Status of resistance of *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Diparopsis castanea* (Lepidoptera: Noctuidae) to Bt cotton in South Africa

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Abstract

Genetically modified (GM) cotton expressing Cry1Ac proteins was released in South Africa in 1997 for control of the bollworm complex on this crop. No reports of the failure of Bollgard® cotton to control these pests have yet been made. Throughout the world there are concerns about the development of resistance of target pests to Bt cotton due to the use of only one Bt gene. The aim of this study was to determine if *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Diparopsis castanea* (Lepidoptera: Noctuidae) developed resistance to Bt cotton in South Africa. To determine if *H. armigera* developed resistance, laboratory experiments were conducted to determine the levels of larval survival and development time when feeding on Bt and non-Bt cotton. Bollworm populations were collected on maize and cotton at different sites in South Africa and reared on Bt and non-Bt cotton under laboratory conditions. Results showed that some populations survived on Bt cotton and that a significant proportion of the individuals successfully completed their life cycles on Bt cotton. Surveys were also conducted amongst cotton farmers to determine the levels of compliance to the refuge strategy that has to be implemented by farmers as an insect resistance management (IRM) strategy to delay resistance development. The levels of compliance to refugia requirements were low and farmers generally only started planting refugia several years after they planted Bt cotton for the first time. The development of resistance of *H. armigera* to Bt cotton in South Africa can possibly be ascribed to non-compliance to the prescribed refuge requirements. No conclusions can be made on resistance of *D. castanea* to Bt cotton but the relatively long time to mortality of larvae could indicate development of tolerance to Cry1Ac proteins. The new generation Bollgard II® cotton, expressing both Cry1Ac and Cry2Ab2 proteins, has been released in South Africa during the 2010/11 growing season and field observations showed effective control of the bollworm complex at several sites in the country. Monitoring of refuge compliance levels as well as resistance development in the bollworm complex to Bollgard II® cotton is necessary to ensure the future success of GM cotton.

Keywords: *Helicoverpa armigera, Diparopsis castanea*, Bt cotton, Lepidoptera, resistance.

Opsomming

Geneties gemodifiseerde (GM) katoen wat Cry1Ac-proteïen uitdruk is in 1997 vir die eerste keer in Suid-Afrika vir die beheer van die bolwurm-kompleks vrygestel. Geen aanmelding van die mislukking van Bollgard® katoen teen die teikenplae in Suid-Afrika is beskikbaar nie. Wêreldwye kommer bestaan aangaande die ontwikkeling van weerstand teen Bt-katoen wat slegs een geen bevat. Die doel van hierdie studie was om te bepaal of *Helicoverpa armigera* (Lepidoptera: Noctuidae) en *Diparopsis castanea* (Lepidoptera: Noctuidae) weerstand teen Bt-katoen in Suid-Afrika ontwikkel het. Om vas te stel of *H. armigera* weerstand ontwikkel het, is eksperimente in die laboratorium uitgevoer om die persentasie larwale oorlewing te bepaal. Larwes het op beide Bt- en nie-Bt katoen gevoed. Bolwurmbevolkings is op mielies, sorghum en katoen op verskillende plekke in Suid-Afrika versamel en op Bt en nie-Bt katoen onder laboratoriumtoestande grootgemaak Die 1ste generasie larwes is willekeurig op Bt- en nie-Bt katoen bolle geplaas. Resultate het getoon dat sommige populasies op Bt-katoen oorleef en dat 'n beduidende deel van die individue hulle lewensiklusse suksesvol op Bt katoen voltooi. Opnames is ook met katoenboere uitgevoer om te bepaal of hulle aan die vereistes voldoen deur toevlugareas te plant vir insekweerstandsbestuur (IRM) strategie om die ontwikkeling van weerstand te vertraag, te bepaal. Die plant van toevlugsareas was aanvanklik laag. Die ontwikkeling van weerstand van *H. armigera* teen Bt-katoen in Suid-Afrika kan moontlik toegeskryf word aan die feit dat daar nie aan die voorgeskrewe toevlugarea-vereistes voldoen is nie. Geen afleidings kan gemaak word oor die weerstand van *D. castanea* teenoor Bt-katoen nie, maar die relatief lang tydperk voor afsterwe van die larwes dui moontlik op die ontwikkeling van toleransie teenoor Cry1Ac-proteïene. Die nuwe Bollgard II®-katoen, wat beide Cry1Ac en Cry2Ab2 proteïne uitdruk, is tydens die 2010/11 groeiseisoen in Suid-Afrika vrygestel met die doel om weerstandbiedende bolwurm-populasies te beheer. Veldwaarnemings het effektiewe beheer van die bolwurmkompleks op verskeie plekke in die land bevestig. Monitering van weerstandsontwikkeling van die bolwurmkompleks ten opsigte van Bollgard II® katoen in Suid-Afrika is nodig vir die toekomstige sukses van die gewas.

Sleutelwoorde: *Helicoverpa armigera, Diparopsis castanea,* Bt katoen, Lepidoptera, weerstand.

Table of Contents

Chapter 1: LITERATURE REVIEW

1.1. General background of cotton and production in South Africa

American upland cotton, *Gossypium hirsutum* L. (Malvales: Malvaceae) has been cultivated in South Africa since 1846 (Annecke & Moran, 1982). Cotton is the leading plant fiber crop produced in the world with India being the leading cotton producing country. Transgenic Bt cotton varieties was first introduced in USA, Mexico and Australia during 1996 and since the commercial release of Bt cotton a rapid worldwide increase in the production of Bt cotton occurred (Ismael *et al*., 2001). Bt cotton is cultivated to control the complex of lepidopterous pests that mainly attack the flowering parts of this crop. The most important species in this pest complex are *Heliothis* spp., *Helicoverpa* spp., *Diparopsis* spp., *Earias* spp. and *Pectinophora* spp. (Hill, 1983).

The first Bt cotton that was commercially released was INGARD cotton in Australia and Bollgard® cotton in the United States (Olsen & Daly, 2000). The total area planted to Bt cotton at that stage was approximately 800 000 ha. By 2003, the global area of genetically modified (GM) cotton reached 5.8 million ha which was grown in nine countries (Ismael *et al*., 2001). According to James (2010) a total of 20 million ha GM cotton (insect resistant and herbicide tolerant cotton) was cultivated globally during 2010. Cotton production in 2011/12 is forecast to increase in most of the major producing countries as producers respond to the current high market price of this fibre crop (Department of Agriculture, 2011).

There are different advantages associated with the adoption of transgenic crops. These are higher yields, less labour intensive and a reduction in the use of insecticides that result in higher profits of the crop in the USA, China, South Africa and Mexico (Fernandez-Cornejo & Klotz-Ingram, 1998; Gianessi & Carpenter, 1999; Fernandez-Cornejo *et al*., 1999; Perlak *et al*., 2001; Pray *et al*., 2001; Ismael *et al*., 2001; Traxler *et* *al*., 2001; Huang *et al*., 2002; Bennett *et al*., 2004; Shankar & Thirtle, 2005). There are also some possible negative effects and fears of adverse effects to the environment such as a reduction in biodiversity and the possible development of resistance that can result in economical losses to farmers. Another possible environmental threat associated with GM crops is that genes may be transferred to congeneric plants that could then become weedy (Ismael *et al*., 2001).

Bt cotton and Bt maize was approved for cultivation in South Africa, during 1997 and 1998 respectively. In South Africa, the commercialization and introduction of GM crops is facilitated by the Genetic Modified Organism Act (GMO Act, Act 15 of 1997) (Government Gazette, 1997) which was implemented in 1999 (Ismael *et al*., 2001). This act promotes the safe use of Bt crops that are introduced into South Africa, and was developed to promote the responsible development, production, use and application of genetically modified organisms and to ensure that activities are carried out in such a way as to limit possible harmful consequences to the environment and human health. The act requires regular monitoring and reporting on the effect of GM crops on target and non-target organisms.

Many different GM crops have been approved for field trials in South Africa, but only GM cotton, soybeans and maize are grown on a commercial basis (Gouse *et al*., 2005). South Africa and Burkina Faso are the only countries in Africa that released Bt cotton on a commercial scale (Ismael *et al*., 2001). The first insect resistant cotton has been planted in South Africa in 1997 (Cotton SA, 2006) and herbicide tolerant cotton has been available in this country since 2001 (Andow *et al*., 2006; Brookes & Barfoot, 2006; Cotton SA, 2006).

Cotton production systems in South Africa can be divided into two groups: small-scale farmers that are resource-poor and grow cotton under dry-land conditions and large-scale farmers that produce cotton under irrigated as well as dry-land conditions (Gouse *et al*., 2004). Cotton is mainly grown in high rainfall areas and most large-scale cotton production, including Bt cotton takes place in five production regions in South Africa (Fig. 1.1). These regions are: Vaalharts (Northern Cape Province), Loskop irrigation scheme (Groblersdal and Marble Hall in the Limpopo Province) (Annecke & Moran, 1982), Weipe next to the Limpopo River (Limpopo Province) (Gouse *et al*., 2003), Jacobsdal (Free State Province) and Douglas (Northern Cape Province). Bt cotton has, however, only been planted for the first time during the 2010/11 growing season in the Douglas and Jacobsdal areas. The main small-scale cotton production area in South Africa is the Makhathini Flats in KwaZulu-Natal.

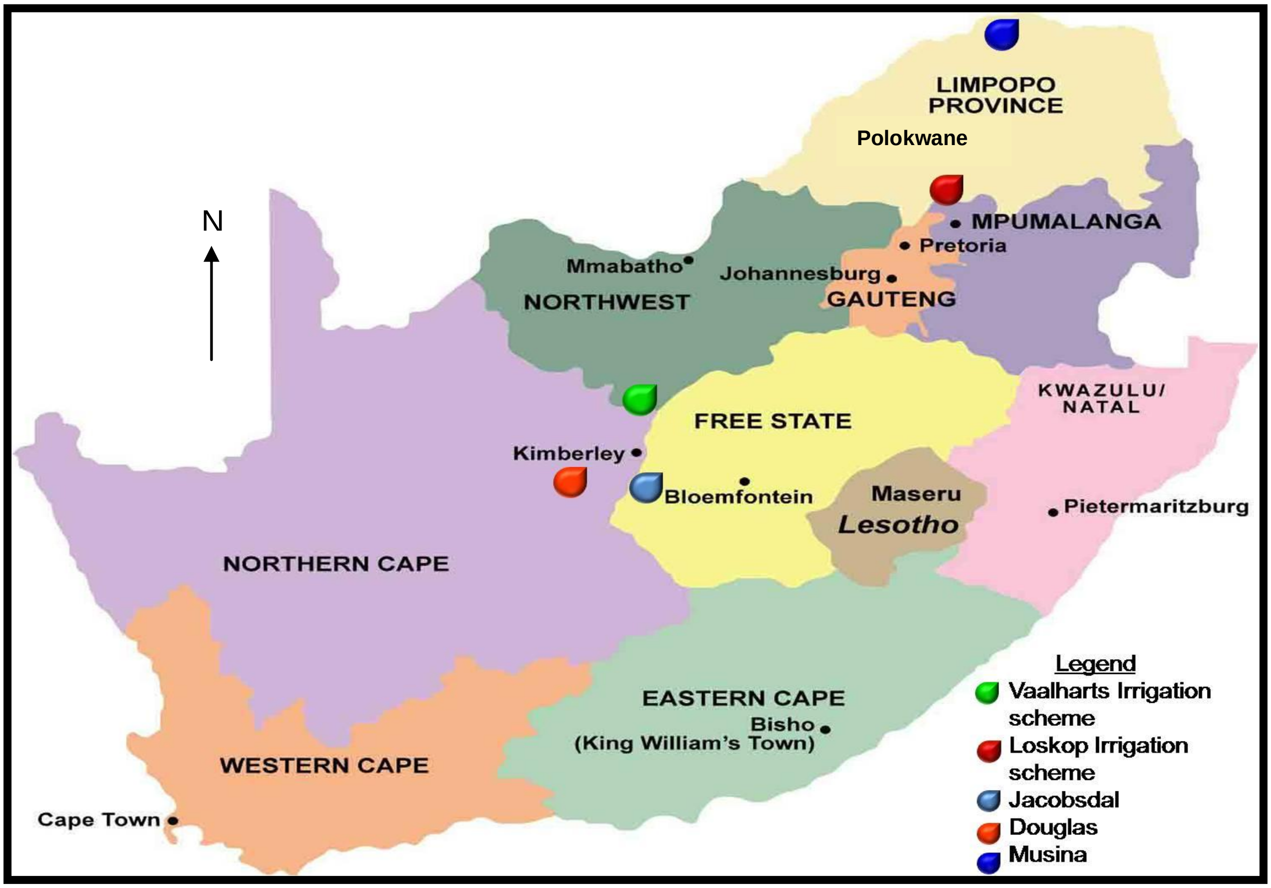

Figure 1.1: The five major cotton production regions of South Africa.

The Makhathini Flats is a small-scale farming area where farm size range between one and three hectares (Ismael *et* al., 2001; Morse *et al*., 2006). In the1998/99 season, Bt cotton was commercially released to smallholders in Makhathini Flats and by 2001/02 more than 90 % of the approximately 3500 farmers in the area had adopted Bt cotton varieties (Ismael *et al*., 2001; Bennett, 2002; Morse *et al*., 2006). Approximately 4500 cotton farmers could have potentially been active in the Makhathini area (Gouse *et al*., 2003).

The introduction of Bt cotton (Bollgard®) in the Makhathini Flats was successful in the sense that it provided many advantages for the small-scale farmers. The introduction of Bt cotton created new pest management opportunities for small-scale farmers in rural communities. Farmers that adopted Bt cotton experienced great success such as a decrease in the amount of insecticides used to control cotton pests, one of the reasons for the high adoption rate of Bt cotton in this area (Ismael *et al*., 2001; Morse, *et al*., 2006). Furthermore, cultivation of Bt cotton improved the income of the farmers and lowered production costs to such an extent as to offset the higher seed cost (Gouse *et al*., 2003). Cotton was an important crop for these farmers because compared to large-scale farmers that can rotate cotton with maize and other crops, small-scale cotton farmers were dependent on cotton, because of low, irregular rainfall and a lack of production credit for other crops. The amount of cotton that was produced in the Makhathini area depended on the availability of production credit and the price of cotton (Bennett, 2002; Gouse *et al*., 2003). During 2003 most farmers on the Makhathini Flats planted Bollgard® cotton. Green *et al*. (2003) therefore highlighted the fact that for the technology to be preserved, the development of resistance to the Bt-toxin expressed in the transgenic cotton plant had to be prevented.

Vunisa Cotton Company was responsible for the management of the cotton industry on the Makhathini Flats where they supplied seed, chemicals, credit and information to farmers as well as to buy the cotton harvest from the farmers. All farmers in the region delivered cotton to Vunisa Cotton where they weighed and graded the cotton and the farmers were then paid accordingly (Ismael *et al*., 2001; Gouse *et al*., 2003; Morse *et al*., 2006). However, because Vunisa cotton was deregistered (CIPRO, 2011) as a company no cotton was planted in the Makhathini Flats during the 2010/11 growing season.

The cultivation of Bt cotton in the Makhathini Flats was important in the context of small scale farming in South Africa as well as the rest of Africa. It contributed to the control of different bollworm species in this area and resulted in reduced insecticide use. These positive attributes together with the resources made available through the particular private enterprise resulted in a very high adoption rate of Bt cotton. However, production of Bt cotton on 1000's of small fields in this rural area made it difficult to monitor the rate of compliance to the prescribe insect resistance management (IRM) strategy that has to be employed to delay development of insect resistance to Bt cotton. The refuge strategy (discussed below) which is compulsory with the planting of Bt crops in South Africa implies that a certain area of a cotton field should also be planted to non-Bt cotton. It is therefore not known to what extent farmers in these areas planted refugia and resistance monitoring have never been done in this area. Although cotton is not planted by small-scale farmers in the Makhathini region any more, it is important that studies are done to determine if there is any resistance of target pests to Bt cotton in the Makhathini Flats.

During the 1999/00 production year in South Africa, a total of 100 000 ha Bt cotton was planted by 1530 commercial farmers and 3000 small-scale farmers mostly under dryland conditions (Ismael *et al*., 2001). During the following growing season, 31503 tons of Bt cotton was produced with an estimated 300 large-scale commercial farmers producing 95 % of South Africa's cotton crop. The other 5 % was produced by about 3000 small-scale farmers on the Makhathini Flats and a further 312 farmers in the Tonga area (Mpumalanga) (Kirsten & Gouse, 2002). Figures indicated that 5200 ha cotton was planted in KwaZulu-Natal under dryland and 1560 ha under irrigation during the 2005/06 production year and decreased to about 490 ha under dryland in the 2010/11 production year (Cotton SA, 2011).

A decrease in both irrigated and dryland cotton in production has been observed in South Africa especially since 1999 (Fig. 1.2). The reduced cultivation of cotton is ascribed to the low product price and the higher prices of competitive crops such as maize and sunflower. A number of cotton gins had been forced to close due to their inability to cover fixed costs and it had a huge effect on cotton production in South Africa (Fok *et al*., 2007; Cotton SA, 2011). While the cotton price remained largely similar between 1999 and 2007 (Fig. 1.3) a tendency of increased product price has been observed over the last 4 years (Fig. 1.3).

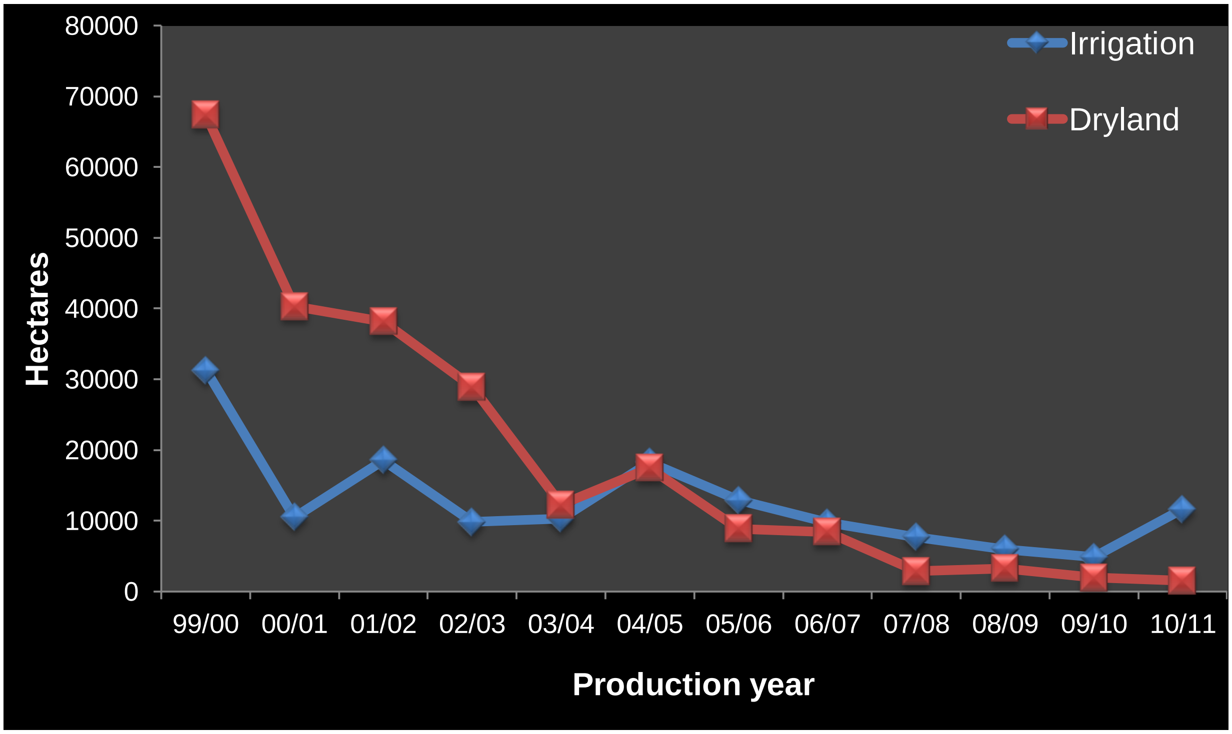

Figure 1.2: Cotton production in South Africa from the 1999 to the 2011 production year on both irrigated and dryland cotton (Cotton SA, 2011).

