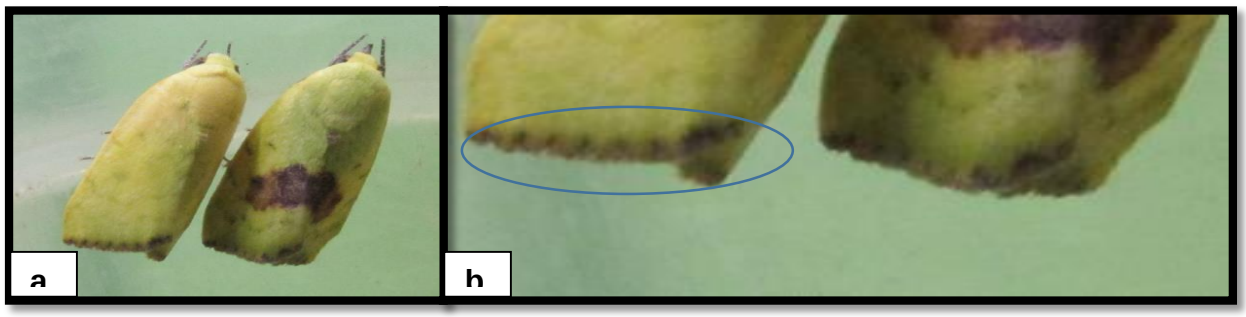
There are seven *Earias* spp. globally, but only two of these species occur in South Africa, namely *Earias biplaga* (Walker) and *E. insulana (*Boisduval) (Bennett, 2015). The spiny bollworm forms part of the bollworm complex in South Africa that consist of three different species of lepidopteran pests, namely the red bollworm *Diparopsis*

castanea (Hampson), African bollworm *Helicoverpa armigera* (Hübner) and spiny bollworm the *Earias spp.* (Bennett, 2015). These pests are common in the cotton growing regions, and the damage caused by the spiny bollworm is often under estimated (Bennett, 2015).

There are very few differences between the larvae of *E. insulana* and *E. biplaga.* Pearson and Darling (1958) speculated that the larvae with an orange-brown appearance are *E. biplaga* and the yellower-green larvae, *E. insulana*. The pattern of the forewing during the adult stage is a better indicator for identification of these species (Bennett, 2015). The colour of the outer fringe of the wing of *E*. *insulana* is the same as the rest of the wing (Figures. 4.1a & b) and may vary from silver green to yellow (Bennett, 2015). The outer fringe of the wings of *E. biplaga* is a darker brown (Figures 4.2a & b). The colour of the wings may vary from metallic green to yellow (Bennett, 2015). Only the colour of the fringe and not colour variation of the rest of the wings can be used for identification purposes. The colouration of the moths is shown in figures 4.1 and 4.2



Figures 4.1 (a) *Earias insulana* moth, (b) The outer fringe of the fore wings is the same colour as the rest of the wing (Photos: Pretorius, J.D.).



Figures 4.2 (a) *Earias biplaga* moths, (b) The outer fringe is a darker brownish colour.

4.2.4 Development of *Earias* spp.

The eggs hatch 2-3 days after oviposition, depending on the environmental conditions and temperature. Neonate larvae are blackish in colour and have a hairy appearance (Bennett, 2007). There are five larval instars followed by the pupal stage that can develop within two weeks into adult moths depending on the environmental conditions (Bennett, 2015) (Figure 4.3).