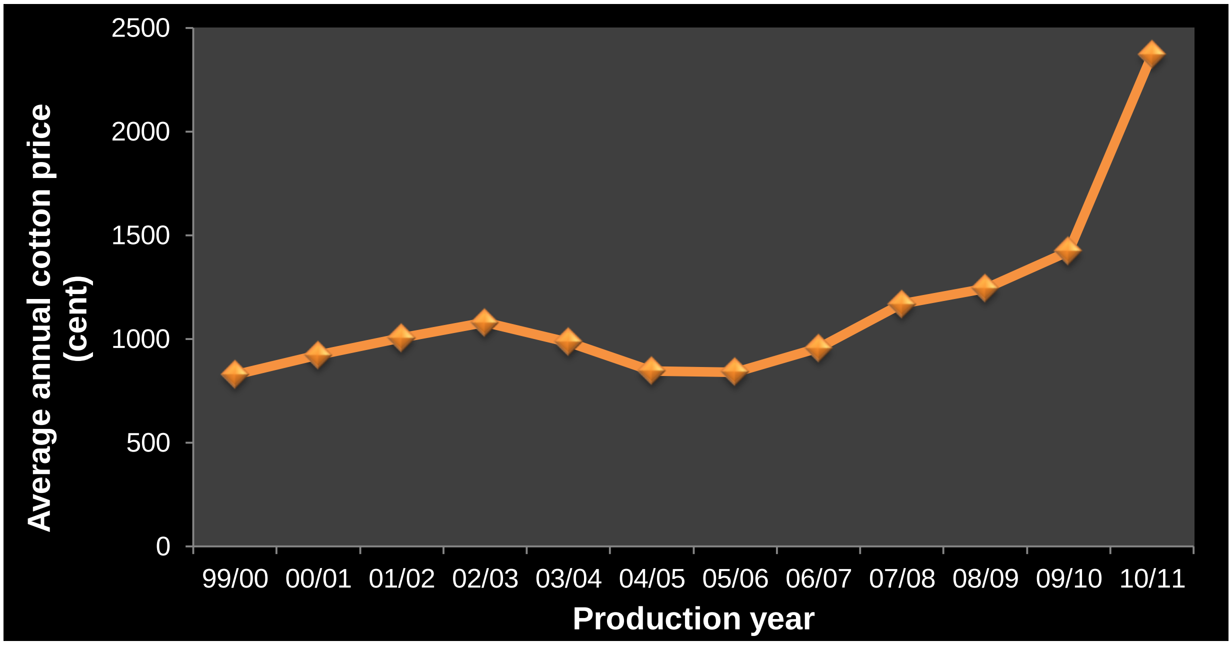

Figure 1.3: The average cotton price in South Africa from the 1999/00 to 2010/11 production yeas (Cotton SA, 2011).

The main reason and the biggest driver of adoption of insect resistant cotton by large-scale farmers was a reduction in the use of insecticides and secondly the increased yield resulting from reduced damage caused by target pests (Gouse *et al*., 2003). One of the biggest advantages that farmers noticed was the increase in populations of beneficial insects that contribute to the control of the target pest (Gouse *et al*., 2004). Van Hamburg and Guest (1997) reported that high numbers of diverse species of natural enemies of pests may occur in cotton fields and that these should be protected in order to enhance natural control. According to large-scale farmers the only disadvantage of Bt cotton is the cost of seed and the technology fee. Seed cost is one of the reasons why some farmers stopped to plant Bt cotton in South Africa (Gouse *et al*., 2003). A study conducted by Gouse *et al*. (2003) indicated the cost of a 25 kg bag of Bt cotton seed to be R210 with an additional R600 technology fee. The indirect cost of bollworm control with the use of Bt cotton is therefore high. Farmers that plant 20 kg seed, therefore spend R480/ha for bollworm control. The current estimate is that the technology fee is about 10 % of a 25 kg bag of seed. Therefore, farmers are currently spending R205/ha to control bollworm infestations, irrespective of whether the pests are present or not. Farmers can control bollworms by means of insecticide applications at a lower cost, but in cases where the bollworm pressure is high the application of insecticides can easily exceed this additional technology fee.

The adoption rate of Bt cotton in South Africa since the first year of commercial release was mainly because of the various benefits that it provided to farmers. These include increase yield as well as associated financial benefits despite the higher seed cost. It also reduces the use of insecticides and therefore leads to a healthier environment and ecosystem (Gouse *et al*., 2004). Bt cotton provides continuous protection against the target pest for the whole growing season (Gouse *et al*., 2003). Despite the increase in the yield of Bt cotton, the demand for cotton in South Africa currently exceeds the domestic production. Cotton is therefore imported to meet the demand.

Before the commercial release of insect resistant cotton in South Africa the only method of bollworm control was by means of insecticide application. Cotton was extensively sprayed to control the most important cotton pest, the African Bollworm (*Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Before 1975 farmers applied insecticides mainly preventatively, it was reported that up to 15 insecticide sprays was applied during a single season (Whitlock, 1973; Morse *et al*., 2006). This high number of sprays contributed significantly to increased production costs and the risk of bollworm resistance development against insecticides (Whitlock, 1973; Morse *et al*., 2006). This could also have negative effects on natural enemies that contribute to pest control which could then result in build-up of the numbers of secondary pests (Van Hamburg & Guest, 1997; Yan *et al*., 2001).

The main purpose for the development of insecticides was to support crop production, to protect crops against pests and to limit crop losses (Waibel, 1986). However, the widespread use of insecticides by farmers started to pose some disadvantages towards the environment. Some disadvantages are listed: (Pingali & Gerpacio, 1997):

- a risk to human health and the environment since it impacts negatively on beneficial insects such as parasitoids that contribute to the control of pests
- contamination of water bodies by means of drift and surface water runoff and seepage
- accumulation up of pesticide residues in crops
- development of resistance by pests to insecticides
- the development of secondary pests.

The most widely used biological insecticide which is often applied as an insecticide spray formulation is *Bacillus thuringiensis* (Bt) which produces different kinds of insecticidal toxins during its sporulation process (Höfte & Whiteley, 1989; Schnepf *et al*., 1998). However, GM plants such as Bt cotton produces proteins which are toxic to Lepidoptera and some Coleoptera (Morse *et al*., 2006). Bt cotton is reported to be relatively target-specific and does not protect the crop against other pests such as aphids. For this reason some insecticide applications may still be required if infestation levels of non-target pests reach economically important levels (Morse *et al*., 2006). Each type of protein has a unique mode of action against specific target pests. These different *cry* genes produce proteins that can be divided into four main groups:

- *CryI* is selective to lepidopteran larvae (Höfte & Whiteley, 1989; Gilliland *et al*., 2002).
- *CryII* genes are selective to lepidopteran larvae such as *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) and *Lymantria dispar* (Linnaeus) (Lepidoptera: Lymantriidae) and larvae of Diptera such as *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) (Höfte & Whiteley, 1989).
- *CryIII* genes are specific to Coleoptera (Höfte & Whiteley, 1989).
- The genes that are Diptera specific are the *CryIV* and *CytA* genes (Höfte & Whiteley, 1989).

Bt cotton expresses the Cry1Ac gene from the soil bacterium *B. thuringiensis* subspecies *kurstaki*. The mode of action of *B. thuringiensis* crystal inclusions in insects is complex. Upon digestion by susceptible insect larvae, the inclusion bodies are solubilised, and the protoxins are converted into toxins. The activated toxins bind to receptors on the surface of mid-gut epithelial cells of susceptible insects, which result in the lysis of the mid-gut epithelial cells and death of the insects (Van Rie *et al*., 1989; English & Slatin, 1992; Gill *et al*., 1992; Ferré & Van Rie, 2002).

There are concerns about the possible development of resistance to Bt cotton as a result of the use of only one Bt gene. It is possible that resistance may develop to the specific *cry* protein produced by the Bt crop in the same way that insects develop resistance to insecticides (Mellet *et al*., 2003). Bt cotton is commercially known as Bollgard® (MON 531) and is the most widely used cotton cultivar in South Africa (Perlak *et al*., 2001). The other registered transgenic cotton event in South Africa is MON 1445 which is herbicide tolerant cotton that allows farmers to spray glyphosate over the cotton to control weeds. Bollgard II® (MON 15985) cotton was commercially released in South Africa for the first time during the 2010/11 cropping season. Bollgard II® is a stacked variety (containing different transgenes) and expresses both the Cry1Ac and Cry2Ab2 proteins. It is expected that the release of Bollgard II® cotton would expand the range of benefits to both growers and the environment (Monsanto, 2003).

1.2. General description of the cotton plant

1.2.1. Stems and leaves

The cotton plant grows into either a small shrub or a shrub like tree several meters high and the length and the number of axial limbs vary according to variety and may be influenced to a large extent by conditions of cultivation and location. There are two types of branches that occur on a cotton plant, namely the vegetative branch and the fruiting branch. The vegetative branches are structurally the same as the main stem and they bear flowers and fruit only after re-branching. The vegetative branches develop from the main stem near the ground and tend to grow in an upright position. The second type of branch is the fruiting branches and can develop from the main stem or the vegetative branches (Bennett, 1991). The vegetative branches are carried at an acute angle to the main stem and the fruiting branches are carried in a more lateral position to the main stem (Brown & Ware, 1958; Eaton, 1955; Tharp, 1960; Cobley, 1957; Jones, 1963).

The flowers and the bolls of the cotton plant are produced on the fruiting branches. The main stem and the vegetative branches must first branch to produce the fruiting branches in order for bolls and the flowers to develop. There is a tendency for the lower branches of the stem to be vegetative and the upper ones to be fruiting branches. The first fruiting branch is usually produced at the sixth or eighth node on the main stem (Brown & Ware, 1958; Eaton, 1955; Tharp, 1960; Cobley, 1957; Jones, 1963).

Figure 1.4: A) Illustration of the upright growth of the main stem and the vegetative branch. B) The fruiting branch has a zigzag growth habit (www.pubs.caes.uga.edu/caespubs/pubcd /B1252/B1252.html).

Leaves are spirally arranged on the main axis and its vegetative branches. The leaves vary in size, shape, texture, as well as the presence of leaf hairs (Kochhar, 1981). The degree of hairiness is usually characteristic of different cotton cultivars (Brown & Ware, 1958; Eaton, 1955; Tharp, 1960; Cobley, 1957; Jones, 1963).

1.2.2. Flowers

Fruiting branches of the cotton plant can produce six to eight flower buds that appear as small green pyramidal structures known as squares (Fig. 1.5). It takes approximately 25 days for a square to develop into an open flower. Flowers open at dawn and withers before the evening of the same day (Brown & Ware, 1958; Eaton, 1955; Tharp, 1960; Cobley, 1957; Jones, 1963; Bennett, 1991). The square consists of the following parts:

- whorl of three triangular-shaped green leaflets known as bractlets. The bractlets completely enclose and protect the tender growing flower parts.
- the inconspicuous cup-shaped calyx, which tightly encloses the basal end of the flower bud.
- inside the calyx are the five conspicuous petals which collectively form the corolla.
- inside the corolla is the staminal column, composed of numerous stamens, each with a two-lobed anther.
- the petals have a narrow base, which widens rapidly to broad flat expanse of the upper part of the petal.

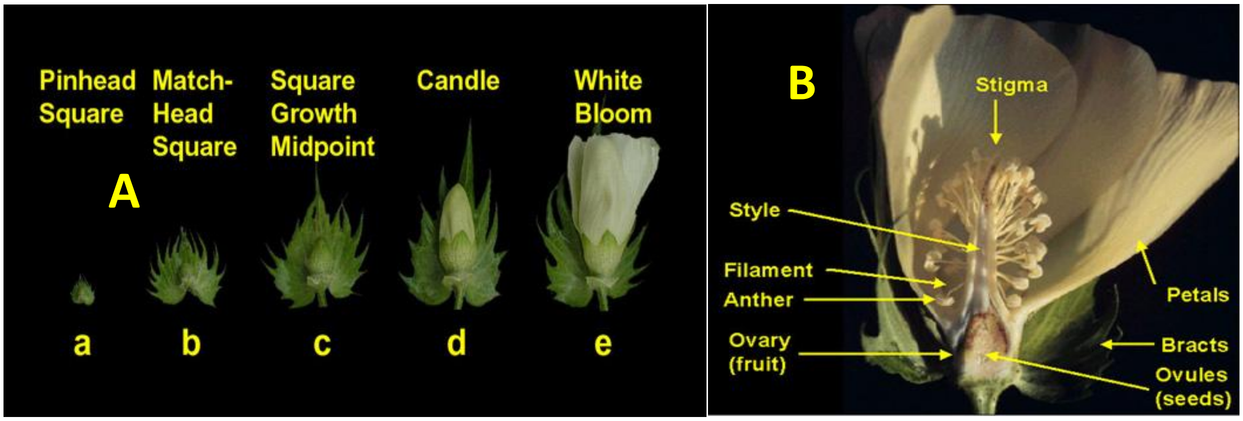

Figure 1.5: A) The different stages in the development of the cotton square. B) The morphology of the cotton flower (pubs.caes.uga.edu/caespubs/ pubcd/B1252/ B1252 .html).

1.2.3. Cotton boll

The fruit of the cotton plant is known as the boll and is a spherical or ovoid capsule varying in form and size (Fig. 1.6). Flowering is determined by temperature, rainfall, sunlight and soil fertility. It takes approximately 40 to 70 days from the first time the plant flowered until the boll opens. The capsules contain the seed, lint and fuzz (Kochhar, 1981). When the bolls are dry they start to crack along the sutures on the boll where the carpels meet. The number of carpels range from four to five and the seeds are arranged in two rows in the locks. The average number of seeds in a lock is about nine (Bennett, 1991). The seed is ovoid, more or less pointed, dark brown and ranges in length from 6 to 12 mm. There are two types of fibre that occur on the epidermis of the seed coat. These are the lint that is the long white fibres and the fuzz which is the short white fibres that are strongly attached to the seed coat (Brown & Ware, 1958; Eaton, 1955; Tharp, 1960; Cobley, 1957; Jones, 1963).

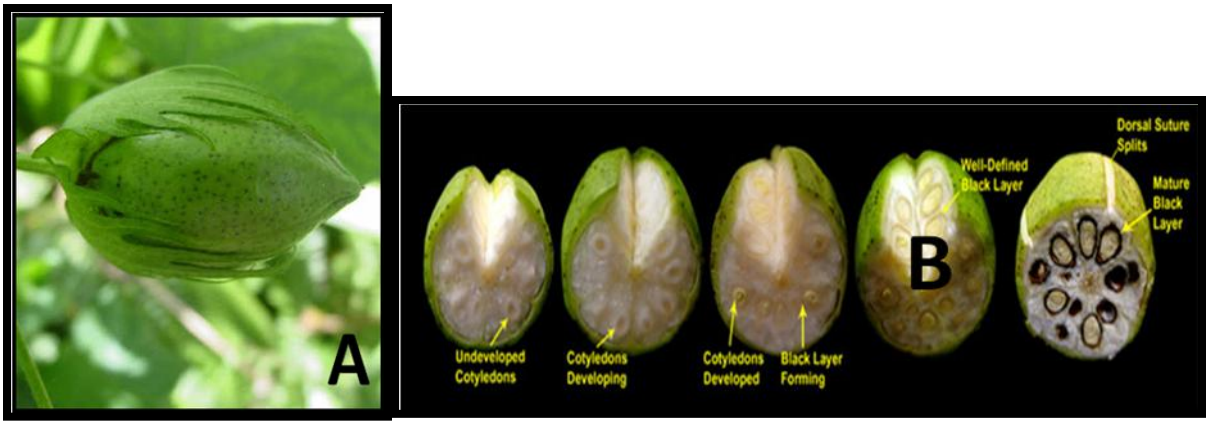

Figure 1.6: The mature boll or capsule of the cotton plant (www.doyletics. com/digest54.htm). b) The stages in the development of the cotton boll (www.pubs. caes.uga.edu/caespubs/pubcd/B1252/ B1252 .html).

1.3 Cotton pests in South Africa

Different lepidopteran species have been recorded as minor or sporadic pests of cotton in South Africa (Annecke & Moran, 1982) and are listed in Table 1. A variety of insects can cause damage to cotton, both quantitative and qualitative. The majority of insect pests on cotton are polyphagous, for example the different bollworm species. The most important lepidopteran pests of cotton are the bollworm complex that feed on the reproductive plant parts of the cotton plant (Van Hamburg & Guest, 1997; Morse *et al*., 2006). Some of the pest species of cotton in South Africa are oligophagous, for example the cotton stainers. Cotton stainers (Hemiptera: Heteroptera) are an important group of insects that stains the fibre and cause a reduction in the quality of the cotton (Basson, 1990).

Table 1: The major lepidopteran pests of cotton in South Africa.

Different groups of lepidopteran pests	**Pest Species**	**References**
Bollworm Complex	African bollworm (*Helicoverpa armigera*)	Annecke & Moran, 1982; Vaissayre & Cauquil, 2000
	Spiny bollworm (*Earias biplaga*)	Annecke & Moran, 1982; Vaissayre & Cauquil, 2000
	Spiny bollworm (*Earias insulana*)	Annecke & Moran, 1982; Vaissayre & Cauquil, 2000
	Red Bollworm (*Diparopsis castanea*)	Annecke & Moran, 1982; Vaissayre & Cauquil, 2000
False Bollworm	False Pink Bollworm (*Sathrobota simplex*)	Annecke & Moran, 1982
Leaf caterpillars	Tomato semi - looper (*Chrysodeixis acuta*)	Annecke & Moran, 1982
	Cabbage semi-looper (*Thysanoplusia orichalcea*)*	Annecke & Moran, 1982
	Cotton semi-looper (*Anomis flava*)	Annecke & Moran, 1982; Vaissayre & Cauquil, 2000
	Leaf worm (*Xanthodes graellsi*)	Annecke & Moran, 1982
	Cotton leaf worm (*Spodoptera littoralis*)	Annecke & Moran, 1982; Vaissayre & Cauquil, 2000
	Leaf roller (*Syllepte derogata*)	Annecke & Moran, 1982; Vaissayre & Cauquil, 2000
	Leaf miner (*Acrocercops gossyppi*)	Annecke & Moran, 1982
	False codling moth (*Cryptophlebia leucotreta*)	Annecke & Moran, 1982
Cutworms	Black cutworm (*Agrotis ipsilon*)	Annecke & Moran, 1982
	Brown cutworm (*Agrotis longidentifer*)*	Annecke & Moran, 1982
	Common cutworm (*Agrotis segetum*)	Annecke & Moran, 1982
	Spiny cutworm (*Agrotis spinifera*)	Annecke & Moran, 1982

*listed by Annecke and Moran (1982) under different scientific names.

1.4. The bollworm complex

In South Africa the bollworm complex consists of three species namely the African bollworm (*Helicoverpa armigera*) (Hübner) (Lepidoptera: Noctuidae), Spiny bollworm (*Earias biplaga*) (Walker) (Lepidoptera: Noctuidae) and Red bollworm (*Diparopsis castanea*) (Hampson) (Lepidoptera: Noctuidae).

1.4.1. African bollworm (Helicoverpa armigera) (Hübner) (Lepidoptera: Noctuidae)

African bollworm is distributed all over Africa, southern Europe, the near and Middle East, India, Central and Southeast Asia, Japan, the Philippines, Indonesia, New Guinea, eastern Australia, New Zealand, Fiji, and some other Pacific islands (Annecke & Moran, 1982).

Helicoverpa armigera (Fig. 1.7) is generally regarded as the most important pest of agriculture throughout the world because of its wide host range (Zalucki *et al*., 1986; Fitt, 1989; Bell & McGeoch, 1996; Van Hamburg & Guest, 1997; Vaissayre & Cauquil, 2000). It is also the most important species of the bollworm complex and is widely distributed throughout Africa (Van Hamburg & Guest, 1997). It was previously known as the American bollworm or *Heliothis armigera*. This species does not occur in Americas and the name was changed to the African bollworm (Du Plessis & Van den Berg, 1999).

Figure 1.7: Damage caused by *Helicoverpa armigera* to a cotton square.

Forewings of the moth have a brownish, yellowish-brown or grayish-brown colour with darker brown markings. Hind wings are pale, grayish-white with dark veins, and a broad dusky apical band that has two distinct pale spots. The head and body is 18 mm in length and the moth has a wingspan of about 40 mm (Fig. 1.8) (Annecke & Moran, 1982). Eggs are almost spherical, up to 0.5 mm in diameter, pale yellowish at first, becoming brown before they hatch (Vaissayre & Cauquil, 2000). There are usually six, sometimes seven larval instars. The first two are yellowish to reddish-brown. In later larval instars the characteristic pattern of three longitudinal dark bands separated by pale ones, develops (Annecke & Moran, 1982). The colours are variable and the pattern may be in shades of green, reddish-yellow, reddish-brown or blackish. Larvae grows to a length of 40 mm and has three pairs of thoracic legs, and fleshy leg like protuberances on each of the third to sixth abdominal segments as well as on the ultimate one. The pupa is dark brown (Annecke & Moran, 1982).

Figure 1.8: Moth of *Helicoverpa armigera.*

Eggs are laid singly near the flowers of the cotton plant, usually on the upper rather than the lower side of the leaves (Vaissayre & Cauquil, 2000). Eggs hatch within three to four days (Pălăgeşiu & Crista, 2007) in late spring and summer. The young larvae, having usually devoured the shell of the egg, go in search of a bud or flower which it will attack and destroy (Eyhorn *et al*., 2005). It takes approximately two to three weeks for the larvae to mature after which it pupates (Annecke & Moran, 1982). Pupae are formed in a flimsy cocoon up to 170-180 mm deep in the soil. In mid-summer the pupal stage may be as short as 15 days but becomes longer with the onset of cool weather in late summer, autumn and early winter, and the duration of the pupal stages is further protracted because most, but not all of the pupae enter diapause (Annecke & Moran, 1982).

Adult moths fly strongly and are most active from sunset until dark. Eight weeks after germination of the cotton plants, for a period of about 12 weeks, the cotton plants are attractive to moths seeking to lay eggs. Female moths mate approximately four days after emergence and a moth can lay up to 1600 eggs during her two to three week life span. A maximum of 480 eggs can be laid in a single night (Annecke & Moran, 1982). Eggs on cotton and other host plants are laid in large quantities only when buds and flowers are formed. The females are short-lived if deprived of nectar and liquid nourishment. There may be three to four major moth flight periods during the summer season and there are probably five to six generations per year (Annecke & Moran, 1982). Cotton plants are vulnerable to attack by the bollworms for long periods of time because cotton have a long flowering period and bollworms start to attack the plant from flowering onwards (Van Hamburg & Guest, 1997). This long period of vulnerability makes control of bollworms difficult. *Helicoverpa armigera* have many different parasitoids and predators and efficient management of the cotton pest complex is important to preserve these natural enemies (Annecke & Moran, 1982).

1.4.2. Red bollworm (Diparopsis castanea) (Hampson) (Lepidoptera: Noctuidae)

Red bollworms (*D. castanea* and *D. watersi*) (Fig. 1.11) are found only in Africa, *D. castanea* south of the equator and *D. watersi* north of the equator (Hill, 1983; Vaissayre & Cauquil, 2000).

Figure 1.11: Damage caused by *Diparopsis castanea* to a cotton square.

Diparopsis castanea is monophagous and is consequently linked to cotton (Vaissayre & Cauquil, 2000). The moth of the red bollworm has a wingspan of up to 35 mm. The forewing has three curved transverse lines demarcating four areas consisting of a reddish area at the base (Annecke & Moran, 1982). The hind wings and abdomen are largely cream in colour. Moths are active during the night and the females lay approximately between 250-300 eggs, more than half of which are laid in the first two weeks (Annecke & Moran, 1982). Eggs are hard-shelled, usually laid singly, and are pale blue, becoming greyish as they age (Vaissayre & Cauquil, 2000). Eggs are 0.5 mm in diameter and minutely spined (Hill, 1983).

Figure 1.12: Moth of *Diparopsis castanea.*

Eggs are laid on various parts of the plant, mainly on young stems and petioles in the vicinity of buds, less commonly on flowers or bolls. Eggs hatch in about five days at 25ºC and five larval instars take between 18-41 days to complete, depending on temperature (Annecke & Moran, 1982).

It is difficult to determine the precise duration of the larval stage, because the final moult takes place in a cell constructed in the soil (Annecke & Moran, 1982). First instar larvae are creamy white with a dark head but in the later instars characteristic red arrowhead-shaped markings develop on each segment. The basic colour of the older instars is pale green (Annecke & Moran, 1982; Vaissayre & Cauquil, 2000). Larvae bore into the growing tips of cotton plants when they have not yet produced flowers buds (Annecke &

Moran, 1982; Vaissayre & Cauquil, 2000). Larvae that hatch early in the growing season fail to mature unless they find cotton fruit to feed on. The tip-boring injury is of special importance in cotton that is mechanically harvested because it changes the shape of the cotton plant (Annecke & Moran, 1982). Larvae pupate within the top 70 mm of soil. Pupae are therefore protected by a soil casing (Annecke & Moran, 1982; Hill, 1983; Vaissayre & Cauquil, 2000). Pupae that are formed early in the season emerge as moths within a few weeks of pupation. As the season advances an increasing proportion of larvae enter diapause to emerge as moths intermittently over the following year, but with a detectable peak in spring or early summer and another in late summer or early autumn. The diapause period may last for several years but is usually of shorter duration (Hill, 1983).

1.4.3. Spiny bollworm (Earias biplaga) (Walker) (Lepidoptera: Noctuidae)

Distribution of the spiny bollworm (*E. biplaga*) (Fig. 1.9) is confined to Africa south of the Sahara (Hill, 1983; Vaissayre & Cauquil, 2000).

Figure 1.9: Damage caused by *Earias biplaga* to a cotton square.

Seven spiny bollworm species attack cotton all over the world but only two, *E. biplaga* and *E. insulana* (Fig. 1.9), occur in Africa. These two species differ mainly in the colour pattern of the forewing. In *E. insulana* the colour of the forewings vary from silvery green to straw yellow and the outer fringe has the same colour. The colour of the wings of *E. biplaga* varies from a metallic green- to gold with a dark brown outer fringe (Fig. 1.10). The several thin dark lines on the forewings constitute a clear pattern which differs only slightly between the two species (Annecke & Moran, 1982).

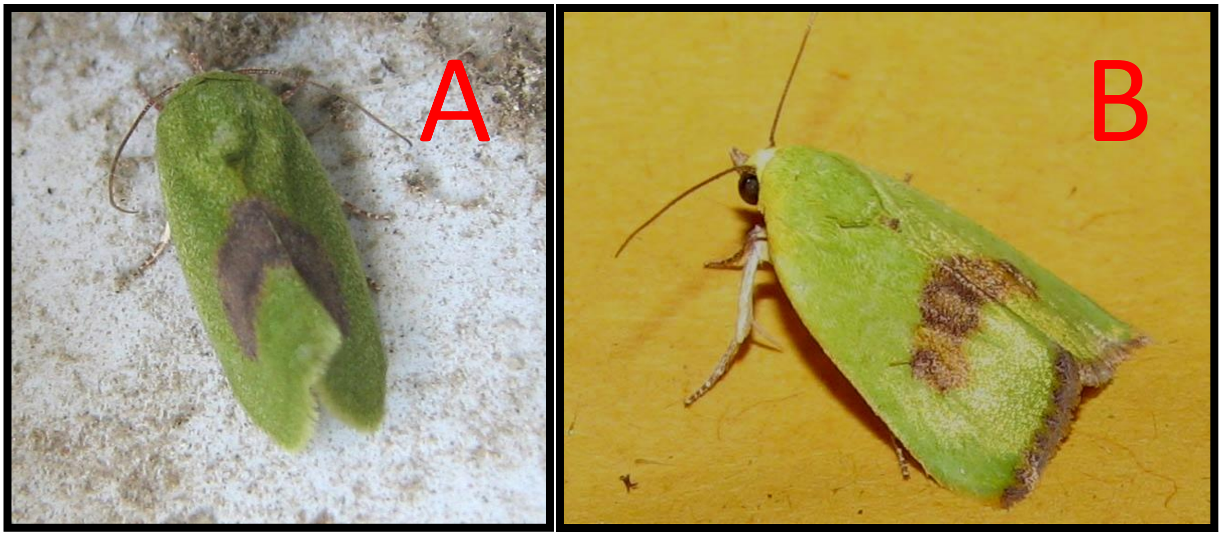

Figure 1.10: The minor difference between the two species of *Earias* that attack cotton in South Africa. A) *E. insulana* with the same colour outer fringe as the rest of the wing. B) *E. biplaga* with a dark fringe on the terminal end of the wing.

The fecundity of the moths has not yet been studied in South Africa, but approximately more than 200 eggs are laid by a single female. Eggs are 0.4 mm in diameter and are blue-green in colour which makes them very difficult to locate. Eggs are laid on any part of the plant but usually on the young shoots or flower buds and bolls. Eggs hatch in about three days in summer and the larvae pass through five moults. Larvae become spindle shaped and attain a length of 18 mm. The larvae feed on soft growing tissue in the growing points or internodes of the plant (Vaissayre & Cauquil, 2000). Larvae also bore into the flower buds and green bolls (Fig. 1.9) where they block the entrance with excreta. The second and third thoracic segments and the abdominal segments each have four fleshy tubercles, one on each side and two above. In summer, development of the larvae may be completed in two weeks and they pupate in a pale to brown cocoon on the plant or in debris beneath it. Larvae usually pupate on cotton stems and petioles, protected by a characteristic cocoon (Vaissayre & Cauquil, 2000). The pupal stage lasts for about two weeks (Annecke & Moran, 1982).

1.5. The importance of Integrated Pest Management (IPM)

A wide range of tactics may be used to manage pests and to reduce the application of insecticides. Some of these components include conservation or augmentation of beneficial insect populations, host plant resistance, application of selective insecticides and implementation of cultural control strategies. Bt cotton varieties should be viewed as a foundation on which to build IPM systems which incorporate a broad range of biological and cultural tactics (Fitt, 2000). IPM therefore plays an important role in a cotton production system, because it forms the basis to manage pests and reduce the use of insecticides that pose a health threat. For example, the reduction in the number of insecticide applications in small-scale farmers in South Africa that adopted Bt cotton decreased from 11.2 to 3.8 sprays per season to control other pests such as aphids, jassids and thrips (Bennett *et al*., 2003).

Conservation of beneficial insect species is an important concept and it can be assumed that survival of these species will be higher in the transgenic cotton in comparison to conventional cotton that is sprayed with insecticides to control the target pest (Berkeley, 2004). Experiments conducted by Fitt *et al*. (1994) in Australia indicated that INGARD cotton had little effect on non-target species, including non-target lepidopterous pests, beneficial insects, and other canopy dwelling and soil dwelling species. It is expected that control of the target pests will be more effective in transgenic cotton and that the beneficial insects will provide some protection against secondary pests such as mites and aphids which are induced pests in insecticide-sprayed cotton (Fitt, 2000).

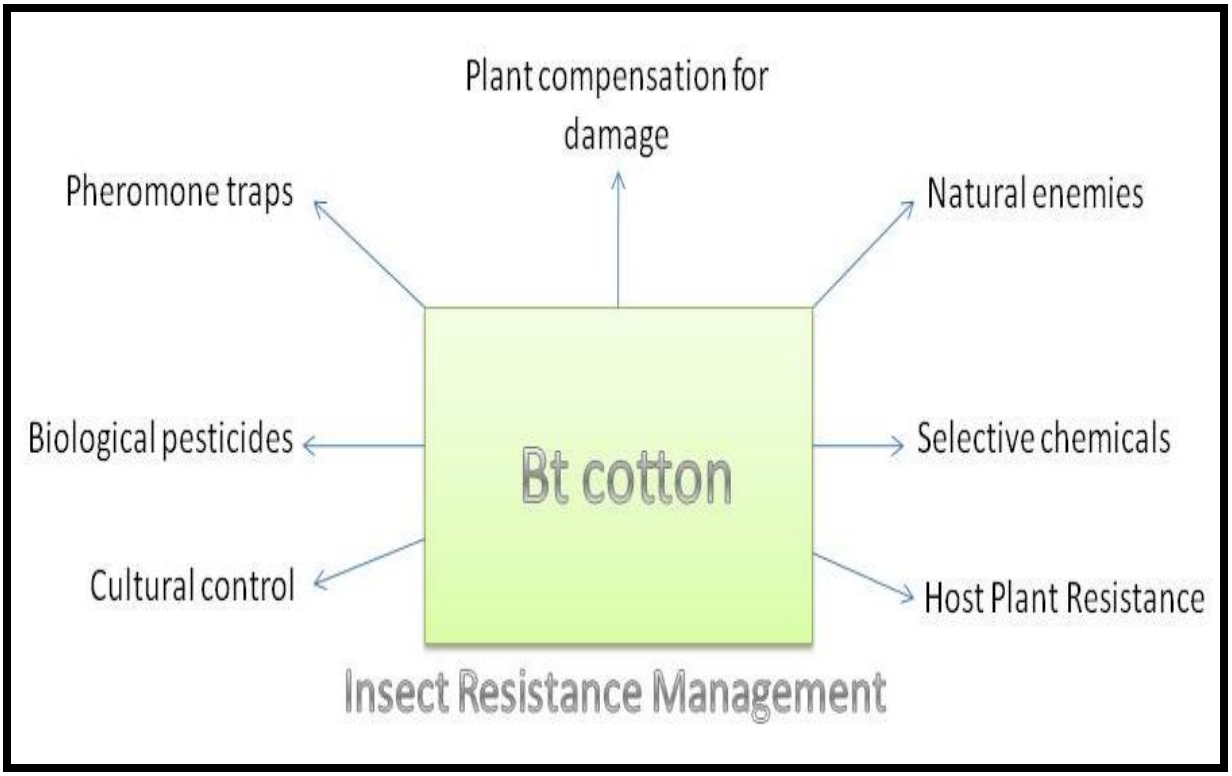

Figure 1.13: Indication of the central role and importance of transgenic cotton in an IPM program to control target pests (Fitt, 2000).

1.6. Insect resistance to Bt crops

Bt crops will only be effective for a short period of time if the target pest is over-exposed to the Bt crop and if the pest adapt to the insecticidal proteins expressed by crops (Tabashnik *et al*., 2008; Gould, 1998; Butler & Reichardt, 1999; Tabashnik, 1994a). Although there are many benefits for large-scale farmers in planting Bt cotton, the usefulness for small-scale farmers in developing countries was questioned (Grain, 2001). It was argued by Grain (2001) that Bt cotton does not have any positive impact on yield and it was suggested that bollworm resistance was already becoming a problem in China shortly after its release.

Since the report by Liu *et al*. (1999) that no reports of resistance to Bt crops under field conditions existed after four years of release, four lepidopteran species have been reported to be resistant to Bt crops. *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) to Bt cotton in southeastern United States (Luttrell *et al*., 2004), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) to Bt maize in Puerto Rico (Matten *et al*., 2008), *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) to Bt maize in South Africa (Van Rensburg, 2007) and *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) to Bt cotton in India (Monsanto, 2010a; Bagla, 2010). The first Coleoptera species that developed resistance to Bt maize in the United States was *Diabrotica virgifera* (LeConte) (Coleoptera: Chrysomelidae) (Gassmann *et al*., 2011).

Bt cotton was developed to reduce the use of insecticides and to prevent the development of resistance of the target pest to synthetic insecticides that were over-used (Akhurst *et al*., 2003). More than 30 % of insecticides that are used worldwide are directed against *H. armigera* and this has resulted in high levels of resistance of this pest (Ahmad, 2007). Insecticide resistance can be defined as the ability of an insect population to survive a dose of poison that is lethal to the majority of individuals in a normal population of the same species (WHO, 1957). *Helicoverpa armigera* is one of the species that show great capacity for developing resistance to synthetic chemical insecticides that are usually used to control this pest on cotton (Forrester *et al*., 1993). This pest already showed high levels of resistance to cypermethrin during 1989 in South India (Armes *et al*., 1992) and moderate resistance to carbamates in Spain during 1995 – 1999 (Torres-Vila *et al*., 2002). African bollworms have evolved resistance to most of the chemical insecticides and resistance evolution resulted in high levels of cross-resistance to insecticides within the same class (Fitt *et al*., 1994). It is expected that if the target species have the ability to develop resistance to the synthetic insecticides they have the ability to develop resistance to Bt cotton if they are over exposed to the insecticidal protein. The over-exposure to the toxins expressed by Bt cotton plants is an example of selection pressure that can result in the development of resistance (Fitt *et al*., 1994).

1.7. Insect Resistance Management (IRM) and the high dose/refuge strategy

As a pre-emptive measure Bollgard® and Bollgard II® cotton must be managed in ways that will prevent the development of insect resistance. The goal of resistance management is to delay the evolution of resistance in pests. In 2001, the Council for Biotechnology Information warned that the successful adoption of the Bt crops by farmers and the resulting widespread use of Bt proteins in crops will lead to development of insect populations that are resistant to these proteins (Alstad & Andow, 1995; Gould, 1998; Tabashnik *et al*., 2008). They further stated that this will render Bt crops and Bt sprays less effective in controlling these pests. It is therefore important that strategies are in place to delay and minimize the potential development of pest resistance. Scientific approaches should be used to establish management practices that will minimize the risk of resistance and sustain the performance of Bt pesticidal proteins. Other practices must also be established because there are already five cases of resistance established towards Bt crops (Council for Biotechnology Information, 2001).

In South Africa the high dose/refuge strategy is the only IRM program used to delay the development of resistance (Bennett *et al*., 2003; Qiao *et al*., 2006).

Concerns regarding the development of resistance of different bollworm species to Bt cotton prompted the U.S. Environmental Protection Agency (EPA) to establish limits on the total hectares planted by individuals, because the bigger the area that a farmer plant, the more difficult it becomes to monitor the development of resistance. This was also done to implement the refuge strategy. The appropriate refuge proportions was difficult to determine because of uncertainty over bollworm genetic resistance potential in the field and the uncertainty over the complex relationship between insecticide resistance and insecticide use in the field (Adkisson & Nemec, 1967).

The high-dose/refuge strategy is based on a combination of transgenic plants producing high doses of toxin, with nearby non-Bt plants or refugia that does not produce any toxins (Gould, 1998; Renner, 1999; Gould, 2000; Shelton *et al*., 2000; Tang *et al*., 2001; Chilcutt & Johnson, 2004). The purpose of the high dose is to kill off as many pest individuals as possible and the refuge is to produce pest individuals that survive on the particular crop. This is to ensure that rare individuals that survive on the Bt crop can mate with the susceptible individuals from the refuge and can reduce the development of resistance (Tabashnik, 1994b; Renner, 1999; Gould, 2000; Tabashnik *et al*., 2008).

Farmers that plant Bt cotton are obligated to sign a license agreement, stating that a non-Bt cotton refuge area will be planted for every 100 ha of Bt cotton (Monsanto, 2010b). Although the planting of refugia is compulsory to limit resistance development (Monsanto, 2007), the level of compliance by farmers in South Africa is not known. The current refuge requirements are either a 20 % refuge planted to conventional cotton which may be sprayed with lepidopteran-active insecticides, or a 5 % refuge area that should not be sprayed with chemical insecticides (Chilcutt, 2007).

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). If the resistance is recessive 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 susceptible plants. Mating between these adults produce hybrid F1 progeny that cannot survive on Bt plants (Liu *et al*., 1999). It is thus very important that farmers comply with the refuge strategy to limit the development of resistance in the target pest.

Insect resistance management plans are implemented through grower agreements and include other special features to assure their effectiveness such as:

- education on the importance of resistance management and how to identify potential resistance problems
- monitoring programs
- compliance with the IRM strategy
- reporting of suspected insect resistance
- taking action in the event of confirmed cases of insect resistance (Council for Biotechnology Information, 2001).

The typical time that it takes for insect pests to develop resistance to the most conventional neurotoxic pesticides in the field have been exceeded by Bt crops (McCaffrey, 1998). The question however remains if this delayed resistance development can be ascribed to only the efficacy of these IRM strategies. It is difficult to answer this question because the increase in resistance to Bt sprays in the field, laboratory and greenhouse demonstrate that resistance to Bt crops most likely remains a question of not ‘if’ but ‘when’ (Frutos *et al*., 1999; Tabashnik *et al*., 2003).

Tabashnik *et al*. (2003) identified several factors that could be possible reasons for the absence of field resistance to Bt crops. These factors are: (1) large fitness costs or other disadvantages suffered by resistant individuals; (2) initial low frequency of resistant alleles; (3) a dilution of resistant alleles with susceptible individuals from non-Bt plants; and (4) a high dose of toxin expressed by plants.

IRM strategies for Bt crops started as a theoretical exercise and resulted in development of e several tactics designed to delay resistance (Tabashnik, 1994b). The strategies that were proposed included the following:

- Moderate toxin dosage. There is only a moderate expression of the toxin in the plant and allow some susceptible larvae to survive. This tactic may result in only a small delay in resistance development (Roush, 1997).
- High toxin dosage to kill insects that can inherit resistant alleles. High doses of toxins are produced that kill all individuals of the target pest (Roush, 1997). This

tactic can contribute to the development of resistance, because if an insect survives exposure to the toxin it has no susceptible insect to mate with. From an IRM perspective, a dose that is high enough to cause mortality to hererozygotes is preferred and from an IPM perspective, a high dose will also ensure that crop damage is maintained below an economic threshold.

- Combination of toxins. This strategy involves the use of stack Bt varieties that express different toxins simultaneously (Tabashnik, 1994a; Roush, 1997).
- Temporal or tissue-specific toxin expression. In this approach the toxin is expressed in the plant at certain times or in specific parts of the plant through the use of temporal, tissue-specific or chemically inducible promoters (Roush, 1997). This strategy can promote the development of resistance where insects move between toxic and non-toxic plants and where they become strong enough to overcome the toxic plant and causes damage to them.
- Provision of non-toxic plants. This strategy is also known as the high/dose refuge strategy where plants that does not express the toxins are planted close to plants that produce toxins to allow susceptible insects to mate with possible resistant insects (Tabashnik, 1994a; Roush, 1997).

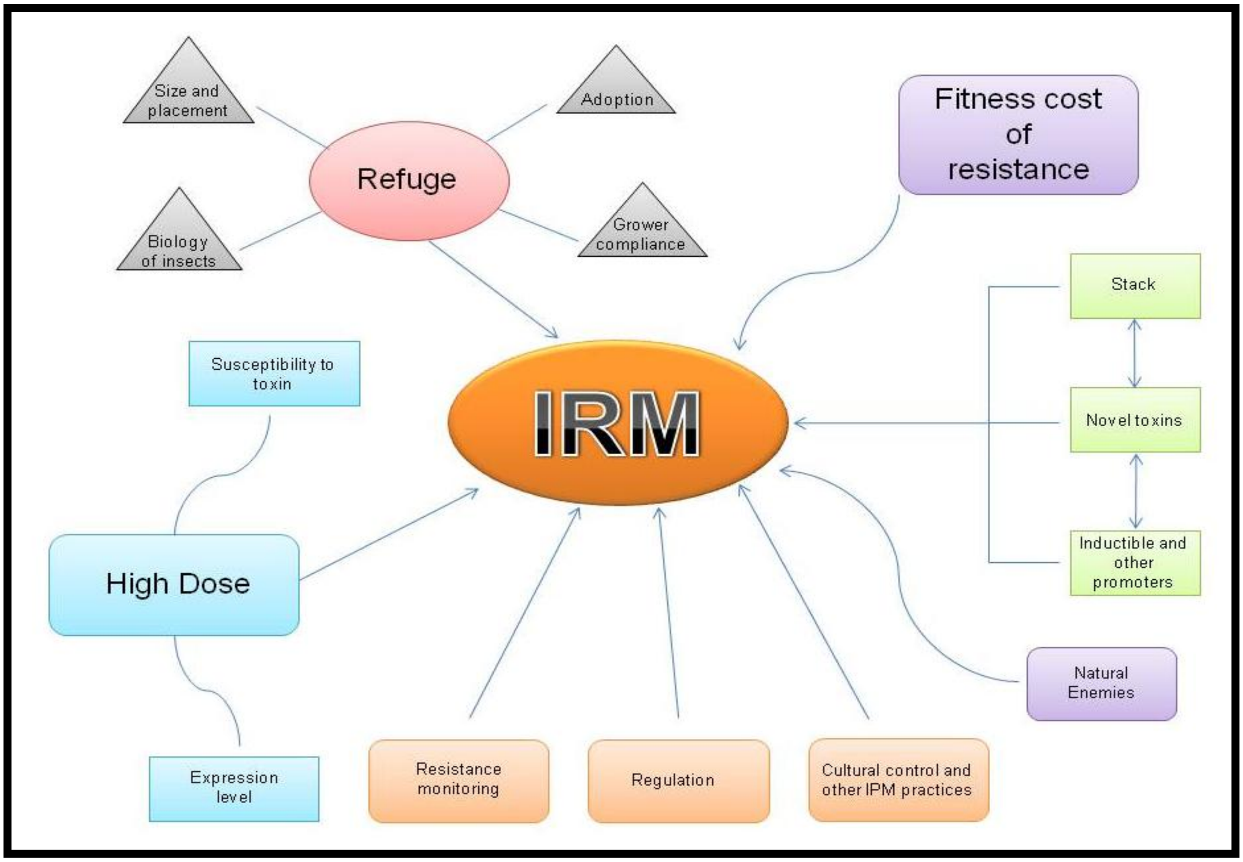

Figure 1.14: Factors affecting the efficacy of IRM strategies for insect resistant transgenic crops (Modified from Bates *et al*., 2005).