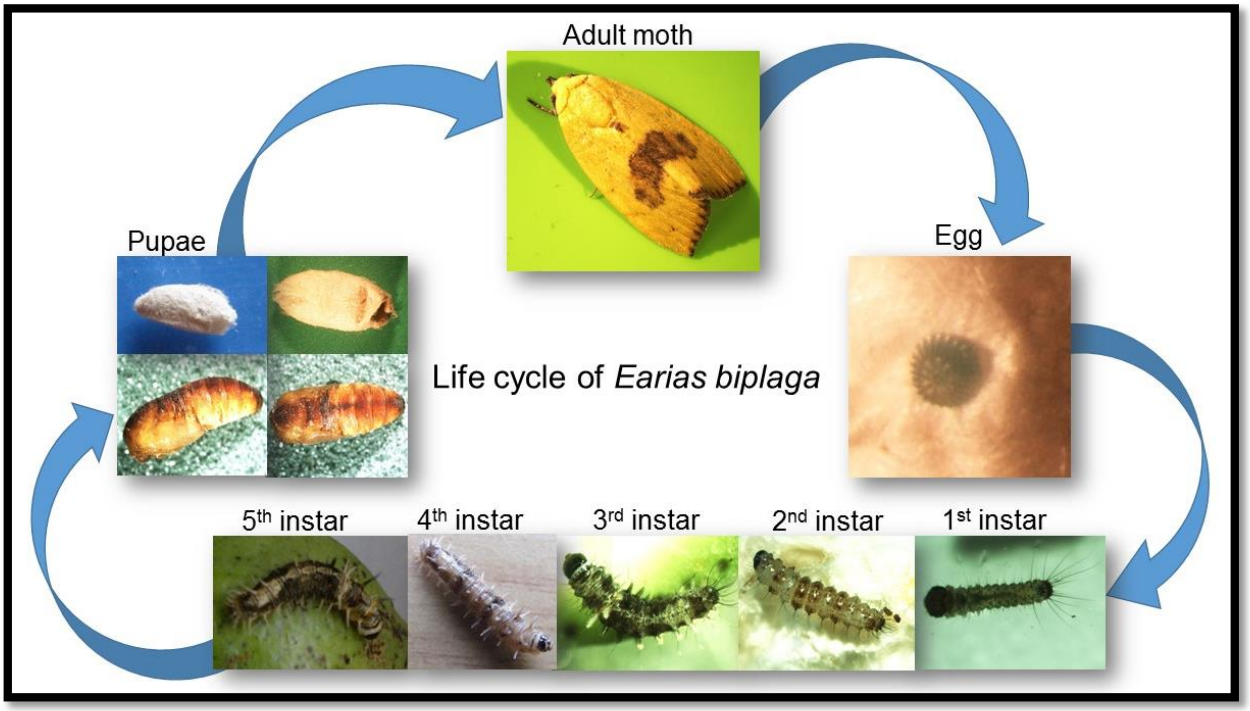


Figure 4.3 Life cycle of *Erias biplaga*.

4.2.5 Objectives

The objectives of this study were to assess the development rate of *E. biplaga* at different constant temperatures, to determine the number of degree-days (°D) required for each stage to complete development as well as for overall egg-to-adult development.

4.3 Material and methods

4.3.1 *Earias biplaga* stock colony

Earias biplaga moths were collected using light traps as described in chapter 2. The moths were kept in plastic containers (40 x 20 x 15 cm) with aerated lids in a rearing room at 26±1˚C and 14L: 10D photoperiod. Sugar water was provided as food and a piece of pipe cleaner (15 mm long), a piece of synthetic cotton wool and wax paper were provided as oviposition substrates. Cotton bolls, petals and leaves were used as stimulus for egg production.

4.3.2 Temperature dependent development

Eggs were collected within 12 hours of oviposition and ±50 eggs were transferred to small plastic containers (55 mm high and 50 mm in diameter) with a steel mesh infused lid and placed into a glass desiccator (150 mm) in which RH was maintained at 70 ± 10 % using a potassium hydroxide solution according to the method of Solomon (1951). The desiccators were kept at constant temperatures of 18, 20, 25, and 30 ± 1°C in incubators with a 14L: 10D photoperiod. There were two plastic containers with eggsper desiccator. Temperature and RH at each regime were recorded at 30-minute intervals using iButtons® from Coldchain Thermo Dynamics (Fairbridge technologies).

The eggs were checked daily until neonate larvae emerge. The number of days for the eggs to develop was recorded. Neonate larvae were collected within 24 hours after hatching and transferred individually to slices of cotton balls in Petri dishes (90 mm in diameter). The larvae were kept at the same temperature regimes in incubators as described above and observed daily. Head capsules and exuviae (shed cuticles) were removed. Daily observation of the larvae was made and moulting as well as survival was recorded. Only data from individuals that completed development to the adult stage were used for the analyses.

4.4 Data analysis

4.4.1 Degree-day model

The relationship between temperature (x) and development rate (y) was determined by using a simple linear regression analysis. The lower threshold temperature (t) and number of degree-days (k) are required to complete development for each of the stages, as well as their standard errors that were calculated using the Campbell *et al.* (1974) equations. The lower threshold temperature was estimated by setting y = 0 and solving x for the regression equation, y = a + bx, where:

y = 1/days

x = temperature,

a = intercept,

b = slope,

the lower temperature threshold: t =-a/b

number of degree-days (ºD): k = 1/b

S.E. of t = ȳ/b $\sqrt{(s^2/Ny^{-2})}$ + [S.E. of b/b]^2

S.E. of k = (S.E. of b)/b^2

The mean degree-days (ºD) for the development of the egg, larval and pupal stage was estimated using the equation of Jackson & Elliot (1988): ºD = T(c - T_{min}), where T is the number of days taken to complete development at a constant temperature (c) and T_{min} is the minimum temperature for development. The thermal constant was used, and the mean number of ºD required for development of each life stage. The different results of the constant temperatures (18, 20, 25 and 30°C) were compared to each other.

The effect of temperature on development was analysed by means of one-way ANOVA using STATISTICA 11 (Statsoft, Inc., 2013), followed by Tukey's HSD test (P = 0.05).

4.5 Results

A preliminary study was done to determine development of *E. biplaga* at 15°C. Mortality rate was very high and almost no development of larvae occurred. A lowest temperature of 18 °C was then included in this study. *Earias biplaga* completed development at all temperatures between 18 and 30 °C (Table 4.1). Development time for all life stages was inversely related to temperatures from 18 to 30 °C. Incubation time for eggs decreased significantly from 8.32 days at 18 °C to 2.4 days at 30 °C ($F_{3,567}$ = 1974.9; P < 0.001). *Earias biplaga* completed its life cycle (22.46 days) significantly faster at 30°C compared to 25°C, 20°C and the slowest development time of 68.89 days at 18°C $F_{3,179}$ = 2383.7; P < 0.001 (Table 4.1). There was a significant difference in development time at 18, 20, 25 and 30°C for first to fifth instar larvae [(first instar: $F_{3,179}$ = 101.1.0; P < 0.001), (second instar: $F_{3,179}$ = 137.0; P < 0.001), (third instar: $F_{3,179}$ = 130.2; P < 0.001), (fourth instar: $F_{3,179}$ = 113.9; P < 0.001) and (fifth instar: $F_{3,179}$ = 118.9; P < 0.001)].

Development time was significantly shorter for instars one and two at 30 °C, but it was similar and did not increase for the later instars three, four and five at 25°C and 30°C (Table 4.1). It can therefore be assumed that the upper threshold temperature for instars three, four and five larval development will be reached once temperature rise above 30 °C. Development time for total larval ($F_{3,179}$ = 552.9; P < 0.001) and pupal development ($F_{3,179}$ = 2625.5; P < 0.001), as well as development time to complete a life cycle ($F_{3,179}$ = 2383.6; P < 0.001) decreased significantly with increasing temperature. The upper threshold temperature to complete a life cycle is higher than 30 °C, since development time for egg, larval and pupal development, as well as development time for completion of a life cycle is significantly faster at 30 °C compared to 25 °C and it still follows a linear trend.

The relationship between temperature and developmental rate of *E. biplaga* was linear between 18 and 30 °C and more rapid development was observed with increasing temperatures (Figure 4.4 and Figure 4.5). The correlation value (r-value) between temperature and development rate ranged between 0.68 for eggs and 0.99 for total

larval development, respectively. Linear regression equations describing these relationships and of the lower developmental threshold temperature (t) and the number of degree days °D for each life stage are summarized in table 4.2. The thermal constants (k) for completion of the egg, larval, pupal and egg-to-adult development are 31.9, 192.1, 154.1 and 392.2, respectively (Table 4.2). The number of degree-days estimated to complete the life cycle of *E. biplaga* at 18, 20, 25 and 30 °C were similar (Table 4.3). Based on linear regression analysis of development rate at all temperatures, a minimum temperature threshold between 8.6 and 15.2 °C was estimated depending on the developmental stage (Table 4.2).