1.8. Monitoring of Bt crops

As part of IRM requirements, companies that seed of GM crops are mandated to implement an annual resistance monitoring program, the goal of which is to detect changes in resistance levels in pest populations. The currently most widely used method for resistance monitoring is a diagnostic or discriminating dose of a particular *cry* protein incorporated into an artificial diet. Such a dose, when carefully selected, will allow only resistant individuals to survive. This relatively inexpensive method allows many individuals to be tested and detects both polygenic resistance and multiple resistance mechanisms (Hawthorne *et al*., 2002). However, monitoring of resistance to Bt cotton is not 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.

It is therefore important to determine and report resistance of *H.* armigera and *D. castanea* to Bt cotton in South Africa. The first field resistance of the pink bollworm to Bt cotton has been confirmed in India during the 2008/09 growing season (Monsanto, 2010a) emphasizing the importance to assess whether resistance also occur in other countries.

1.9. Aims of the study

The general objective of this study was to determine if *Helicoverpa armigera* and *Diparopsis castanea* populations was resistant to Bt cotton in South Africa.

Specific objectives were to:

- assess farmer's perceptions about the use of Bt cotton and development of bollworm resistance and field damage.
- evaluate resistance levels of the African bollworm (*Helicoverpa armigera*) (Lepidoptera: Noctuidae) to Bollgard® cotton.
- evaluate resistance levels of the red bollworm (*Diparopsis castanea*) (Lepidoptera: Noctuidae) from the Makhathini Flats.

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CHAPTER 2

RESISTANCE OF AFRICAN BOLLWORM *HELICOVERPA ARMIGERA* (HÜBNER) (LEPIDOPTERA: NOCTUIDAE) TO BOLLGARD® COTTON IN SOUTH AFRICA

2.1. Abstract

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a key pest of cotton in South Africa. This pest has been controlled by means of Bt cotton since its release in 1997. It is expected that the bollworm will develop resistance to Bollgard® cotton expressing the Cry1Ac toxin if insect resistance management strategies are not followed. The aim of the study was to determine if *H. armigera* populations collected at different localities in South Africa developed resistance to Bollgard® cotton. A laboratory study was conducted during 2011 to evaluate African bollworm resistance to Bt cotton expressing the Cry1Ac protein. Larvae were collected from five localities in South Africa and reared on artificial diet until moths appeared. Neonate larvae deriving from eggs of these moths were used in the experiment. Fifty cotton squares each of Bt and non-Bt cotton were inoculated with two neonate larvae each and reared under controlled conditions. The number of surviving larvae was determined every four days when fresh food was provided in the form of fresh bolls. The Groblersdal population showed resistance to Bt cotton with 24 % larval survival after 20 days. Ten and 14 % survival was observed for larvae of the Parys and Vaalharts populations on Bt cotton compared to 24 % and 44 % survival on non-Bt cotton respectively. A delay in the development of pupae was observed for all populations on Bt cotton. This delay in development ranged between 4 – 12 days for different populations. The observed delay in pupal development may result in non-random mating between moths emerging from Bt and non-Bt cotton refugia, thereby increasing the rate of resistance development.

2.2. Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is the most important pest in agriculture because of its wide host range and damage it causes (Fitt, 1989; Bell & McGeoch, 1996). Bollworms are globally considered as the most important pest of cotton (Basson, 1990; Van Hamburg & Guest, 1997; Mellet *et al*., 2003). Insecticides were mainly used to control bollworms which contributed greatly to the total cost of cotton production. However, the efficacy of Bt cotton and chemical pesticides may gradually decrease as pests develop resistance towards these compounds (Yan *et al*., 2001). *Helicoverpa armigera* show great capacity to develop resistance to chemical insecticides that are usually used to control the pest on cotton (Forrester *et al*., 1993). This pest already showed high levels of resistance to cypermethrin during 1989 in South India (Armes *et al*., 1992) and moderate resistance to carbamates in Spain during 1995 – 1999 (Torres-Vila *et al*., 2002). As mentioned African bollworms showed great capacity for resistance development to chemical insecticides and have developed resistance to most chemical insecticides that resulted in high levels of cross-resistance to insecticides within the same class (Fitt *et al*., 1994). It is expected that if this species develop resistance to the synthetic insecticides they also have the ability to develop resistance to Bt cotton if they are over-exposed to the insecticidal protein (Fitt *et al*., 1994). Akhurst *et al*. (2003) indicated that *H. armigera* already attained a level of resistance to Bt cotton expressing Cry1Ac toxins in Australia, but that it was low in comparison to that reported for *H. virescens* in the USA (Gould *et al*., 1995).

Bt cotton contains the Cry1Ac gene from the soil bacterium *Bacillus thuringiensis* subspecies *kurstaki* (Halcomb *et al*., 1996; Pannetier *et al*., 1997; Hilder & Boulter, 1999; Peck *et al*., 1999). The 1st generation Bt cotton that was commercially in South Africa since 1997 is known as Bollgard® (MON 531). Bt cotton had been released to control the major Lepidopteran pests that attack cotton in South Africa (Perlak *et al*., 2001). Concerns about the development of resistance of target insect species if they are exposed to the Bt toxins for extended periods of time was raised two decades ago by

Tabashnik *et al*. (1991) as well as by McGaughey & Whalon (1992). The increase in adoption of Bt cotton since its first release can therefore result in development of resistance towards Cry1Ac toxin since the large-scale planting of Bt cotton increases selection pressure on the target pest (Bates *et al*., 2005). Evolution of insect resistance to Cry1Ac could limit the value of Bt cotton in future if it is continued to be planted. Monitoring of Bt crops and development of resistance is important. Through monitoring increase in the frequency of resistant alleles will be detected and correct resistance management strategies can be implemented (Kranthi *et al*., 2005).

Several studies demonstrated that transgenic plants expressing two Bt toxins can delay insect resistance evolution (Tabashnik *et al*., 2002; Zhao *et al*., 2003; Bird & Akhurst, 2004). Pyramiding different Bt genes in cotton is valuable for managing resistance evolution. Bollgard II® (MON 15985) cotton expressing both Cry1Ac and Cry2Ab proteins has been developed to delay resistance development (Zhao *et al*., 2003). Bollgard II® cotton was approved for cultivation in South Africa during 2005 (James, 2005) and was planted for the first time during the 2010/11 growing season. A study conducted by Luo *et al*. (2007) indicated no cross-resistance of Cry1Ac resistant *H. armigera* larvae towards Cry2Ab. They concluded that transgenic cotton expressing Cry1Ac and Cry2Ab genes may be deployed for management of Cry1Ac resistant *H. armigera* in China. Toxins expressed by the pyramided Bt-genes should have different modes of action to ensure that there is a low probability of cross-resistance between two toxins (Luo *et al*., 2007). Laboratory bioassays in which artificial diets were used did however show an increase in the frequency of resistance alleles of *H. punctigera* (Wallengren) (Lepidoptera: Noctuidae) to Cry2Ab2 protein expressed by Bollgard II cotton (Dowes *et al*., 2010). This could be an indication of early stages of resistance evolution of this pest.

As Bt cotton plants mature through the growing season, their insecticidal activity decreases and some *H. armigera* larvae are able to complete development on these plants. It is therefore important not to accept that bollworms are resistant to Bt cotton just because damage is observed under field conditions at late plant growth stages (Fitt *et al*., 1994). Prior to this study, resistance of *H. armigera* on Bt cotton in South Africa has not been studied or monitored.

Bollgard® cotton contains one of the genes that are also present in Bollgard II® cotton. It is important to know if resistance to Cry1Ac has already been achieved since its release, if this is the case, the possibility exists that the evolution of resistance to Bollgard II® may be more rapid. If *H. armigera* populations with resistance to Cry1Ac do exist in South Africa, it can be expected that resistance could evolve rapidly since this would then again come down to the use of a single-gene Bt cotton with only one novel gene being present in the pyramidal transgenic crop that can protect the plant from the target pest.

The objective of this study was to determine if *H. armigera* populations collected at different localities in South Africa shows resistance to Cry1Ab protein expressed by Bollgard® cotton.

2.3. Materials and method

2.3.1. Bollworm population collection

Helicoverpa armigera larvae were collected from five sites in South Africa (Fig. 2.1) during the 2010/11 growing season. The locality and crop from which larvae were collected are indicated in Table 2.1. Cotton is cultivated at only two of these sites, *i.e*. Groblersdal in the Loskop irrigation scheme and at the Vaalharts irrigation scheme. While cotton is not cultivated at Rustenburg any more, this area was known for large scale cotton production up to 15 years ago. Parys and Potchefstroom have no history of cotton cultivation with maize being the dominant crop.

The sites for bollworm collection were selected to represent areas that had a history of cotton production and some areas with no history. Rustenburg was chosen because the area had a history of cotton production which stopped about 15 years ago. No Bt cotton have been planted in the region. This locality can be considered unique in the sense that the target pests were not previously exposed to any GM cotton. Parys and Potchefstroom were chosen because they have no cotton planting history. Groblersdal and Vaalharts have been chosen because they have a long cotton planting history and cotton is still planted in these areas. The selection of the different localities was done mainly to determine if the populations that were collected from areas with a long cotton history and where Bt cotton is still planted have higher levels of resistance than the areas with no or some cotton history.

Table 2.1 Sampling sites and crops from which *Helicoverpa armigera* was collected in South Africa.

Site	**GPS coordinates**	**Crop**	**Plant part**	**History of cotton cultivation in area**
Groblersdal	S25º12'312 E29º16'505	Sweet corn	Maize ear	Decades-long
Vaalharts	S27º44'436 E24º47'025	non-Bt maize	Maize ear	Decades-long
Parys	S26º58'071 E27º22'01	Sorghum	Sorghum panicle	No history of cotton cultivation
Potchefstroom	S26º46'584 E27º08'171	Sorghum	Sorghum panicle	No history of cotton cultivation
Rustenburg	S25º43'372 E27º17'454	Organic cotton	Flowers and bolls	Large scale production until 15 years ago

Approximately 100 *H. armigera* larvae (F_0-generation) were collected in commercial crop fields at each site. These larvae were reared under laboratory conditions on the crop that they were collected until pupation. Pupae were maintained in plastic containers (52 x 55 mm) at 26 + 1 °C and a 14L: 10D photoperiod in an incubator. Once the moths emerged they were placed together in a large plastic container (40 x 20 x 15 cm) with an aerated lid. Moths were provided with pipe cleaners as oviposition substrate and plant material was used as stimulus for egg production. Neonate larvae (F_1-generation) emerging from these eggs were used in the feeding study. Approximately 50 male and female moth pares were present and allowed to mate. Each female laid approximately 250 eggs and from these eggs larvae that emerged were randomly selected and placed on Bt and non-Bt cotton bolls.

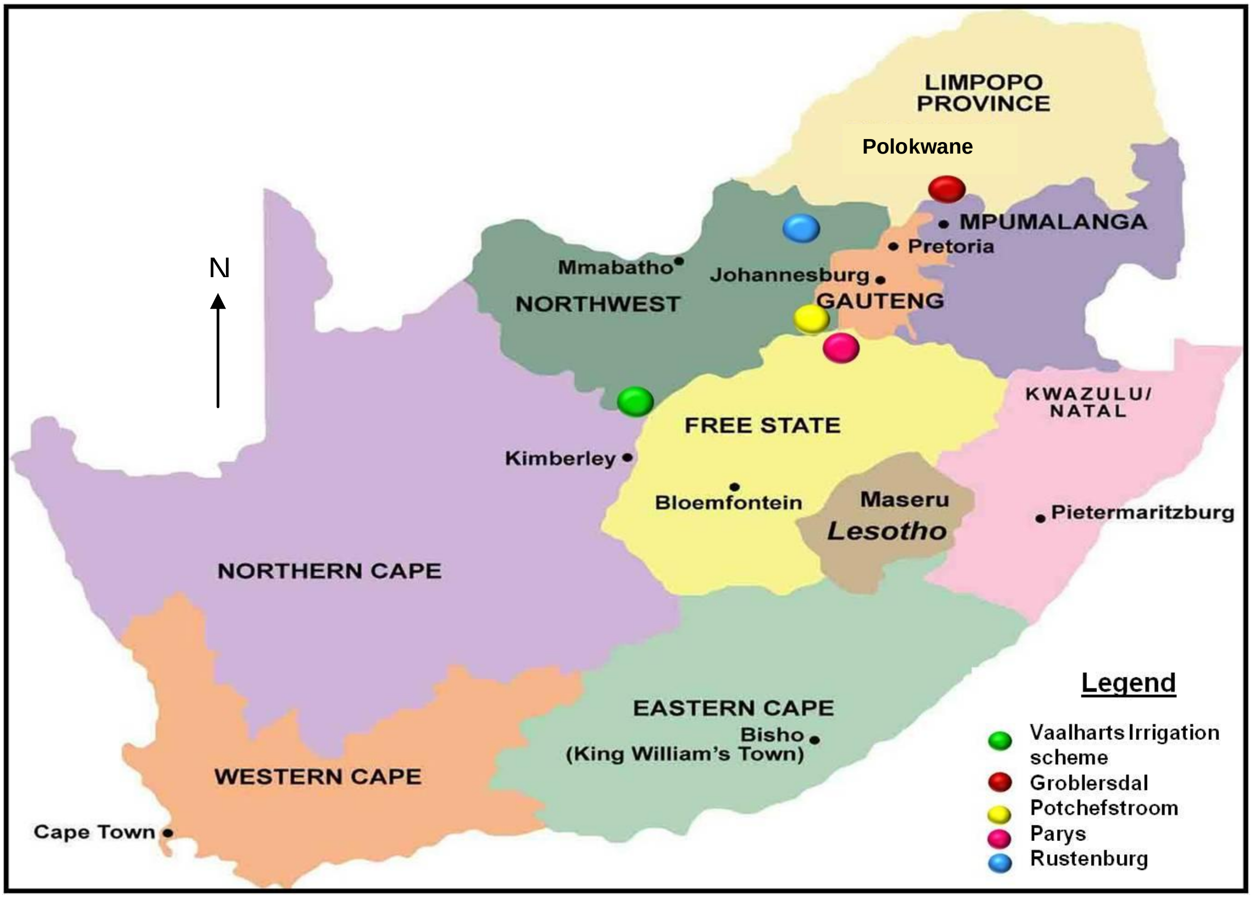

Figure 2.1: Collection sites of *Helicoverpa armigera* populations from different localities in South Africa.

2.3.2. *Experiment 1: Feeding study*

Survival of larvae (F_1-generation) collected from different localities was determined on Bt and non-Bt cotton. Each treatment was replicated 50 times.

Squares (from candle stage to mature boll) (Fig. 2.2) were used during the study since larvae prefer to feed on these plant parts and this also represents the scenario of developing squares that occurs under field conditions. A camel-hair brush was used to place two 1st instar larvae on a square (candle development stage). Two larvae were used to compensate for possible larval mortality due to handling. The squares and larvae were then placed in a 100 ml plastic container covered with steel mesh to provide aeration.

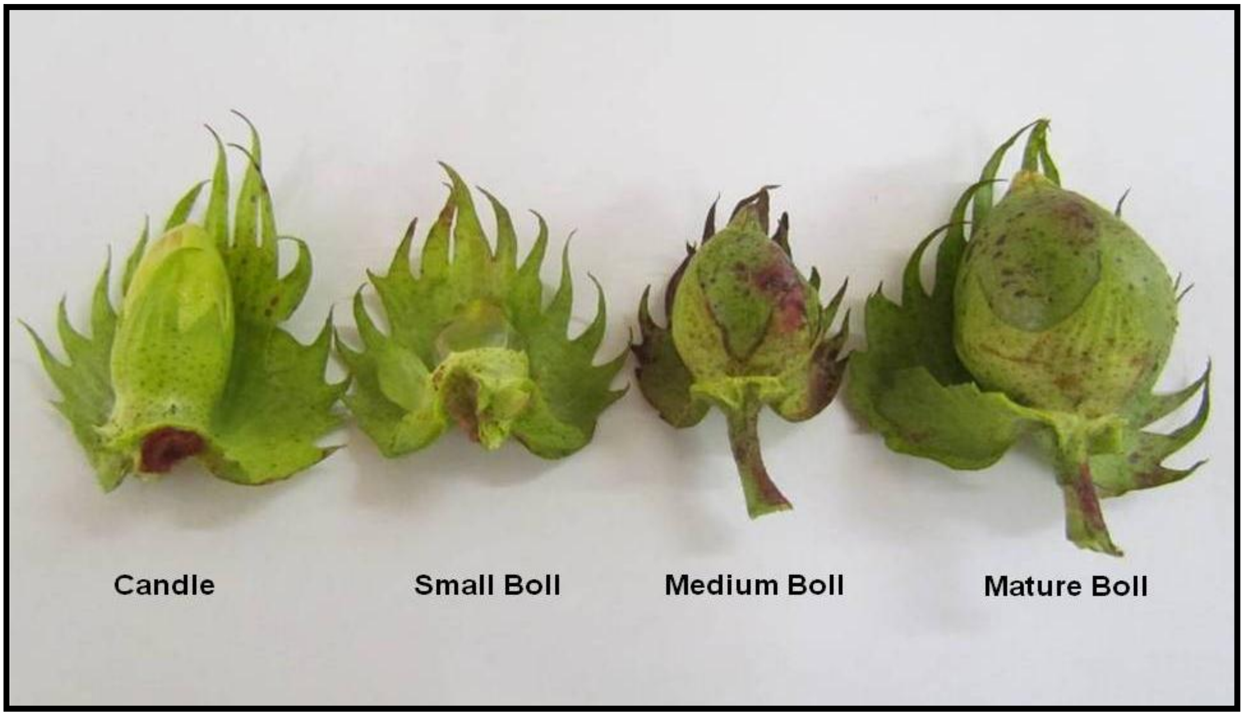

Figure 2.2: Examples of reproductive stages of the cotton plant that was used during this study.

Squares and bolls used during this study (Fig. 2.2) were from Bt and non-Bt cotton plants grown in a field at ARC - Grain Crops Institute in Potchefstroom. Bt and non-Bt cotton plants were also planted at weekly intervals in a greenhouse to ensure availability of squares of different developmental stages that may be needed at different times during the study. Nu-opal and Delta-opal cotton varieties were planted for the purpose of the study.

Larvae were allowed to feed for four days on squares in the candle stage, after which containers were cleaned. Larvae were then placed on larger bolls as they developed. If more than one larva was present after the first four days they were separated and placed in separate containers, because they tend to become cannibalistic as they increase in size. Containers were placed at a 14L: 10D photoperiod in an incubator at 26±1 ºC.

The numbers of surviving larvae were determined every 4th day when containers were cleaned and fresh food was provided. The number of surviving larvae at each date was expressed as a percentage of the initial number of larvae. Larvae started to pupate on day 16. Repeated measures analysis of variance was therefore done using data collected on day 16 to indicate the level of larval survival before pupation commenced. Previous experience showed that large variation occurred in data if the mass of pre-pupae and pupae were used.

2.4. Data analysis

Data were analysed using STATISTICA version 10 (StatSoft, Inc., 2011). T-tests were s used to determine if there were significant differences between survival of larvae from specific populations (sites) sites on non-Bt and Bt cotton. T-tests were also used to determine if there was a significant difference between the pupal mass of larvae reared on Bt and non-Bt cotton, for each locality. Repeated measures analysis of variance (ANOVA) was used to compare the percentage survival of *H. armigera* larvae that fed on Bt and non-Bt cotton. Lethal time (LT50), indicating the time (number of days) until 50 % mortality was reached was calculated by using logistic regressions of larval survival over time. The 95 % Fiducial Limits was also calculated to determine overlapping between the mortality of larvae between Bt and non-Bt cotton.