4.6 Discussion

The effect of temperature on an insect pest can differ from species to species (Nair, 2007). Temperature, amongst other factors such as the presence of natural host plants, at which a species can develop and adapt will determine whether or not it can invade other geographical habitats, where conditions will also be suitable for its development (Nair, 2007). Climate change and temperature differences in the environment can therefore affect the successful adaptation of species from year to year (Carmmell & Knight, 1992). Temperature significantly affects the development of *Earias vittella* (Fabricius) (Lepidoptera: Noctuidae) in Saudi Arabia (Al-Mehmmady, 2000). The shortest development time to complete a life cycle of *E. vittella* was 31.82 days at 32.9°C (Al-Mehmmady, 2000), which is comparable to the results found in this study for *E. biplaga,* of 29.92 days at 30 °C.

Development time at 30°C was still linear indicating that 30°C is below the optimum temperature for development of this species. The upper temperature limit for development of the larval stage of *E. vitella* was reported to be 40°C (Ahmad & Ullah, 1939). Although information on temperature dependent development of the *Earias* spp. is limited, results from the study of Al-Mehmmady (2000) on *E. vitelli* and the current study on *E. biplaga*, suggest the optimum temperature for development to be higher than 30°C.

The development rate determined for *E. biplaga* in this study at 18°C was slow and almost not existing at 15 °C. These findings are in accordance with that of Ahmad & Ullah (1939) that exposure of *E. vitella* larvae to 13°C or below is fatal. Entwistle (1967) also found the larval period of *E. biplaga* to vary with temperature. The longest development period of 16.0-16.5 days occurred during the wet-season months when lower temperature and shorter days prevailed. And faster development of 9.69 days was reported during the dry season when temperatures were higher and days were longer. The minimum threshold temperature for the larval stage was lower than for the egg stage. Eggs will therefore hatch at temperatures which is also suitable for larval development.

The lower temperature threshold for cotton (host plant) is 18°C (Gipson, 1986), which overlaps with the lower threshold level of *E. biplaga*. Cotton production in Egypt is the best at a temperature of 32°C (Balls, 1919), but 35°C proofed to be unsuitable for cotton development (Balls, 1919). The optimum temperature for cotton production of 32°C and the rapid development of E*. biplaga* at 30°C determined in this study therefore partially explains the pest status of *E. biplaga* on cotton.

4.6 References

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Table 4.1 Mean development time (days ± S.E.) of different life stages of *Earias biplaga* at constant temperatures. Range of numbers of days to develop is shown in brackets.

Developmental stage	Temperature (± 1°C)			
	18	20	25	30
Egg	8.32 ± 0.12a (7-9)	5.85 ± 0.05b (5-6)	4.43 ± 0.08c (4-6)	2.38 ± 0.12d (1-4)
Instar 1	6.24 ± 0.20a (4-10)	5.45 ± 0.14b (4-10)	3.61 ± 0.09c (3-5)	2.95 ± 0.09d (2-5)
Instar 2	4.81 ± 0.10a (3-6)	3.37 ± 0.11b (2-5)	2.33 ± 0.13c (1-3)	1.51 ± 0.09d (1-3)
Instar 3	5.05 ± 0.12a (4-7)	4.10 ± 0.15b (2-8)	2.16 ± 0.10c (1-5)	1.84 ± 0.09c (1-3)
Instar 4	5.95 ± 0.25a (4-12)	4.67 ± 0.18b (2-9)	2.43 ± 0.11c (1-5)	1.86 ± 0.06c (1-2)
Instar 5	7.16 ± 0.31a (4-13)	5.27 ± 0.18b (4-12)	2.84 ± 0.11c (1-4)	2.57 ± 0.12c (1-3)
Larvae	29.87 ± 0.69a (24-37)	22.85 ± 0.25b (19-29)	13.37 ± 0.23c (11-18)	10.73 ± 0.18d (9-13)
Pupae	30.70± 0.29a (24-37)	22.68 ± 0.18b (18-26)	12.12 ± 0.10c (11-14)	9.35 ± 0.09d (9-11)
Egg-to-adult	68.89 ± 0.80a (63-78)	51.38 ±0.29b (48-58)	29.92 ± 0.24c (27-34)	22.46 ± 0.18d (21-26)

Means within the same row followed by the same letter do not differ significantly at P=0.05 (Tukey's HSD).

Table 4.2 Linear regression between the development rate (1/days) and temperature (18-30°C) at the different development stages of *Earias biplaga*. k is the thermal requirements in degree-days and t is the estimated lower temperature threshold.

Development stage	Regression model (y=bx-a)	k ± S.E.	t ± S.E.	r- value
Eggs	y= 0.0314x – 0.4766	31.89±1.44	15.20 ± 0.25	0.68
First instar	y= 0.0161x – 0.1261	59.74 ± 2.96	8.55 ± 0.62	0.83
Second instar	y= 0.0432x – 0.5643	23.16 ± 1.41	13.07 ± 0.60	0.77
Third instar	y= 0.0362x – 0.4459	27.61 ± 1.86	12.31 ± 0.69	0.74
Fourth instar	y= 0.0342x – 0.4367	29.21 ± 1.76	12.76 ± 0.64	0.78
Fifth instar	y= 0.0268x – 0.3275	37.25 ± 2.99	12.20 ± 0.63	0.68
Larval stage	y = 0.0327x – 0.4166	192.09 ± 4.61	11.31 ± 0.28	0.81
Pupal stage	y = 0.0064x – 0.0831	154.13 ± 2.01	12.88 ± 0.13	0.98
Egg-to-adult	y= 0.0026x – 0.0312	392.16 ± 4.29	12.80 ± 0.08	0.99

Table 4.3 Mean development time in days and degree-days (°D) for *Earias biplaga* at constant temperatures from 18 – 30 °C.

Development stage	Temperature (°C)	n	Development time (days ± S.E.)	Range	°D ± S.E.
Egg	18	37	8.32 ± 0.12	7-9	23.30 ± 0.17
	20	60	5.85 ± 0.05	5-6	28.13 ± 0.17
	25	49	4.43 ± 0.08	4-6	43.33 ± 0.48
	30	37	2.38 ± 0.12	1-4	35.50 ± 0.75
Mean					34.31 ± 0.45
Larvae	18	37	29.87 ± 0.69	25-43	199.80 ± 4.60
	20	60	22.85 ± 0.25	19-29	198.57 ± 2.13
	25	49	13.37 ± 0.23	11-18	183.00 ± 3.11
	30	37	10.73 ± 0.18	9-13	200.54 ± 3.37
Mean					195.05 ± 1.66
Pupae	18	37	30.70 ± 0.29	28-35	157.20 ± 1.48
	20	60	22.68 ± 0.18	18-26	161.50 ± 1.31
	25	49	12.12 ± 0.10	11-14	146.92 ± 1.21
	30	37	9.35 ± 0.09	9-11	160.10 ± 1.52
Mean					156.45 ± 0.81
Egg-to- adult	18	37	68.89 ± 0.80	62-82	358.24 ± 4.14
	20	60	51.38 ± 0.29	48-58	369.96 ± 2.09
	25	49	29.92 ± 0.24	27-34	365.00 ± 2.97
	30	37	22.46 ± 0.18	21-25	386.30 ± 3.17
Mean					369.57 ± 1.63

Degree-days were calculated using the lower threshold temperature for development determined for each developmental stage (eggs = 15.20 °C, larvae = 11.31 °C, pupae = 12.80 °C and egg-to-adult = 12.23 °C).