2.5. Results

2.5.1. Experiment 1: Feeding study

Percentage survival on non-Bt cotton ranged between 20 and 44 % for the different populations. Larvae of the Groblersdal, Parys, Potchefstroom and Vaalharts populations developed to the pupal stage on Bt cotton. When fed on Bt cotton, between 20 and 30 % larvae from the Vaalharts and Groblersdal populations survived until pupation (Fig. 2.3). Less than 10 % larvae from the Parys population survived and only 2 % larval survival was observed for the Potchefstroom population on Bt cotton (Fig. 2.4). No larvae from the Rustenburg population survived until pupation (Fig. 2.3). There was a rapid decline in survival of both Vaalharts and Rustenburg populations on Bt cotton over the first four days compared to non-Bt cotton with the Rustenburg population reaching 100 % mortality on day 20 (Fig. 2.3). Larval survival decreased rapidly over the first eight days for the Parys and Potchefstroom populations when fed on Bt cotton and larvae reached 10 % and 2 % survival respectively after 16 days of feeding on Bt cotton. Larval survival on non-Bt cotton was 26 and 20 % for the Parys and Potchefstroom populations respectively at the end of the experiment (Fig. 2.4).

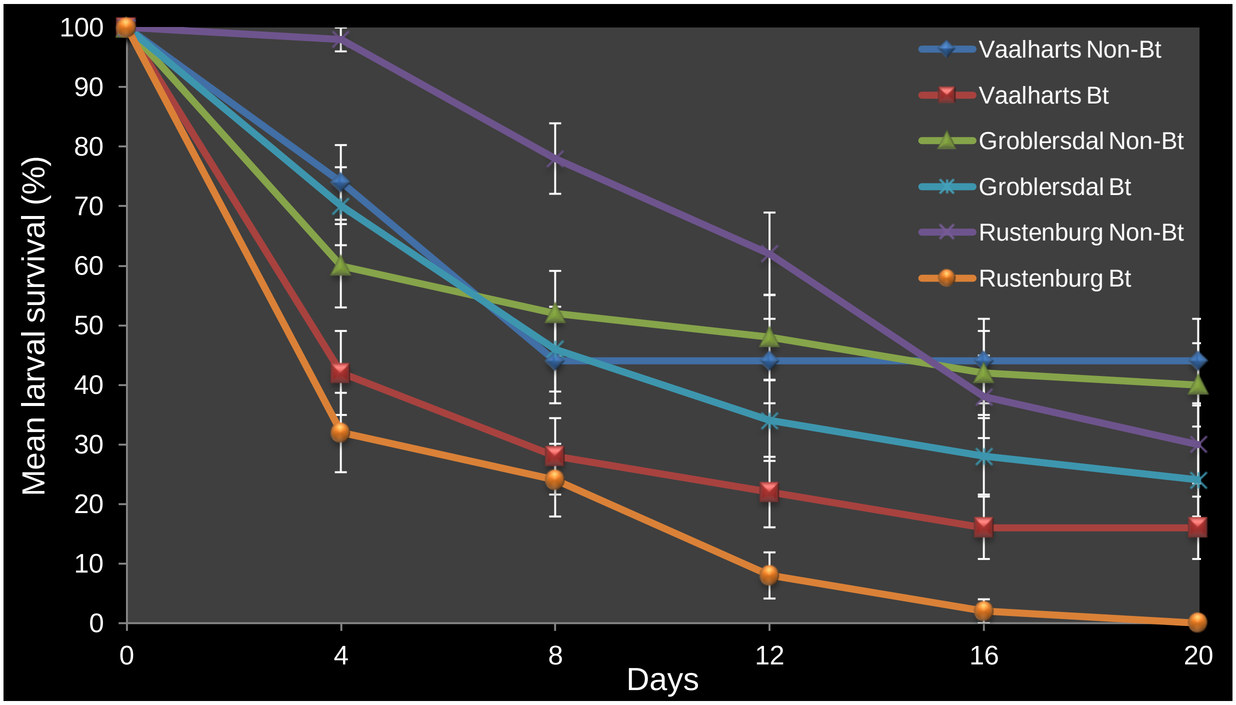

Figure 2.3: Mean percentage survival (±S.E.) of *Helicoverpa armigera* larvae feeding on Bt and non-Bt cotton from 1st instar onwards under laboratory conditions.

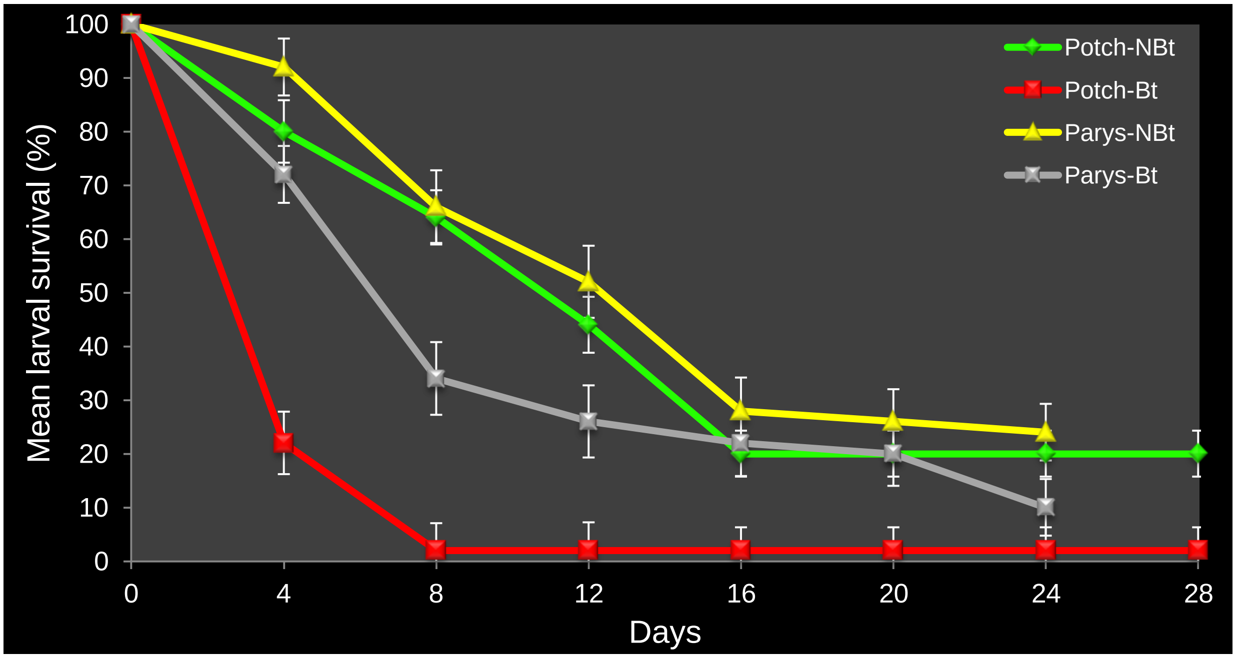

Figure 2.4: Mean percentage survival (±S.E.) of *Helicoverpa armigera* larvae populations that had no history on cotton. Larvae fed on Bt and non-Bt cotton from 1st instar onwards under laboratory conditions

Percentage larval survival was significantly higher on non-Bt cotton compared to Bt cotton for the Vaalharts, Rustenburg and Potchefstroom populations ($P < 0.05$) on day 16 (Table 2.2). There was no significant difference between the percentage larval survival of larvae that fed on Bt and non-Bt cotton for the Groblersdal ($P = 0.14$) and Parys ($P = 0.480$) populations at day 16 when pupae started to form (Table 2.2). Similar data were obtained at the end of the experiment for the different populations when larvae pupated. There was no significant difference in percentage pupae between Bt and non-Bt cotton for Groblersdal ($P = 0.088$) and Parys ($P = 0.063$) (Table 2.2).

LT50 values and the fiducial limits are provided in Table 2.2. Results indicated that there was a significant difference in the rate of mortality between the Bt- and non-Bt cotton (Table 2.2). There was no overlapping between the 95 % Fiducial Limits of any of the Bt and non-Bt cotton populations.

Table 2.2: Percentage survival after 16 days and percentage survival and LT50 values on the last day of the experiment of larvae of different populations of *Helicoverpa armigera* on Bt and non-Bt cotton under laboratory conditions

Population	**Treatment**	**Mean survival (%) (± SE)**	**t-value**	**P-value**	**F-ratio**	**LT50 (Days)**	**95% Fiducial Limits**	**Mean survival (%) (± SE)**	**t-value**	**P-value**	**F-ratio**
Vaalharts	Non-Bt	44 ± 7.09	3.176	0.00199	1.83	17.1	14.2 – 20.7	44 ± 7.09	3.467	0.00078	2.047
	Bt	16 ± 5.24				2.7	0.6 – 4.6	14 ± 4.95			
Groblersdal	Non-Bt	42 ± 7.05	1.469	0.14510	1.21	12.9	10.7 – 15.6	40 ± 6.99	1.723	0.0879	1.316
	Bt	28 ± 6.41				7.4	5.1 – 9.4	24 ± 6.1			
Rustenburg	Non-Bt	38 ± 6.93	4.988	0.000003	12.02	20.3	18.1 – 22.5	30 ± 6.55	4.583	0.00001	0.00
	Bt	2 ± 2				3.1	1.8 – 4.1	0 ± 0			
Potchefstroom	Non-Bt	20 ± 5.71	2.973	0.00371	8.16	12.9	11.2 – 14.7	20 ± 5.71	2.973	0.0037	8.163
	Bt	2 ± 2				1.7	0.5 – 2.8	2 ± 2			
Parys	Non-Bt	26 ± 6.27	0.708	0.48093	1.20	13.8	12.4 – 15.2	24 ± 6.10	1.878	0.0634	2.027
	Bt	20 ± 5.71				6.5	4.9 – 7.8	10 ± 4.29			

2.5.2 Pupal mass and development

A general delay in development time of larvae to the pupal stage was observed for larvae that fed on Bt cotton. Larvae that fed on non-Bt cotton, started to pupate from 10 to 18 days after egg hatch with the majority of pupae forming between 18 and 24 days (Fig. 2.5). Larvae of different populations that fed on Bt cotton, started to pupate between 18 to 30 days after egg hatch with the majority of pupae forming between 22 and 32 days. This delay occurred in all populations except the one from Rustenburg (Fig. 2.5). Pupal mass of the different F_1-population was not affected by exposure of the larvae to Bt cotton (Table 2.3).

Table 2.3: Mean pupal mass of F_1-generation *Helicoverpa armigera* originating from field collected (F_0) larvae that were reared on Bt and non-Bt cotton.

Population	Treatment	Mean mass (mg) (±SE)	df	t-value	P-value	F-ratio
Groblersdal	Non-Bt	296.5 ± 0.02	29	1.22	0.234	2.34
	Bt	255.92 ± 0.02				
Vaalharts	Non-Bt	251.42 ± 0.02	27	0.61	0.547	2.94
	Bt	226.14 ± 0.02				
Rustenburg	Non-Bt	210.68 ± 0.01	14	2.77	0.015	0.00
	Bt	0 ± 0				
Potchefstroom	Non-Bt	256.58 ± 0.04	9	0.02	0.985	0.00
	Bt	254.3 ± 0				
Parys	Non-Bt	398.42 ± 0.04	13	0.299	0.199	4.79
	Bt	298.1 ± 0.03				

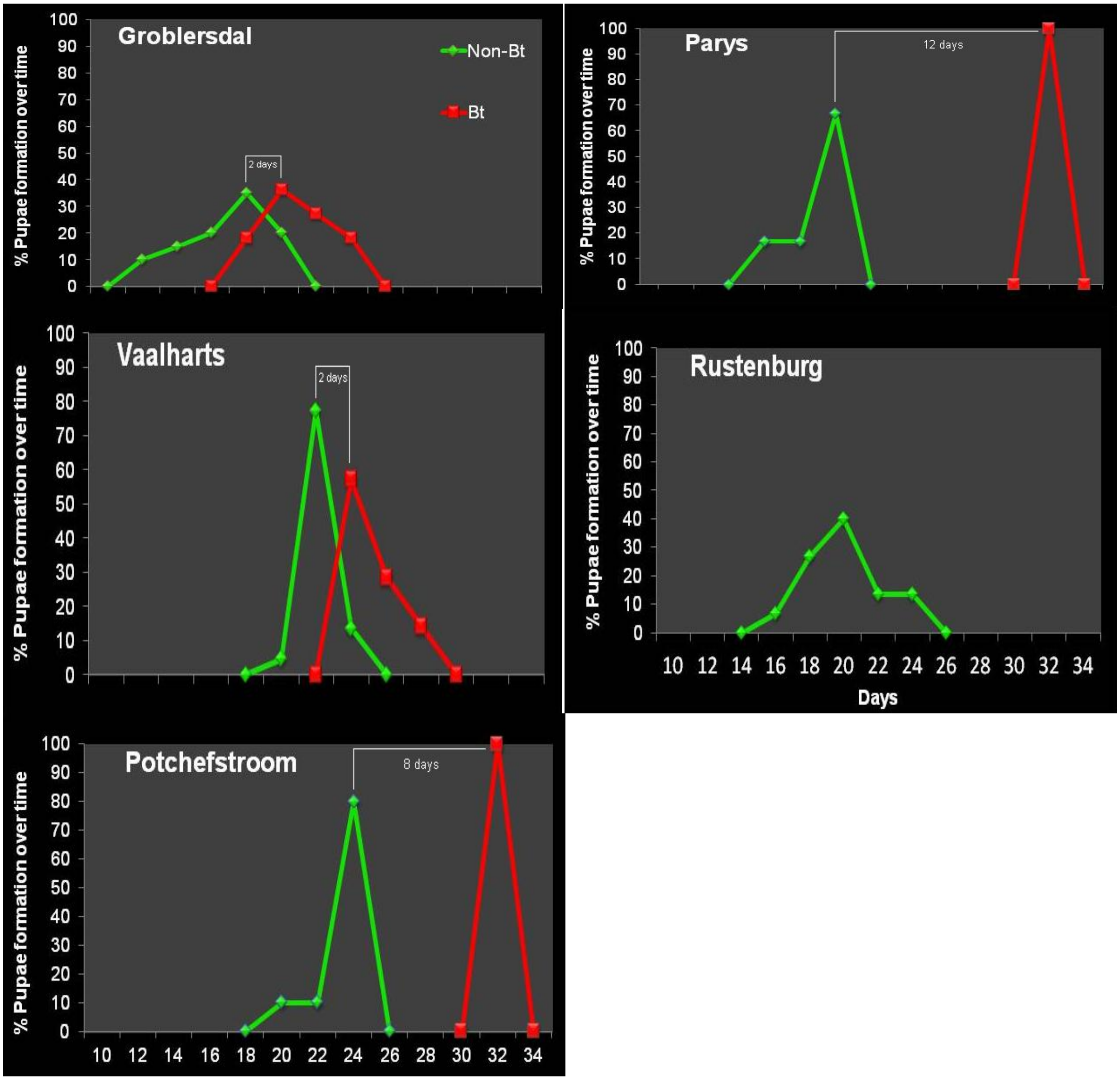

Figure 2.5: Percentage of *Helicoverpa armigera* pupae (F_1) developing over time from larvae feeding on Bt and non-Bt cotton.

2.6. Discussion

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). According to Tabashnik *et al*. (2009) laboratory selected resistance occurs when exposure to a toxin in the laboratory causes a heritable decrease in susceptibility. The term tolerance is often used when comparative survival of different stem borer species on a Bt protein is evaluated as well as when survival rates of different strains of a single species is compared. The general use of 'tolerance' in this regard describes a significant level of survival on a Bt crop whereas 'resistance'' is used to describe the phenomenon of a genetically determined resistance.

Environmental factors had no effect on the results obtained in the experiment since all experiments were conducted under controlled conditions. Although expression levels were not determined we expect that the expression of Bt toxins were high during the stage of boll collection.

Results indicated that some *H. armigera* populations were resistant while others were tolerant to Bollgard® cotton. Percentage survival, pupal mass and LT50 values indicated that the Groblersdal, Vaalharts and Parys populations were resistant while the Rustenburg and Potchefstroom populations were tolerant to Bt cotton.

Although larvae from Rustenburg did not complete the larval stage on Bt cotton, they survived for prolonged periods (20 days), indicating tolerance to the Cry1Ac toxin. The low percentage survival of larvae feeding on non-Bt cotton is ascribed to infections of a nuclear polyhedrosis virus in the field-collected larvae. This virus is well known as biological control agent in the environment. It is common in polyphagous lepidopteran species such as *H. armigera* (Chen *et al*., 2001). The virus can be transmitted by the adults to their offspring (Olofsson, 1988; Kukan, 1999), and could explain the high mortality of larvae feeding on non-Bt cotton and could also be a reason for high mortality rate in Bt cotton. These viral infections could, however, not be prevented since F_0 larvae derived from field collected individuals should be used for resistance studies.

There are different factors that affect the development of resistance in different insect populations. Metapopulation simulation models indicate that resistance evolution is affected by the distribution and abundance of Bt fields and refuges (Carrière *et al*., 2010). It is also affected by the management of refugia that is planted each year. There is a potential that resistance of different species can spread if the total amount of Bt cotton that is planted in a region increases, therefore increasing the selection pressure for the development of resistance (Peck *et al*., 1999; Caprio, 2001; Storer *et al*., 2003; Sisterson *et al*., 2004; 2005). The simplest explanation for evolution of resistance of *H. armigera* is the intensive planting of Bt cotton expressing Cry1Ac toxins (Zhang *et al*., 2011). However, in regions where there is limited planting of Bt cotton the selection pressure or selection for resistance will be lower and therefore the evolution of resistance will be delayed (Zhang *et al*., 2011). Different crops that are cultivated in a particular region can therefore also act as a refuge for pests of Bt crops and through this, selection pressure on target pests may be reduced. The possibility of using other crops as refugia for *H. armigera* when planting Bt cotton seems viable, mainly because this pest is highly polyphagous. A study of the population dynamics of *H. armigera* in China showed that other crops can be used as refugia for *H. armigera* in Bt cotton growing areas (Shengjiang *et al.*, 2001). Different crops such as maize, soybean, sorghum and groundnut can act as refugia for bollworm populations that attack Bt cotton (Wu *et al*., 2004). However, the inter-planting of maize and Bt cotton may cause negative effects on the release of Bt maize in the future. This is because, in some cases, Bt cotton and Bt maize expresses the same toxin gene, which will result in exposure of the majority of the pest population in an area to similar *cry* toxins, even if different generations of the pest alternate between crops. This continued exposure may accelerate the development of resistance in *H. armigera* populations (Shengjiang *et al.*, 2001).

A significant decrease in area planted to cotton was observed in South Africa over the past decade. When Bt cotton was commercially released the total area planted to cotton during the 1999/00 production year was 100 000 ha (Cotton SA, 2011b) with approximately 20 % (20 000 ha) of the total amount being Bt cotton. During the 2000/2001 growing season the area planted to Bt cotton increased to about 80 % (40 000) of 50 768 ha. The total amount of transgenic cotton planted during 2001/02 (increased to 95 %) was 54 000 ha of 56692 ha with the release of herbicide-tolerant cotton (Gouse *et al*., 2005; Gouse, 2005). The production of cotton during the 2010/11 season was approximately 17190 ha (Cotton SA, 2011a). Zhang *et al*. (2011) indicated that intensive planting of Bt cotton can select for Cry1Ac resistance. There may be other factors that contributed to the evolution of resistance because the planting of cotton in South Africa however, cannot be described as intensive and the isolated areas in which cotton is still produced are largely separated by vast areas in which maize is the main crop (Fig. 2.1). These factors should be investigated further.

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Although no significant difference was observed in larval survival on Bt and non-Bt cotton for the Parys population, 10 % of the larvae did complete their development on Bt cotton, indicating the presence of resistant individuals in the population. Although no cotton have been cultivated in this area, this study showed that Cry1Ac resistant individuals occurred in this population which was collected on sorghum. In the Potchefstroom population of which most larvae died after eight days some individuals survived on Bt cotton. This area has also no history of cotton cultivation. The high level of survival of the Groblersdal population on Bt cotton indicated high levels of resistance to Bt cotton in this area where Bt cotton have been cultivated since 1999.

This is the second report of resistance of a target pest to Bt crops in South Africa. The first report was that of *B. fusca* that developed resistance to Bt maize expressing Cry1Ab protein (Van Rensburg, 2007). The decline in production of cotton in South Africa could have reduced the selection pressure on *H. armigera* and therefore delayed the development of resistance. The development of *H. armigera* resistance to Bt cotton in South Africa was surprisingly slow which may be partly ascribed to the small areas under cotton production in South Africa.

The delayed development of pupae from larvae that fed on Bt cotton may have contributed to the observed resistance development. There was an eight and 12 days difference in larval development time to pupation between populations reared on non-Bt and Bt for Potchefstroom and Parys respectively. The difference between the peak pupal development period on Bt and non-Bt cotton at the other localities was two days. Refugia are planted to reduce the numbers of resistant alleles in the population. The principle is that the few individuals that survive on Bt crops will mate with the many surviving individuals from the refuge (Tabashnik & Croft, 1982; Gould, 1998; Renner, 1999; Shelton *et al*., 2000; Tang *et al*., 2001). This strategy delays the development of resistance by providing susceptible insects that can mate with the resistant insects and reduces the amount of offspring that may be resistant (Tabashnik, 1994; Liu *et al*., 1999; Halcomb *et al*., 2000; Tabashnik *et al*., 2008). For this strategy to function optimally peak moth flights of Bt resistant and susceptible populations should occur simultaneously. The delay in peak pupation period may therefore have negative implications for the refuge strategy. If environmental conditions are favourable pupation of the larvae on non-Bt cotton will occur a few days earlier than those on Bt cotton which can result in limited overlap between resistant moths emerging from Bt cotton and susceptible moths from the refugia. However, since bollworm generations often overlap, larvae of different instars may occur simultaneously in a single cropping system. Pupation and moth flights therefore occur throughout the production season in a specific field but on a limited scale. If this synchronisation between appearance of susceptible and resistant moths is poor it may result in increased selection pressure since few susceptible moths will be available to mate with resistant moths.

Pupal mass can be used as an indication of moth fecundity (Gilbert, 1984). Since no differences in pupal mass were observed between any of the populations on Bt and non-Bt cotton, it is also highly likely that susceptible and resistant moths produce similar amounts of eggs. This can be a contributing factor to resistance development since the fitness of resistant individuals is the same as those of susceptible individuals.

2.7. Conclusion

The Groblersdal, Vaalharts, Potchefstroom and Parys populations were identified to have developed resistance to Bollgard® cotton in South Africa. This study indicated that the period to pupation was delayed for larvae feeding on Bt cotton. The introduction of Bollgard II® cotton which produces both Cry1Ac and Cry2Ab2 proteins will contribute to improved management of the resistant *H. armigera* populations.

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CHAPTER 3

EVALUATION OF RESISTANCE OF RED BOLLWORM *DIPAROPSIS CASTANEA* (HAMPSON) (LEPIDOPTERA: NOCTUIDAE) TO BT COTTON

3.1. Abstract

Diparopsis castanea (Hampson) (Lepidoptera: Noctuidae) and *Earias biplaga* (Walker) (Lepidoptera: Noctuidae) are the major pests of cotton in the Makhathini Flats area in KwaZulu-Natal, South Africa. Cultivation of genetically modified Bt cotton expressing the Cry1Ac protein commenced in this area during the 1998/99 growing season. The aim of this study was to evaluate whether *D. castanea* in the Makhathini Flats developed resistance to Bt cotton. A laboratory study was conducted during 2011 to evaluate red bollworm resistance to Bt cotton. Non-Bt cotton bolls infested with larvae were collected on the Makhathini Flats and maintained until moths emerged. Neonate larvae emerging from eggs laid by these moths were used in this experiment. Fifty cotton squares each of Bt and non-Bt cotton were inoculated with two neonate larvae. The number of surviving larvae was determined every four days when fresh food was provided in the form of new bolls. Survival curves and Lethal Time (LT50) values were calculated. Results indicated 100 and 52 % larval mortality on Bt and non-Bt cotton after 12 days respectively. This may indicate tolerance of larvae to the Cry1Ac protein expressed by Bt cotton. The LT50 values on Bt cotton indicated that 50 % mortality was achieved at 1.07 and 15.3 days on Bt and non-Bt cotton respectively. None of the larvae did however survive on Bt cotton. This result indicated that the Bt event (Bollgard®) used for control of the bollworm complex was still effective against *D. castanea* but that tolerance to the *cry* protein seems to be developing.

3.2. Introduction

The most important lepidopteran pests of cotton in South Africa are the noctuid species, *Helicoverpa armigera* (Hübner), *Diparopsis castanea* (Hampson) and *Earias biplaga* (Walker), known as the bollworm complex. These species cause serious damage to cotton when they attack the bolls and flowers (Pearson & Darling, 1958). A study conducted by Green *et al*. (2003) indicated that the red bollworm (*D. castanea*) and the spiny bollworm (*E. biplaga*) are the major pests of cotton in the Makhathini Flats (KwaZulu-Natal, South Africa).

Before the introduction of Bt cotton expressing Cry1Ac in South Africa, insecticide application was the only control measure used to keep these pests below the economic threshold level. Farmers sprayed their fields up to 12 times during each growing season depending on the severity of pest attack. Such intensive use of insecticides poses a great threat to the environment and health of farmers. Most important advantages associated with cultivation of Bt cotton is higher yields, lower levels of labour and pesticide use, and higher producer prices for cotton (Fernandez-Cornejo & Klotz-Ingram, 1998; Gianessi & Carpenter, 1999; Fernandez-Cornejo *et al*., 1999; Gouse *et al*., 2003; Bennett *et al*., 2004; Shankar & Thirtle, 2005). Although the introduction of Bt cotton reduced the use of insecticides the level of Bt toxin in the plant also declines towards the end of the season and it might still be necessary to apply insecticides for bollworm control at later stages of crop growth (Mayer, 2003; Morse *et al*., 2006). Prior to the introduction of Bt cotton approximately a fifth of all global insecticides was applied on cotton each year (Mayer, 2003) anything which reduces this toxic load in the environment would appear to be beneficial (Huang *et al*., 2003).

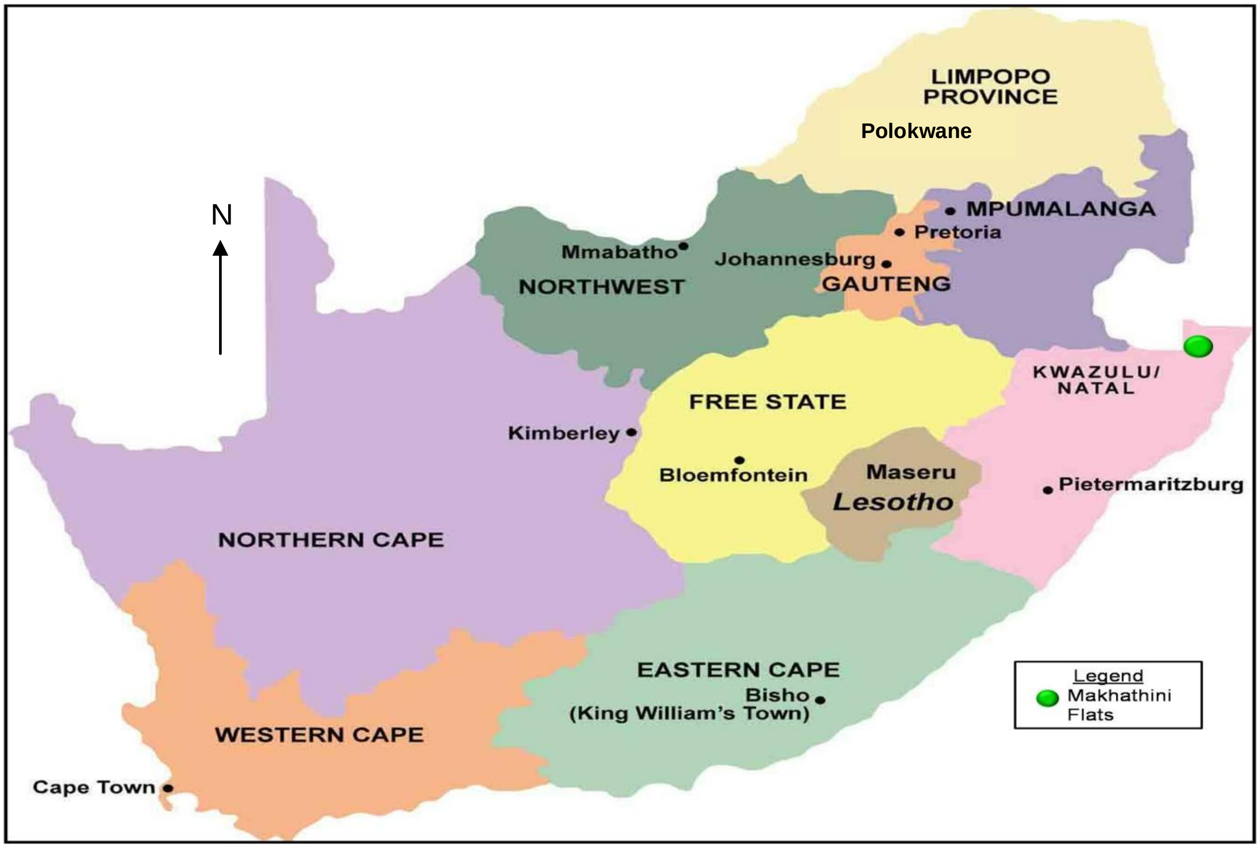

Figure 3.1: Collection site of *Diparopsis castanea* larvae in Makhathini Flats in South Africa.

The Makhathini Flats comprise an area of 1800 km^2 in the most Northern region of Kwazulu-Natal (Fig. 3.1). During the 1998/99 season, Bt cotton was commercially released to smallholders in this area and by 2001/02 more than 90 % of the approximately 3500 farmers in the area had adopted Bt cotton varieties (Bennett, 2002). However, the area under cotton production and the number of cotton producers largely depended on the availability of production credit and the price of cotton. These factors contributed to the large area planted to cotton which ranged between 2 500 and 10 000 ha. Farmers in this area planted an average of between one and three hectares of dry land cotton annually (Bennett, 2002). According to Cotton SA (2011) only 490 ha cotton was planted in Kwazulu-Natal during the 2011 growing season because the approximate 3500 small-scale farmers in the Makhathini Flats stopped planting cotton.

The introduction of Bollgard® (Bt cotton) created new pest management opportunities for small-scale farmers in rural communities. The introduction of Bt cotton in Makhathini Flats was successful in the sense that it provided many advantages for the small-scale farmers. For the technology to be preserved, development of resistance to the Bt-toxin expressed in the transgenic cotton plant has to be delayed (Green *et al*., 2003).

According to Mallett & Porter (1992), the susceptible genes in the bollworm population can be conserved by planting refugia or non-transgenic cotton plants in close proximity to non-transgenic cotton, preferably on the same field. If pests develop resistance to toxins expressed by Bt crops, this technology will no longer be effective and target insect species will again become more prevalent (Forrester, 1994; Riebe, 1999; Tabashnik *et al*., 2000). There are a number of factors that contribute to the rate of resistance development in insect populations.

- pest population dynamics (Wu *et al*., 1999)
- frequency of resistance alleles in the pest population (Tabashnik, 1994)
- genetic mode and stability of resistance (Peck *et al*., 1999)
- fitness of resistant individuals (Alstad and Andow, 1995)
- distribution of the pest on different host plants (McGaughey & Whalon, 1992)
- gene flow among different geographical populations (Wu & Guo, 1997).

Farmers that plant Bt cotton are obligated to sign a license agreement, stating that a non-Bt cotton refuge area will be planted for every 100 ha of Bt cotton (Monsanto, 2010). Although the planting of refugia is compulsory to delay resistance development (Monsanto, 2007), the level of compliance by cotton farmers in South Africa is not known. The current refuge requirements are either a 20 % refuge planted to non-Bt cotton which may be sprayed with lepidopteran-active insecticides, or a 5 % refuge area that should not be sprayed with chemical insecticides (Chilcutt, 2007).

The objective of this study was to determine if *Diparopsis castanea* (Hampson) (Lepidoptera: Noctuidae) is resistant to Bt cotton in the Makhathini Flats.

3.3. Material and methods

3.3.1. Bollworm colony establishment

Approximately 100 *Diparopsis castanea* larvae (F_0-generation) were collected at the Makhathini Flats research station (S27º23`575 E32º10`538) KwaZulu-Natal province during the 2010/11 growing season. Damaged non-Bt cotton bolls with larvae inside were removed from plants and placed singly in containers (52 x 55 mm^2). Larvae were then reared on non-Bt cotton bolls in the laboratory at ambient temperatures ranging between 15 and 25 ºC. Cotton bolls were maintained until larvae pupated. Pupae were maintained in these containers at 26 ± 1 °C and a 14L: 10D photoperiod in an incubator. Once the moths emerged they were transferred to a larger plastic container (40 x 20 x 15 cm) with an aerated lid, allowing the female moths to mate and to lay eggs on pipe cleaners. Plant material was used as stimulus for egg production. Approximately 35 male and female moth pares were present and allowed to mate. Each female laid approximately 250 eggs and from these eggs larvae that emerged were randomly selected and placed on Bt and non-Bt cotton bolls. Neonate larvae (F_1-generation) emerging from these eggs were used in the feeding study.

3.3.2. Experiment 1: Survival study

To determine the survival of larvae (F_1-generation) on Bt- and non-Bt cotton, squares (from candle stage to mature boll) (Fig. 3.2) were used during the study since larvae prefer to feed on these plant parts and this also represents the scenario of developing squares that occurs under field conditions. Larvae prefer to feed on candles when they are small and on bigger bolls as they mature. A camel-hair brush was used to place two first instar larvae on a square in the candle development stage. Two larvae were used at the beginning of the experiment to compensate for larvae that might die due to handling. The squares and larvae were kept in 100 ml plastic vials covered with steel mesh to provide aeration (Fig. 3.3). Each treatment (Bt cotton and non-Bt cotton) was replicated 50 times.

Squares and bolls used (Fig. 3.2) during this study were taken from Bt and non-Bt cotton plants grown in a field at the ARC-Grain Crops Institute in Potchefstroom (26º43'S, 27º06'E). Bt- and non-Bt cotton plants were also planted at weekly intervals in a greenhouse to ensure availability of squares of different developmental stages that may be needed at different times during the study. Nu-opal and Delta-opal cotton varieties were planted for the purpose of the study.

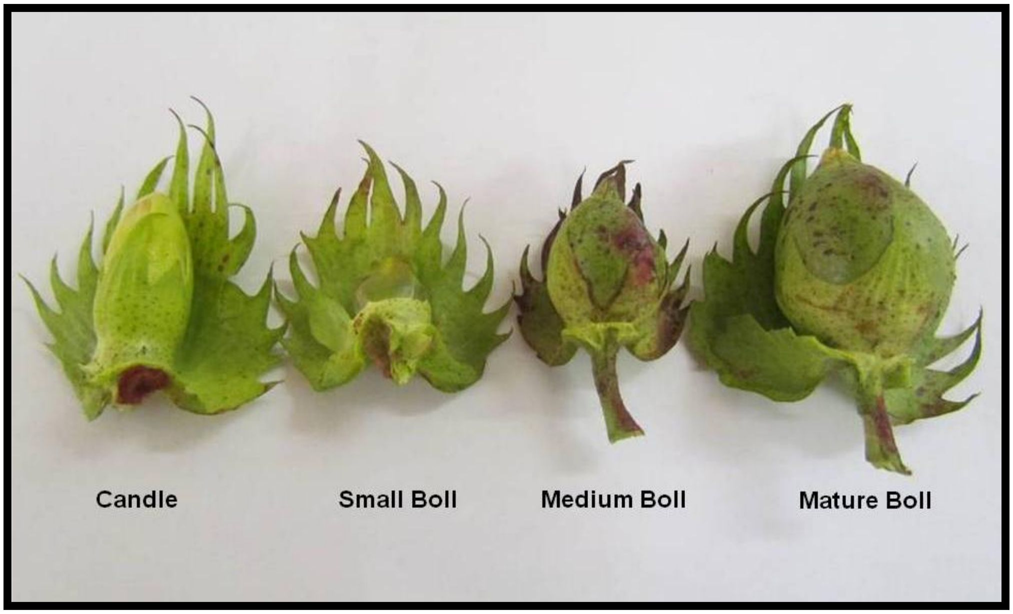

Figure 3.2: Examples of reproductive stages of the cotton plant that were used during this study.

Figure 3.3: Containers used to rear *Diparopsis castanea* larvae in the laboratory.

Larvae were allowed to feed for four days on squares in the candle stage, after which containers were cleaned. Larvae were then placed on larger bolls as they developed. Where both larvae survived the first four days, they were separated and placed singly in containers. Containers were kept in an incubator at 26 ± 1 ºC and a 14L: 10D photoperiod to assure that the experimental conditions were constant throughout the study. The number of surviving larvae was expressed as a percentage of the initial number. The experiment was terminated when 100 % mortality of larvae occurred on Bt cotton.

3.4. Data analysis

Data were analysed using STATISTICA version 10 (StatSoft, Inc., 2011). Survival curves were constructed to compare larval survival on Bt and non-Bt cotton. A t-test was used to determine if there was a significant difference between the percentage survival on the final day of the experiment. Repeated measures analysis of variance (ANOVA) was used to compare the percentage survival of larvae that fed on Bt- and non-Bt cotton over time. Lethal time (LT50)-values, indicating the time (number of days) until 50 % mortality was observed, was calculated by using logistic regressions of larval survival over time.

3.5. Results

3.5.1. Experiment 1: Survival study

Percentage larval survival was significantly higher on the non-Bt cotton than the Bt cotton ($F_{(3,\ 294)} = 20.719$, $P < 0.000001$) for the duration of the experiment (Fig. 3.4). There was a rapid decline in the percentage survival of the larvae that fed on Bt cotton. Only 26 % of the population survived at the end of day four compared to 86 % survival on non-Bt cotton.

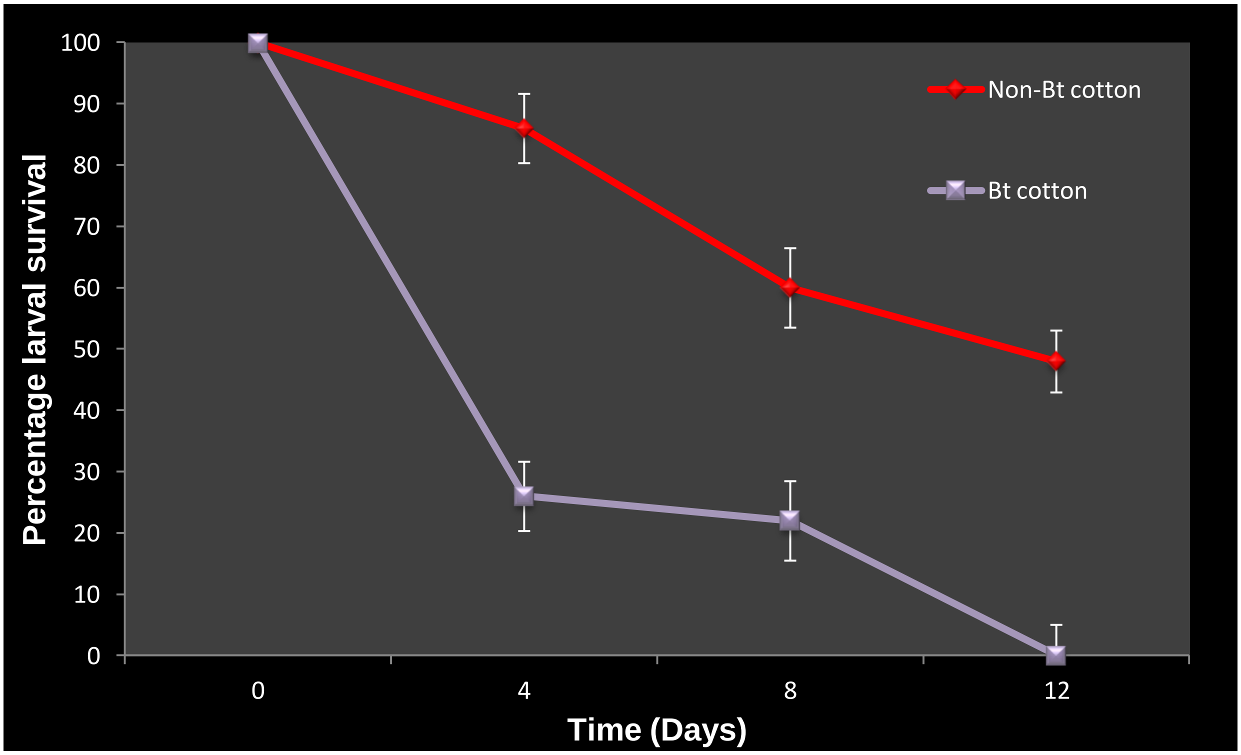

Figure 3.4: Percentage survival of *Diparopsis castanea* larvae reared on Bt- and non-Bt cotton under laboratory conditions (Bars indicate standard errors).

LT50-values and fiducial limits are provided in Table 3.1. LT50-values indicated a significant mortality rate of larvae feeding on Bt- and non-Bt cotton (Table 3.1).

Table 3.1: Lethal time (LT50)-values of *Diparopsis castanea* on Bt cotton expressing Cry1Ac protein and non-Bt cotton under laboratory conditions.

Population	LT50 (Days)	95% Fiducial Limits
Bt cotton	1.07	0.6 – 1.3
Non-Bt cotton	15.3	13.5 – 17.4

3.6. Discussion

The LT50-value of *D. castanea* larvae on Bt cotton was 1.07 days. The fact that it took a further eight days for the population to reach 100 % mortality may be an indication that this population is developing tolerance to Bt cotton. Since the commercial release of Bt cotton in the Makhathini Flats during the 1998/99 season until 2010/11 season (13 years), no resistance of *D. castanea* have been recorded. The extended survival of some larvae on Bt cotton pose no immediate threat for resistance development. This study confirms the findings of Morse *et al*. (2006) who reported no survival of *D. castanea* on Bt cotton in the Makhathini Flats between 1998 and 2002. Bt crops that only expresses one insecticidal gene is potentially vulnerable to development of resistance and therefore a reduction in effectiveness (Forrester, 1994; Riebe, 1999; Tabashnik *et al*., 2000).

Occurrence of *D. castanea* at the Makhathini Flats was only observed on non-Bt cotton (personal observation, 2011). It was therefore assumed that Bollgard® cotton cultivars used against the bollworm complex was effective against *D. castanea*. These observations were confirmed by this laboratory study and it can be concluded that *D. castanea* in this area has not developed resistance to the Cry1Ac toxin.