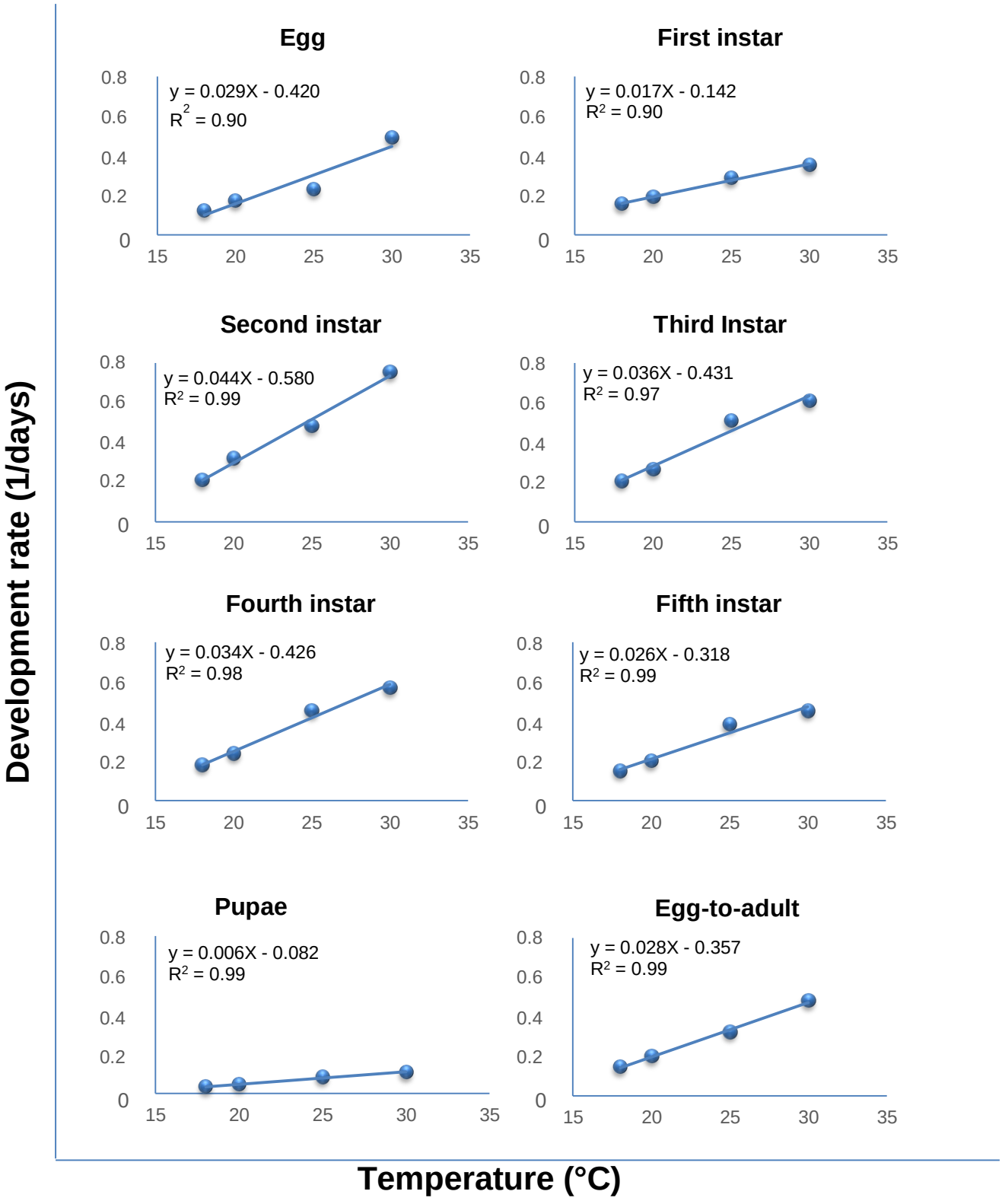

Figure 4.4 Linear regression table of the development rate of *Earias biplaga* at different stages (eggs, larvae, pupae and egg-to-adult) at controlled temperatures (18, 20, 25 and 30°C).