A study conducted by Green *et al*. (2003) indicated that there are indigenous plants and weeds in the Makhathini Flats that may serve as natural refugia for the pests of transgenic crops that are planted in this area. Small-scale farmers in this area, however, may neglect planting refugia, which according to Andow (2008) that may result in the rapid development of resistance to the transgenic crop by the target pest. Alternative host plants include weeds and natural vegetation (Gregory *et al*., 2002). These alternative hosts in a survey by Green *et al*. (2003), larvae and eggs of *D. castanea* were counted on the natural vegetation and non-Bt cotton plants in the Makhathini area and indicated that alternative host plants were present for all the bollworm species that attack cotton. The two plant species that were identified as hosts of red bollworm in this area were *Abutilon austro-africanum* Hochr. (Malvaceae) and *Cienfuegosia hildebrandtii* Garcke (Malvaceae). Survey data indicated that bollworm numbers on weeds were similar to those on non-transgenic cotton (Green *et al*., 2003). However, *D. castanea* has previously been recorded on a very confined host range (Pearson & Darling, 1958). Its hosts are limited to the genera *Gossypium* (Malvaceae) (*G. hirsutum* L.*, G. herbaceum* L. and *G. barbadense* L.), *Gossypioides* (Malvaceae) (*G. kirkii* Mast.) and *Cienfuegosia* (Malvaceae) (*C. hildebrandtii* Garcke). *Gossypium herbaceum* L. (Malvaceae) subsp. *africanum* is indigenous to the eastern lowveld areas of South Africa (Hutchinson *et al.* 1947). Weeds and wild host plants could therefore serve as an alternative refuge or a natural refuge for transgenic cotton.

3.7. Conclusion

Data indicated that there is currently no resistance to Bollgard® in the *D. castanea* population collected in the Makhathini Flats. However, tolerance of larvae towards Bt cotton was present since larvae survived on Bt cotton for 12 days.

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CHAPTER 4

FARMERS' PERCEPTIONS ABOUT THE DEVELOPMENT OF BOLLWORM RESISTANCE AND FIELD DAMAGE TO BT COTTON IN SOUTH AFRICA

4.1. Abstract

Bt cotton (Bollgard®) expressing Cry1Ac protein have been released in South Africa for the first time in 1997. Bollgard II® cotton was commercially released in South Africa during the 2011 cropping season and expresses Cry1Ac and Cry2Ab2 proteins. *Helicoverpa armigera* developed resistance to Bollgard® cotton in South Africa (chapter 2). The planting of non-Bt cotton refugia to sustain bollworm individuals that are susceptible to Bt cotton, is compulsory. The level of compliance to this component of the insect resistance management (IRM) strategy in South Africa is not known. The objectives of this study were to evaluate farmers' perceptions about the cultivation of Bt cotton and to determine the levels of compliance to refuge requirements and the incidence of damage to cotton in the major producing areas in South Africa. Twenty four farmers in five cotton production areas were interviewed. The questionnaire covered approximately 30 % of the cotton production area of the country. Bt- and non-Bt cotton fields were surveyed during the 2010/11 growing season to record damage caused by *H. armigera* to cotton bolls. Scouting was done in the areas where farmers completed questionnaires. The adoption of Bt cotton was low for the first five years ranging from 15 % in 1997 to 25 % in 2002, but increased to 100 % in 2010/11. Only 15 % of farmers complied with refuge requirements for the first eight years after which a rapid increase to 100 % was observed in 2010/11 season. Farmers indicated that the main advantages associated with the planting of Bt cotton were reduced production costs, reduced insecticide use and higher yields.

4.2. Introduction

A large reduction in the amount of cotton planted in South Africa was observed after the 200 000 ha planted during 1989 (Fok *et al*., 2007; Cotton SA, 2011). During the 2010/11 growing season only approximately 13000 ha cotton was planted in the country (Cotton SA, 2011). Many different genetically modified (GM) crops have been approved for field trials in South Africa, but only GM cotton, soybeans and maize are grown on a commercial basis (Gouse *et al*., 2005; Venter, 2008). There are currently three countries in Africa in which GM crops have been approved, Burkina Faso, Egypt (James, 2008; 2010) and South Africa (Ismael *et al*., 2001; James, 2008; 2010) The first insect resistant cotton has been planted in South Africa in 1997 (Cotton SA, 2006) and contained Event MON531 expressing Cry1Ac protein. Herbicide tolerant cotton has been available in this country since 2001 (Andow *et al*., 2006; Brookes & Barfoot, 2006; Cotton SA, 2006). Prior to this study, no research was done on farmers' perceptions about the development of resistance of bollworm to Bt cotton in South Africa. It is, however, important that monitoring of Bt crops is done to evaluate changes in the survival of target species and to evaluate changes in management practices associated with cultivation of Bt crops. The concentration of the Cry1Ac protein that is expressed in Bt cotton plants declines as plants mature allowing 5 to 20 % survival of susceptible *Helicoverpa armigera* larvae towards the end of the growing season (Wu & Guo, 2005; Olsen *et al*., 2005). It is therefore important to assess the levels of bollworm damage to cotton under field conditions in order to detect possible resistance since this may influence the sustainable use of the technology (Gouse *et al*., 2008).

Prior to this study (chapter 2) no *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) resistance to Bt cotton expressing Cry1Ac protein was reported in South Africa. .

Different resistance management strategies have been proposed in an attempt to address concerns over the potential for resistance development and to preserve the usefulness of Bt crops (McGaughey & Whalon, 1992; Tabashnik, 1994; Alstad & Andow, 1995). The strategy that is most widely used is the high dose/refuge strategy. The strategy is based on a combination of transgenic plants producing high doses of toxin, with nearby non-Bt plants (refugia) that does not produce any toxins (Gould, 1998; Renner, 1999; Gould, 2000; Shelton *et al*., 2000; Tang *et al*., 2001; Chilcutt & Johnson, 2004). The purpose of the high dose of toxin is to kill as many individuals of the target pest as possible, whereas the purpose of the refuge is to sustain Bt-susceptible individuals that survive on that particular crop (Renner, 1999; Gould, 2000). This strategy delays development of resistance by providing susceptible insects that can mate with the resistant insects and reduces the amount of offspring that could be resistant (Tabashnik, 1994; Liu *et al*., 1999; Halcomb *et al.*, 2000; Tabashnik *et al*., 2008). The current refuge requirements are either a 20 % refuge planted to non-Bt cotton which may be sprayed with lepidopteran-active insecticides, or a 5 % refuge area that should not be sprayed with insecticides (Chilcutt, 2007).

Although the planting of refugia to delay resistance development is compulsory (Gouse, *et al*., 2008), the level of compliance by farmers in South African is not known. Field surveys are generally accepted as an integral tool to measure the success of Bt crops. However, monitoring should also be conducted to determine refuge compliance levels and to follow changes in the susceptibility of pest populations (Wu *et al*., 2002). Another useful tool is the use of surveys to assess farmers' perceptions about transgenic crops (Grieshop *et al*., 1988). Through surveys the success or failure of a technology that has been introduced and used by farmers can generally be determined (Pilcher & Rice, 1998). Information from farmers can be useful and information can be beneficial to extension specialists, crop consultants, and for developing educational information for farmers (Pilcher & Rice, 1998). Questionnaire surveys are useful to determine levels of compliance to regulatory requirements such as planting of refugia, as was indicated by Kruger *et al*. (2009; 2011).

The objectives of this study were to evaluate farmers' perceptions about the production of Bt cotton and to determine the levels of bollworm damage to Bt cotton in South Africa.

4.3. Materials and methods

4.3.1. Study areas

The study was conducted in five different geographical areas in the cotton production regions of South Africa. Twenty four farmers in these five areas were interviewed. Five farmers were interviewed in each of the Groblersdal (Limpopo province), Jacobsdal (Free State province) and Douglas areas (Northern-Cape province). Eight farmers were interviewed at the Vaalharts area (Northern-Cape province) and only one farmer was interviewed at Musina (Limpopo province). The number of farmers interviewed in each area depended on the participation and availability of farmers.

4.3.2. Farmer survey

The survey was conducted between May and August 2011 and was designed as a self-administered questionnaire. Farmers were randomly selected from a list of producers in each area. Each farmer in the region had an equal chance of being selected to participate in the study.

The questionnaire addressed basic questions on cotton farming and the importance of genetically modified (GM) cotton as a crop in their farming system. The questionnaire was divided into four major categories that addressed the history of cotton production in the area, the compliance with the refuge strategy, pest management strategies and farmers' perceptions of Bt cotton.

4.3.3. Determining the incidence of bollworm damage under field conditions

A preliminary study was done on Bollgard® cotton in the Vaalharts irrigation scheme during the 2009/10 growing season to determine the incidence of damage caused by *H. armigera* to cotton squares. Three cotton fields were inspected. Twenty four plants were randomly selected inside the Bt- and non-Bt section (refuge) of each field and the total number of cotton bolls determined on each plant. Each boll was carefully inspected and the number of bolls with bollworm larvae or damage symptoms (Fig. 4.1) was determined. Percentage bollworm damage per cotton plant was calculated and a mean value calculated for each field. Damage symptoms of *H. armigera* can be determined from *Diparopsis castanea* and *Earias biplaga*. Both *D. castanea* and *E. biplaga* usually remains in a single boll for the whole duration of the larval stage where they totally consume the boll. *Helicoverpa armigera* consume more than one boll and the whole is usually large in comparison to the other two species, because they penetrate the boll when the larvae are small and grow inside the boll.

During the 2010/11 growing season 28 cotton fields (each with its refuge planting) were inspected for the incidence of damage caused by *H. armigera* to cotton squares. Five fields were inspected at Groblersdal, Jacobsdal, Musina and Douglas while eight fields were inspected at in the Vaalharts area. All the fields that were inspected were Bollgard II® cotton. These field surveys were done in the five areas where the questionnaires were done.

Figure 4.1: Symptoms of *Helicoverpa armigera* damage to a cotton boll.

4.3.4. Data analysis

Data from the questionnaires were summarized and expressed as percentages that were calculated based on the total number of farmers that responded to a particular question. Due to the low number of farmers involved in the questionnaires, data collected at 4 of the localities were combined for analysis. The data for the Groblersdal locality is however presented separately because, during a previous study (chapter 2), this locality was identified as an area in which resistance occurred and where refuge compliance levels were low. Data collected from the one farmer at Musina were excluded from analysis in some cases. Adoption rate, refuge compliance and signing of licensing agreements by farmers were expressed as a percentage of the numbers of farmers. The mean number of bollworm damaged squares per plant was calculated and expressed as a percentage of the mean number per Bt- and non-Bt fields at each locality.

4.4. Results and Discussion

A summary of the survey data is provided below in four major categories. The total area planted to Bt cotton by the 24 farmers interviewed was approximately 3952 ha with an approximate 197 ha planted to non-Bt cotton refugia. This survey during the 2010/11 season therefore covered approximately 30 % of the total cotton production area of South Africa (Table 4.1).

4.4.1. History of cotton production

Farming experience with cotton cultivation ranged between one and 37 years with the majority of farmers (75 %) having less than ten years experience in cotton cultivation (Table 4.1). Bt cotton was planted for the first time in South Africa in 1997 when approximately 15 % of the farmers planted it (Fig. 4.2). None of the farmers that participated in this survey adopted Bt cotton before 1999 and adoption was slow until 2002 (Fig. 4.2). There was a 77 % adoption rate of Bt cotton in the 2003/04 season with market penetration of 100 % reached in the 2010/11 growing season (Fig. 4.2).

Table 4.1: Farming experience and cotton area cultivated per farmer in South Africa during the 2010/11 growing season.

		Percentage of farmers
Farmer experience in cotton cultivation (years)	1-10	75
	11-20	12.5
	21-30	8.3
	> 30	4.2
Area planted with Bt cotton (hectares)		**Percentage of farmers**
	1-20	8.3
	21-40	20.8
	41-60	8.3
	61-80	12.5
	81-100	16.7
	101-150	12.5
	151-200	8.3
	> 201	8.3

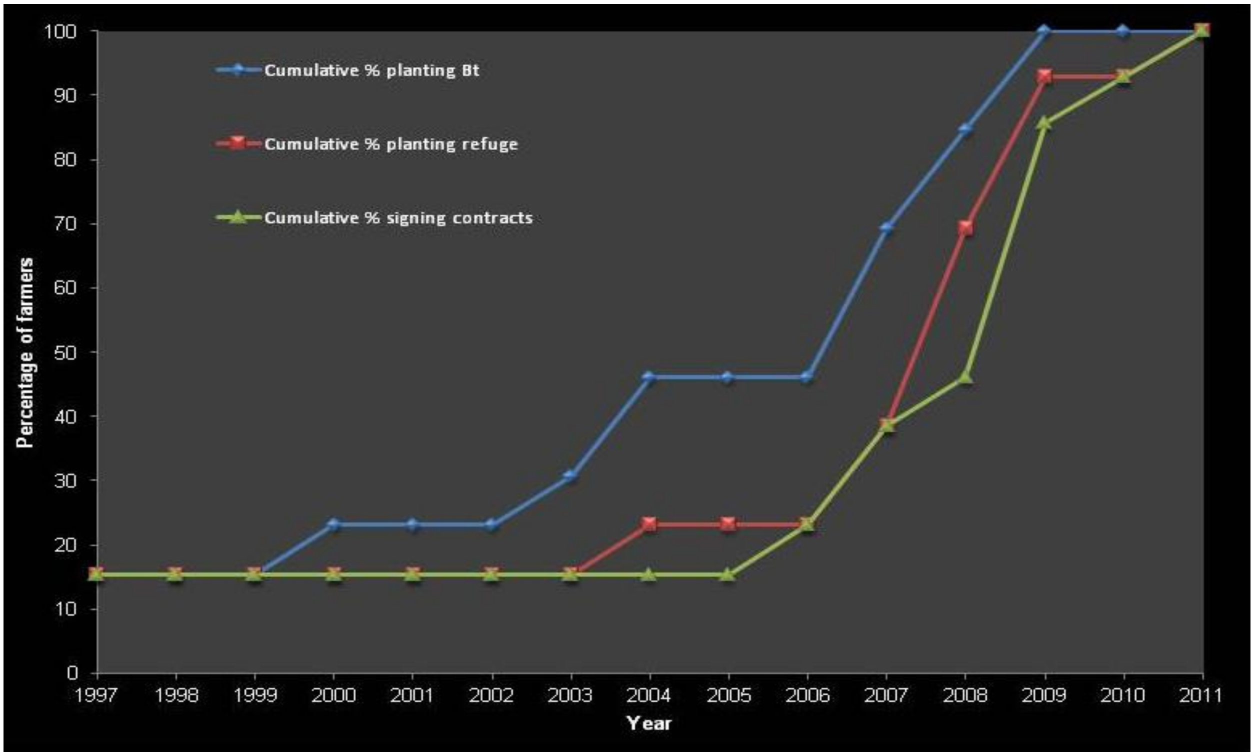

Figure 4.2: Percentage of farmers (combined data) planting Bt cotton, refugia and signing contracts since Bt cotton was released in South Africa.

All of the farmers interviewed indicated that the reason for the adoption of Bt cotton was an increase in yield and 66 % of the farmers indicated the reason for adoption is the ease of management of the crop (Data not shown). Data from Gouse *et al*. (2003) indicated an increase in the yield of 18.5 % for large-scale irrigated farmers, 13.3 % increase for large-scale dryland farmers and a 45.8% increase for small-scale dryland farmers that adopted Bt cotton. Gouse *et al*. (2003) also indicated that farmers planting non-Bt cotton, sprayed only when infestation levels reached certain levels at which time larvae have already started to cause economically important damage (Gouse *et al*., 2003).

During the 2010/11 growing season, farmers in the Douglas and Jacobsdal areas planted cotton for the first time since approximately 1991. Since no cotton was cultivated in these areas since the release of Bollgard® cotton in South Africa (1997) these localities can be considered unique in the sense that the target pests were not previously exposed to any GM cotton. The deployment of Bollgard II® cotton in this area during the 2010/11 season represents a case where the first exposure of the target pest to Bt cotton did not involve a single gene event expressing Cry1Ac only, but pyramid genes expressing both Cry1Ac and Cry2Ab2. It can therefore be expected the time to development of significant levels of resistance to Bollgard II® cotton in this area will be significantly longer than that observed at other localities where Bollgard® was planted previously. The introduction of Bollgard II® cotton in South Africa will therefore be a solution to control the resistant *H. armigera* populations (chapter 2) and delay the resistance development of the tolerant populations to Bt cotton in South Africa.

4.4.2. Compliance to refuge requirements

From the first time that Bt cotton was planted, the percentage of farmers planting non-Bt cotton refugia was low (Fig. 4.2). Farmers failed to comply with this requirement in nearly all the surveyed areas. The percentage farmers planting refugia remained low (<15 %) for the first eight years until the 2005/06 growing season when a rapid increase in the numbers of compliant farmers was observed (Fig. 4.2). The lowest rate of compliance was in the Groblersdal area where farmers only started to plant refugia eight years after the first planting of Bt cotton (Fig. 4.3). However, the compliance rate in the Groblersdal area reached 90 % during the 2010/11 season. A study conducted by Bates *et al*. (2005) indicated that the economic damage caused by pest to plants in the refuge area may be a reason for the lack of compliance amongst farmers growing Bt crops. The incidence of bollworm damaged bolls per plant was the highest at Groblersdal. This was also the area where the highest level of resistance was observed in laboratory studies (chapter 2). The study therefore provides evidence of a strong relationship between development of resistance and slow adoption of refugia.

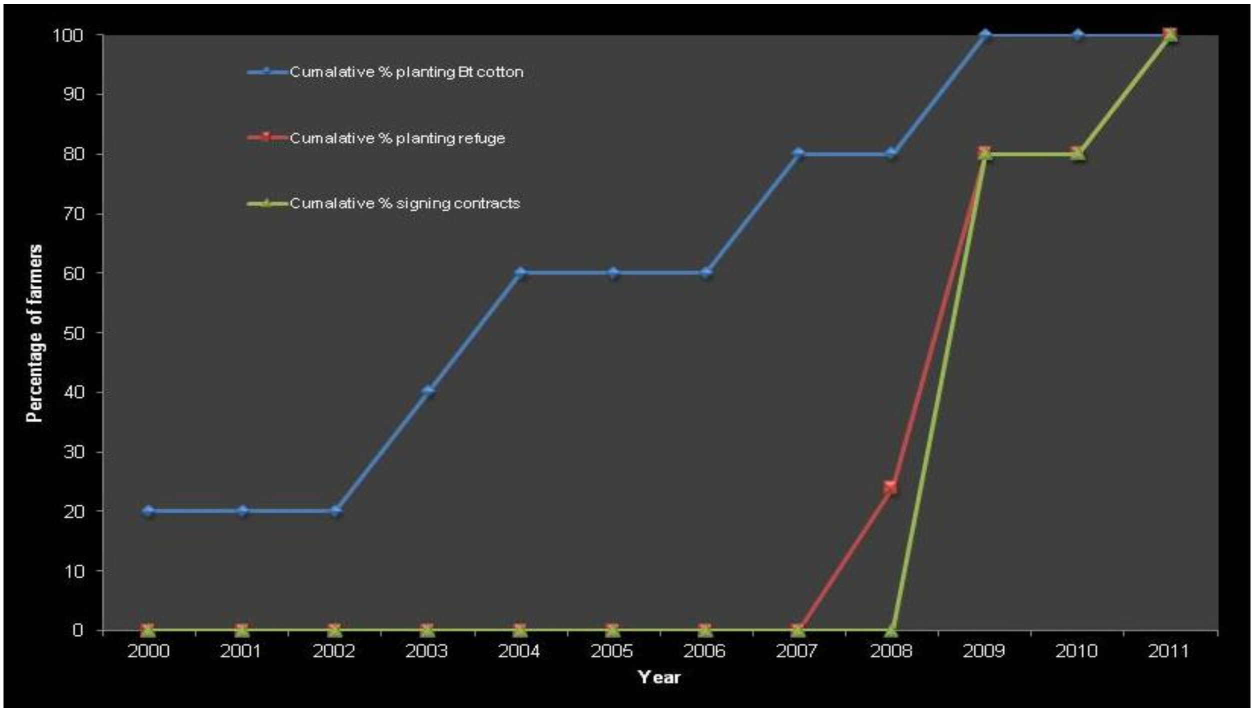

Figure 4.3: Percentage of farmers at Groblersdal planting Bt cotton, refugia and signing licensing agreements since 2000.

It is highly likely that the low levels of compliance with refuge requirements observed in this study contributed to development of resistance. Several examples of such relationships exist. For example, *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) developed resistance in the United States to Bt cotton (Luttrell *et al*., 2004). Luttrell *et al*. (2004) indicated that the general susceptibility of *H. zea* on conventional crops or refugia suggested that factors were functioning in the system to dilute selection for Bt resistance genes. Another example of the relationship between lack of refugia and resistance development is that of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) in South Africa (Van Rensburg, 2007). A study conducted by Kruger *et al*. (2009) indicated that the failure of farmers to comply with the refuge strategy in South Africa was one of the reasons for the development of resistance of the target pest to Bt maize. Farmer surveys done in this study showed the slow adoption of refugia to have contributed to the development of resistance of target pests. A similar study conducted by Kruger *et al*. (2011) on farmers' perceptions about Bt maize cultivation in South Africa showed the same trend amongst farmers for signing contracts and compliance to the refuge strategy.

Various strategies are used to manage resistance development of *H. armigera*. While structured refugia is recommended in Australia (Fitt, 2002) and the USA (Kelly, 2000; Turner, 2000; USEPA, 2006) this is not the case in China. The refuge strategy employed in China relies on the use of natural vegetation that acts as the refuge that sustains susceptible individuals of the highly polyphagous *H. armigera*. The size of the areas with suitable alternative host plants of this pest do however differ in different regions, resulting in selection pressure for resistance being higher in regions with fewer wild host plants (Wu *et al*., 2002; Wu & Guo, 2005; Wu, 2007).