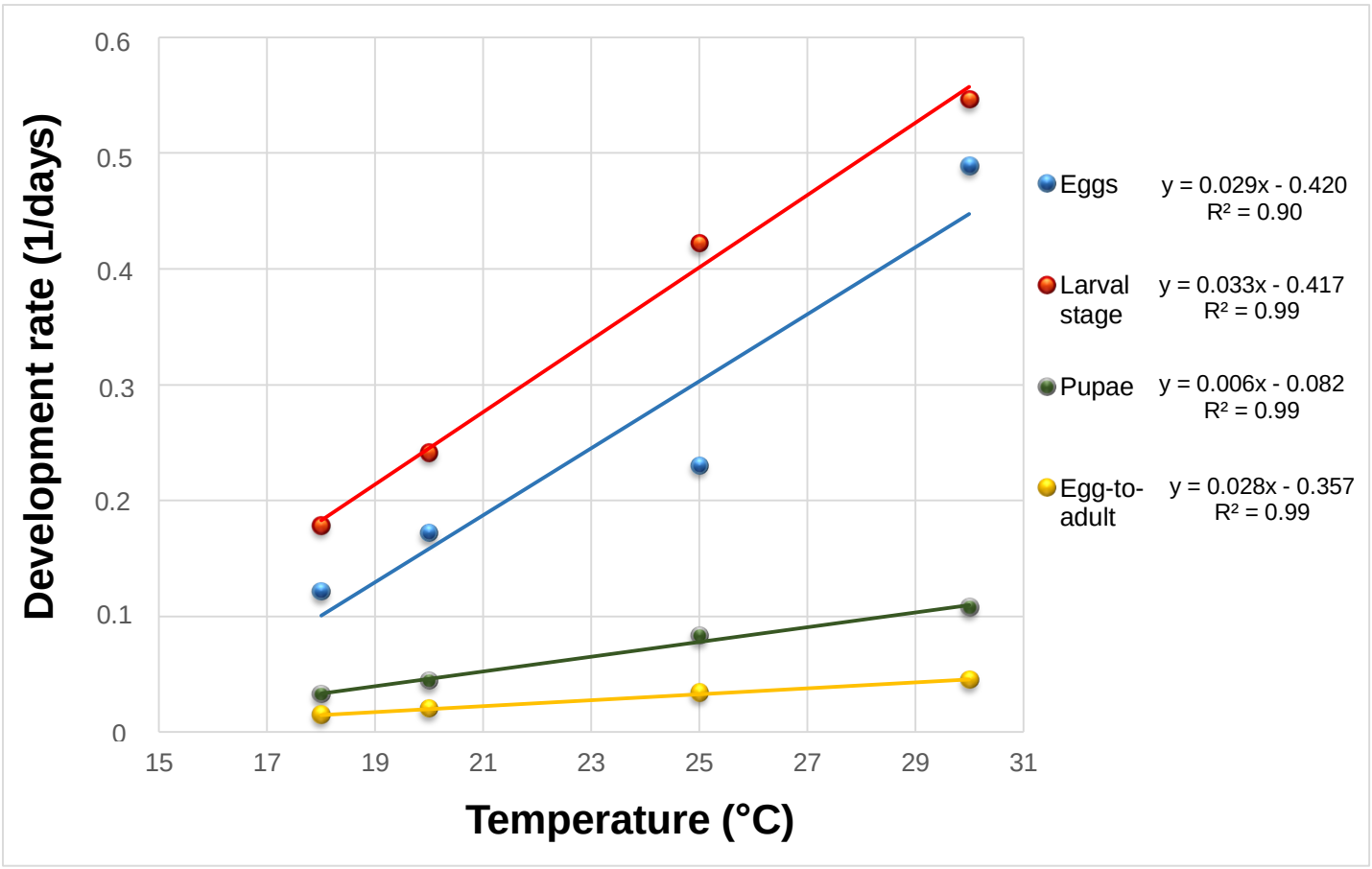

Figure 4.5 Development rates of *Earias biplaga* at constant temperatures for different life stages.

Chapter 5
Conclusion

5.1 Conclusion

The bollworm complex consisting of the African bollworm *Helicoverpa armigera* (Hübner) (Noctuidae), red bollworm, *Diparopsis castanea* Hampson (Noctuidae), and spiny bollworms, *Earias biplaga* Walker (Noctuidae) and *Earias insulana* Boisduval (Noctuidae) is responsible for the most insect damage to cotton in South Africa (Bennett, 2007; Malinga, 2010). Damage by *Earias* spp. to cotton in South Africa, is often underestimated (Bennett, 2007). Larvae tunnel into the flowering parts, cotton squares and cotton bolls causing little damage to the outside surface area, but substantial damage feeding inside the cotton boll (Abro *et al*., 2004).

Bt cotton for control of the bollworm complex is cultivated in South Africa since 1998 (Cotton SA, 2012). Bt cotton is an example of a plant incorporated pesticide (PIP). It is genetic material that were added to a plant's genetic structure, enabling it to produce substances that can act as a pesticides to a specific pest group or pest (Gupta & Dikshit, 2010). The Cry protein in Bt cotton acts as a self-producing insecticide. The control of certain insect pest is therefore made possible without spraying many insecticides that might be harmful to the environment, and which poses a health risk to the people handling it (Perlak *et al*., 2001). The development of resistance to Bt cotton is, however, of great concern. It is important to evaluate the possibility of resistance development in insect pests regularly. It can then be noted in an early stage and the necessary research and control measures can be implemented (Tabashnik, 2008). Although Bt sprays are not registered for control of spiny bollworm in South Africa, it is registered for control of *H. armigera* on apples, pears, beans, citrus, cruciferae, herbs, peas, tomatoes, ornamentals, flowers and lawns (Van Zyl, 2013) The use of Bt sprays is an alternative pest control option to chemical insecticides without the negative effects on the environment. The active components of Bt-based

microbial insecticides is Cry proteins, which have been used as foliar sprays in agriculture and forests for several decades (Reed *et al*., 2001).
Bt spray such as Dipel® as well as PIPs such as Bollgard® and Bollgard II® have to be ingested to have any effect on the larvae (Andow, 2008). It is possibile for insect pests to develop resistance to insect-tolerant crops (McGaffery, 1998; Pray *et al*., 2002; Bennett-Nell *et al*., 2005; Alvi *et al*., 2012). The status of resistance of *E. biplaga* to Bt proteins expressed by plants and contained in Bt sprays in South Africa was therefore investigated in this study to contribute to sustainable management. Neonate *E. biplaga* larvae were 100% controlled by Bollgard® expressing Cry1Ac and Bollgard II® expressing both Cry 1Ac and Cry 2Ab2, showing no resistance these two cotton cultivars. There was no resistance development in both the Potchefstroom and Rustenburg populations evaluated (Chapter 2). Bollgard® is already removed from the South African market, but Bollgard II® is still effective in controlling *E. biplaga* in South Africa. Future studies should also include the determination of Bt expression levels in different plant parts of cotton.

DiPel, used in this study is the most used Bt kurstaki (HD-1 strain) product and contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab (Valent biosciences, 2014). It is used for the control of more than 100 species of Lepidoptera globally (Navon, 1993). In this study (Chapter 3), neonate *E. biplaga* larvae were exposed to the different concentrations of DiPel®, *viz*. the recommended dosage rate for *H. armigera* control on cotton, half, twice and five times this recommended dosage rate. Survival of larva was evaluated 4, 7 and 10 days after application. Half the dosage rate proofed to be ineffective in controlling *E. biplaga* larvae. Bt spray applications at the recommended dosage rate and rates higher than the recommended rates provided effective control of *E. biplaga* (Chapter 3), but chances of survival on conventional cotton sprayed with Bt is better than on Bt cotton expressing the Cry proteins. Bt sprays break down if exposed to UV light and rain can reduce the efficacy, final instar larvae will not be controlled by Bt sprays and effective coverage with the spray application is essential for successful control. Bt is ever-present in Bt transgenic plants, is continuously expressed and a farmer does not have to be specific on the timing of insecticide application (Navon, 2000).

The effect of temperature on the development of *E. biplaga* was also studied, because temperature is considered as one of the most important abiotic factors affecting various life history parameters of insects (Hallman & Denlinger, 1998; He *et al*., 2003). This study provides information about the effect of temperature on the development of *E. biplaga* (Chapter 4). It was conducted at four constant temperatures, *viz*. 18, 20, 25 and 30 ±1 °C and a 14L: 10D photoperiod. Temperature affected development of *E. biplaga* significantly. Mortality was very high and almost no development of larvae occurred in a preliminary study to determine development of *E. biplaga* at 15°C, indicating that a constant temperature of 15 °C is too low for *E. biplaga* development. Development time for all life stages was, however, inversely related to temperatures from 18 to 30 °C. The relationship between temperature and developmental rate of *E. biplaga* was linear between 18 and 30 °C and more rapid development was observed with increasing temperatures The shortest total development time to complete a life cycle (29.92 days) was recorded for *E. biplaga* at 30°C. The total development period was 68.9 to 22.5 days at 18 and 30 days, respectively. The thermal thresholds for *E. biplaga* were 15.2, 11.3, 12.8 and 12.2°C and the thermal constants were 34.3, 195.1, 156.45 and 369.6 °D, for the completion of the egg, larval, pupal and egg-to-adult stages, respectively. The optimum temperature for cotton production of 32°C, and the rapid development of *E. biplaga* at 30°C determined in this study therefore, partially explains the pest status of *E. biplaga* on cotton in South Africa.

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