Global resistance monitoring indicated that refugia have delayed pest resistance to Bt crops, especially when plants have met the high dose criterion and refuges have been abundant (Tabashnik *et al*., 2008; Tabashnik *et al*., 2009). It is therefore important that farmers comply with the planting of refugia.

A relationship between the signing of licensing agreements and compliance to refuge requirements was observed (Fig. 4.2). Farmers that plant Bt cotton are obligated to sign a licensing agreement with the seed company, indicating that a non-Bt cotton refuge area will be planted for every 100 ha of Bt cotton (Monsanto, 2010).

The United States Environmental Protection Agency (USEPA) adopted the refuge strategy for managing the evolution of Bt resistance in 1996 when Bt crops were first released. Other countries such as Canada and Australia (Kelly, 2000; Turner, 2000) as well as most developing countries have also adopted this strategy (Pray, 2001; Qiao *et al*., 2006). The planting of refugia is the only practice employed in South Africa to delay the development of resistance of target insect species to Bt crops (Bennett *et al*., 2003). The development of a resistant gene pool in a specific region becomes much smaller since the interaction between resistant and susceptible moths could cause sufficient dilution of resistant genes to counteract selection for resistance against the effect of the toxin produced by Bt cotton plants (Green *et al*., 2003). In this study all the farmers preferred to plant the 5 % refuge option. The 5 % option that the farmers preferred may not be sprayed with chemical insecticides. Refuge requirements for Bt grown in the 'corn-belt' in the USA are a 20% non-Bt field maize refuge. For Bt maize grown in cotton growing areas, a 50% non-Bt maize refuge is required (USEPA, 2006). The purpose of the 50 % refuge for Bt maize in Bt cotton growing areas is to reduce the selection pressure for development of resistance. Selection pressure in such mixed farming systems is high since Bt cotton and Bt maize expresses the same toxin gene (Shengjiang *et al.*, 2001).

All farmers interviewed during 2010/11 indicated that they planted a refuge for each Bt cotton field, except for the Musina area where only one farmer participated in the survey. Farmers that planted refugia indicated that they used the prescribed refuge layout options. The majority (80 %) of farmers in the Groblersdal area preferred the block-refuge option (Fig. 4.4). In the Douglas and Jacobsdal areas most farmers preferred split-block refuge designs while in the Vaalharts area several of these refuge designs were used (Table 4.2). Farmers indicated that their choice of refuge design was based on what was most suitable and practical in their farming system.

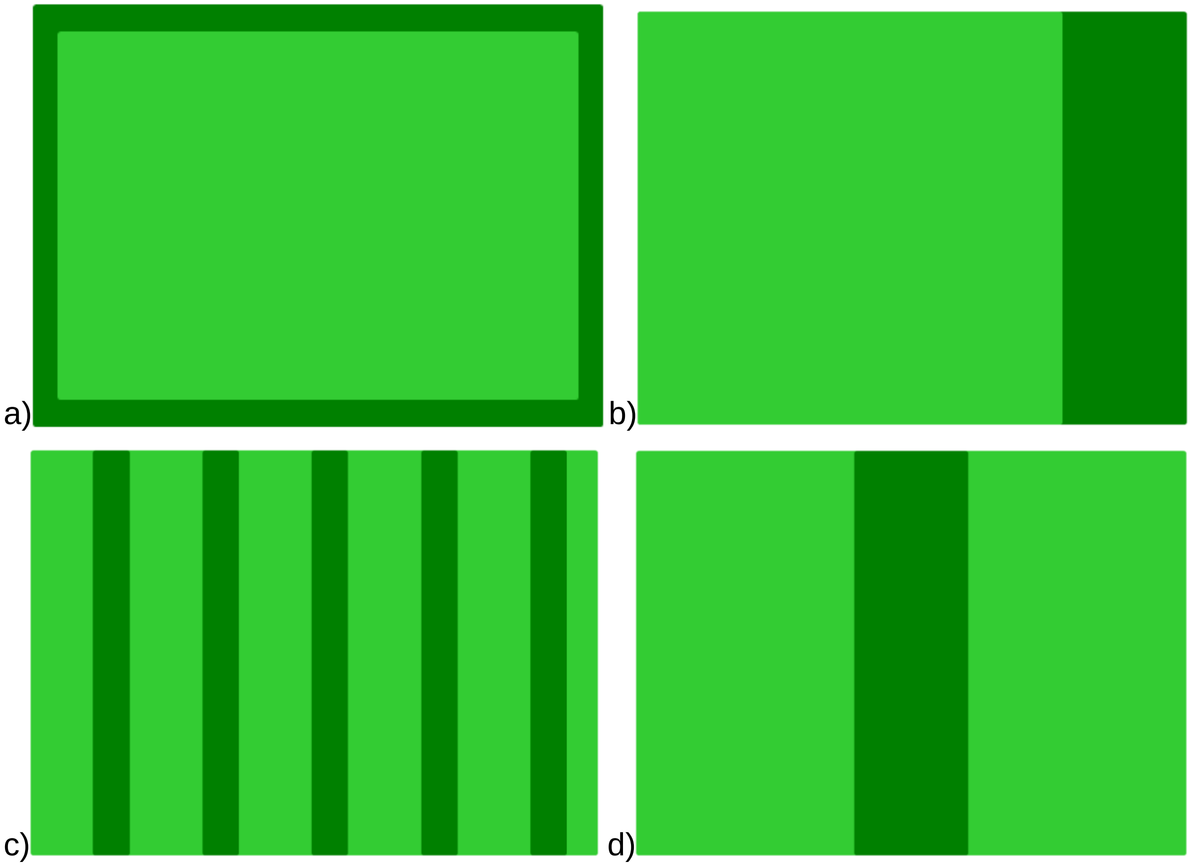

Figure 4.4: Lay-out options for refuge areas. Legend: Dark green areas indicate non-Bt cotton as refuge while light green areas indicate Bt cotton, a) border refuge, b) block refuge, c) strip refuge, d) split field refuge (Monsanto, 2007).

Table 4.2: Layout-options chosen by farmers for refuge area.

Question posed to farmers		**Farmers' response (%)**			
		Groblersdal	Douglas	Jacobsdal	Vaalharts
Layout options for refuge area	Border refuge	20	0	0	37.5
	Block refuge	80	40	40	50
	Strip refuge	0	0	0	0
	Split field refuge	0	60	60	12.5

4.4.3. Pest management practices

Only farmers from the Vaalharts area (20 %) regarded bollworms as a pest on Bt cotton. Most of the farmers (87.5 %) in this area applied insecticides to Bt cotton and they indicated that their Bt cotton crops were also attacked by pests such as cotton stainers, aphids and bollworms (Table 4.3). Farmers in Vaalharts applied broad spectrum pyrethroids on their Bt cotton to kill non-target pests. Through this practise they indirectly apply insecticides that also control bollworms (Table 4.3). In the preliminary study done in 2009/10 farmers indicated they applied insecticides on Bt cotton to control bollworms. Farmers in Groblersdal, however, indicated that bollworm infestation levels became higher during the 2008/09 growing season and that insecticides were applied to control bollworms on Bt cotton. In the Groblersdal, Douglas and Jacobsdal areas a high proportion of farmers (60 – 80 %) indicated that they experienced no pest problems on Bt cotton (Table 4.3). Farmers in Groblersdal indicated that they sometimes applied chemical control against bollworm until the 2009/10 season but that this was not necessary during the 2010/11 growing season. This is ascribed to the planting of Bollgard II® in the latter season.

The main pests indicated by farmers to be of importance on Bt cotton were the cotton stainers, *Dysdercus fasiatus* (Signoret) (Hemiptera: Pyrrhocoridae) and aphids, *Aphis gossypii* (Glover) (Hemiptera: Aphididae) (Fig. 4.5). Farmers from all surveyed areas indicated that all the pest species listed on Bt cotton also occur on non-Bt cotton (Table 4.3). However, between 62 and 100 % of farmers in all surveyed areas made use of broad spectrum insecticides that also kill bollworms on the refuge area, except at Douglas where farmers applied no insecticides on the non-Bt cotton refugia. The farmers indicated that they did not apply insecticides on the refuge to control the pests, but due to the method of application they use on the Bt cotton to control pests that are not killed by the Bt toxins (Table 4.3). The majority of farmers' used broad spectrum pyrethroids to control the hemipteran pests on Bt cotton and applied insecticides once or twice per growing season.

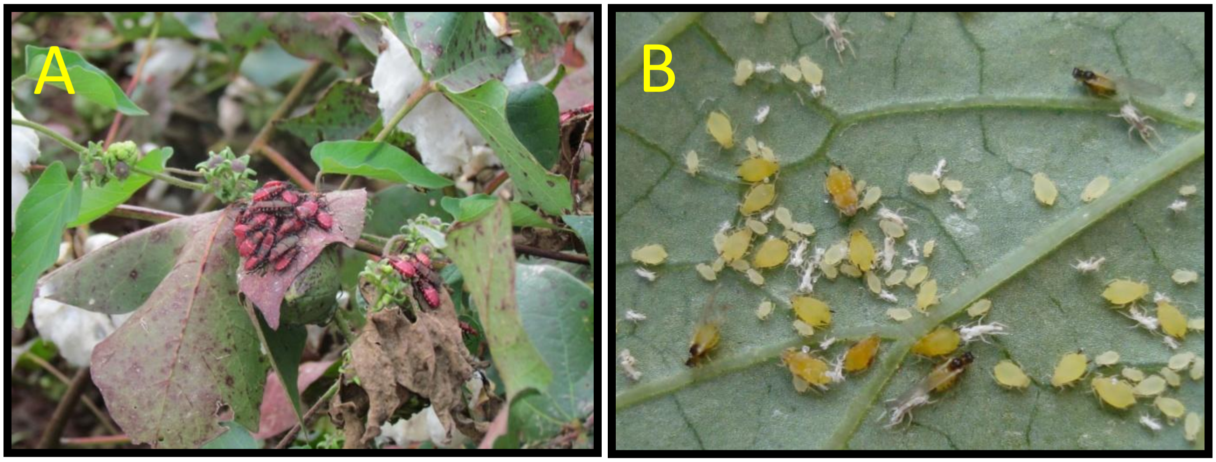

Figure 4.5: The two major pest species that farmers sprayed for on Bt cotton A: The cotton stainer *Dysdercus fasiatus* and B: *Aphis gossypii.*

The reason for these insecticide applications on refuge areas was that it was done through the centre-pivot irrigation systems (40 % of farmers), making it impossible to exclude these areas from application. Other farmers applied insecticides aerially (20 % of farmers) which also prevented exclusion of refuge areas from application.

Gouse *et al*. (2008) concluded that South African farmers needed to apply insecticides for control of sucking pests on Bt cotton. Bt cotton enables farmers to reduce the number of insecticide applications with the added benefit that it increases the useful life of pyrethroids and other chemical insecticides (Mellet *et al*., 2003). Only herbicides are applied on non-Bt maize refugia in South Africa and farmers do not apply any insecticides to control other pests on both Bt and non-Bt maize (Kruger *et al*., 2009). The main concern about the application of pyrethroids on refugia where farmers use the 5 % option is that insecticide application reduces the number of susceptible individuals that the non-Bt refuge is supposed to sustain and therefore also the chance that mating will take place between susceptible individuals and the few resistant individuals that may have survive on Bt cotton. Application of insecticides on the refuge areas therefore increases the selection pressure for resistance development since a comparatively large number of resistant individuals may complete their life cycles inside the Bt crop field, resulting in a higher probability of mating between individuals emerging from the Bt crop. Sucking insects can be controlled with environmentally less damaging organophosphates that are less effective on bollworms (Gouse *et al*., 2008). Pyrethroids also kill the beneficial insects such as lacewings and ladybirds and increase the risk of secondary pest development such as red spider mites, *Tetranychus urticae* (Boisduval) (Acari: Tetranychidae) (Gouse *et al*., 2008; Annecke & Moran, 1982).

Table 4.3: Pest problems farmers experienced on Bt- and non-Bt cotton and pest management practices they used to control different pest species.

Question posed to farmers (Pest management)		**Farmers' response (%)**			
		Groblersdal	Douglas	Jacobsdal	Vaalharts
Do you have any pest problems on Bt cotton?	Yes	40	40	20	75
	No	60	60	80	25
Name type of pests:	Bollworms	0	0	0	20
	Cotton stainer	100	100	100	70
	Aphids	0	0	0	10
Do you apply insecticides on Bt cotton?	Yes	100	40	60	87.5
	No	0	60	40	12.5
What insecticide do you apply?	Lambdacyhalothrin	20	0	0	0
	Deltamethrin	20	0	60	62.5
	Unsure	60	40	0	25
	None	0	60	40	12.5
Do you have any pest problems on non-Bt cotton?	Yes	100	60	60	100
	No	0	40	40	0
Name type of pests:	Bollworms	100	100	100	100
	Cotton Stainer	100	100	100	100
	Aphids	0	0	0	100

Table 4.3 Continued: Pest problems farmers experienced on Bt- and non-Bt cotton and pest management practices they used to control different pest species.

Do you apply insecticides on non-Bt cotton?	Yes	100	0	60	62.5
	No	0	100	40	37.5
What insecticide do you apply?	Lambdacyhalothrin	20	0	0	0
	Deltamethrin	20	0	60	37.5
	Unsure	60	0	0	25
	None	0	0	40	37.5

4.4.4. Farmer's perceptions of the future of Bt cotton and its benefits

Farmers from all areas indicated that they did not perceive Bt cotton to have a negative effect on the environment, except for some farmers (25 %) in the Vaalharts area who indicated that resistance development could indirectly have a negative effect. The reason advanced for this was because alternative techniques such as chemical insecticides will then also have to be used to control resistant individuals (Table 4.4).

All the farmers indicated that there were several advantages to planting Bt cotton (Table 5). These were increase farm productivity and the perception that the technology is, environmentally friendly. Management of the crop is also much easier because Bt cotton provides season-long protection against the target pest (Gouse *et al*., 2003). Farmers regarded this as a great advantage which gave them peace of mind in the control of target pests (Table 4.4). Farmers (40 - 100 %) indicated that cultivation of Bt cotton resulted in reduced overall production costs (Table 4.4).

Some of the advantages indicated by the farmers associated with the cultivation of Bt cotton was reduced labour inputs, a reduction in the use of insecticides and increased profit (Table 4.4). Similar advantages were reported on cotton and maize (Fernandez-Cornejo & Klotz-Ingram, 1998; Gianessi & Carpenter, 1999; Fernandez-Cornejo *et al*., 1999; Carrière *et al*., 2003; Green *et al*., 2003; Bennett *et al*., 2004; Fitt, 2004; Wu *et al*., 2008; Kruger *et al*., 2009). A reduction in the number of insecticide application to cotton largely result in reduced expenditure on diesel and fewer tractor hours (Gouse *et al*., 2004). For small-scale farmers the benefit accrued by cultivation of Bt cotton largely lies in labour saving since most farming activities such as spraying and weeding is done by hand (Gouse *et al*., 2004). Farmers also indicated that a reduction in the use of insecticides results in an increase in beneficial insects that contribute to the control of target pest. These observations are supported by a study done by Van Hamburg & Guest (1997). A study conducted by Kruger *et al*. (2009) indicated the same advantages amongst farmers that adopted Bt maize in South Africa.

Gouse *et al*. (2003) reported farmers perceived high seed costs and the technology fee of GM cotton as a prohibitive factor in decisions regarding planting of this crop. In this study farmers indicated that development of pest resistance to Bt cotton together with low cotton product prizes, could result in them not planting cotton in future (Table 4.4). The reduction in the production of cotton in South Africa is influenced by the poor price prospects during planting time and the higher prices of competitive crops such as maize and sunflower. There was a continuous decline in cotton production since 1989 form more than 200 000 ha (Fok *et al*., 2007; Cotton SA, 2011) to just more than 13000 ha in the 2010/11 growing season (Cotton SA, 2011). Other crops that compete with cotton and that are often used in rotation systems with cotton are potatoes, tobacco, tomatoes, maize, wheat and soybean. If these crops have higher price prospects and the price of cotton declines this may prevent farmers from planting Bt cotton.

Table 4.4: Farmers' perceptions about the impact of Bt cotton on the environment and different advantageous associated with the planting of Bt cotton.

Question posed to farmers		**Farmers' response (%)**			
		Groblersdal	Douglas	Jacobsdal	Vaalharts
Can GM cotton have a negative effect on the environment?	Yes No	0 100	0 100	0 100	25 75
Is it advantageous to plant Bt cotton?	Yes No	100 0	100 0	100 0	100 0
Advantages associated with Bt cotton:					
Increased farm productivity	Yes No	100 0	100 0	100 0	100 0
Environmentally friendly	Yes No	100 0	100 0	100 0	100 0
Convenient management	Yes No	100 0	100 0	100 0	100 0
Reduced production costs	Yes No	100 0	60 40	40 60	62.5 37.5
Is it economically worthwhile to plant Bt cotton in spite of increased seed cost?	Yes No	100 0	100 0	100 0	100 0

Table 4.4 Continued: Farmers' perceptions about the impact of Bt cotton on the environment and different advantageous associated with the planting of Bt cotton.

What may prevent farmers to cultivate Bt cotton in future?	Bollworm resistance	80	80	80	50
	Price of cotton	20	20	0	50
	Technology fee	0	0	20	0

4.4.5. Determining the incidence of bollworm damage under field conditions

The incidence of damage at Vaalharts during the 2009/10 growing season was high. In two of the Bt cotton fields damage was 11 and 15 % on Bt- and non-Bt cotton respectively and 14 and 19 % respectively. In the third field the incidence of damage was higher on the Bt cotton than on the non-Bt cotton, but the percentage damage was low with 3 and 2.5 % on Bt- and non-Bt cotton plants respectively (Fig. 4.6). Farmers indicated that bollworms are a problem on Bt cotton and that they have to apply insecticides to Bt cotton to control larval infestations.

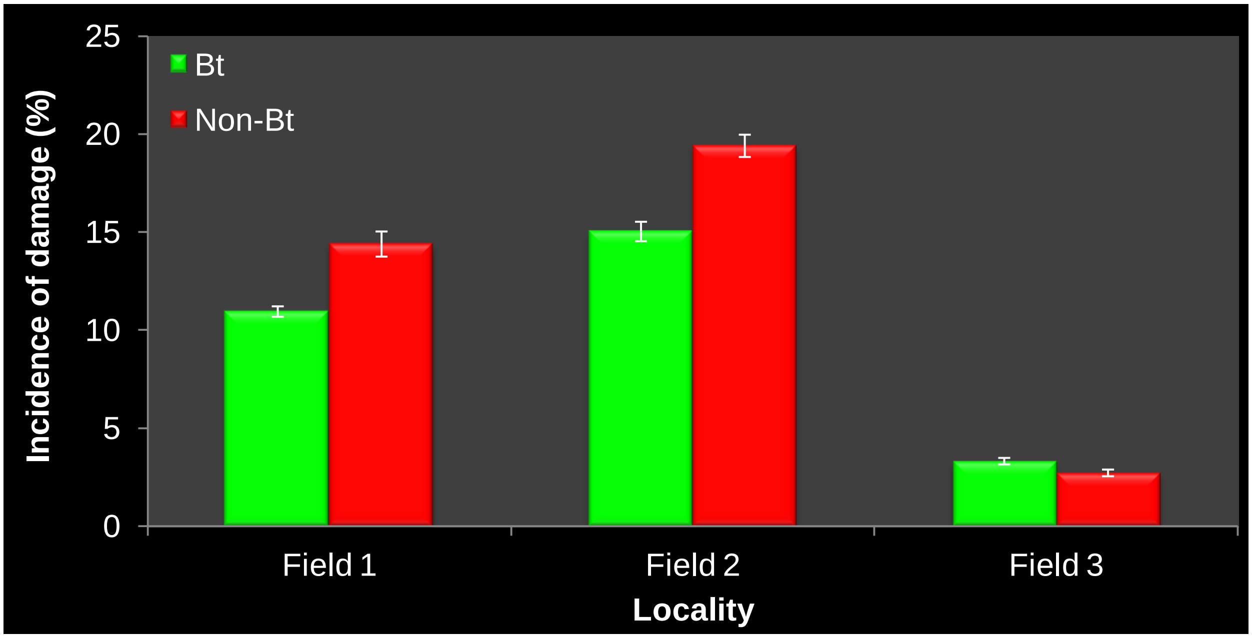

Figure 4.6: The incidence of bollworm damaged bolls on Bt- (Bollgard®) and non-Bt cotton plants at Vaalharts during the 2009/10 growing season (Bars indicate standard errors).

The incidence of bollworm damage on Bt- and non-Bt cotton plants was low in all the areas that were surveyed during the 2010/11 growing season (Fig. 4.7). The highest incidence of damage on non-Bt cotton was in the Vaalharts area (22 %) and is higher than the previous season. The highest incidence of damage on Bt cotton was at Groblersdal (4 %) (Fig. 4.7), which was also the locality where resistance to Cry1Ab producing Bt cotton (Bollgard®) was recorded (chapter 2). The low levels of bollworm damage observed in these surveys could possibly be ascribed to the fact that all surveys were done on fields in which Bollgard II® cotton was planted. Bollgard II® cotton was planted for the first time in South Africa during the 2010/11 growing season. The very low incidence of damage to squares observed in the field surveys does not support the data reported in chapter 2 which showed that *H armigera* was resistant to Bt cotton. These observations are ascribed to that fact that the resistance evaluations reported in chapter 2 were done on Bollgard® cotton which expresses only Cry1Ac proteins while Bollgard II® cotton expresses both Cry1Ac and Cry2Ab2 proteins which makes it more effective in controlling the pest.

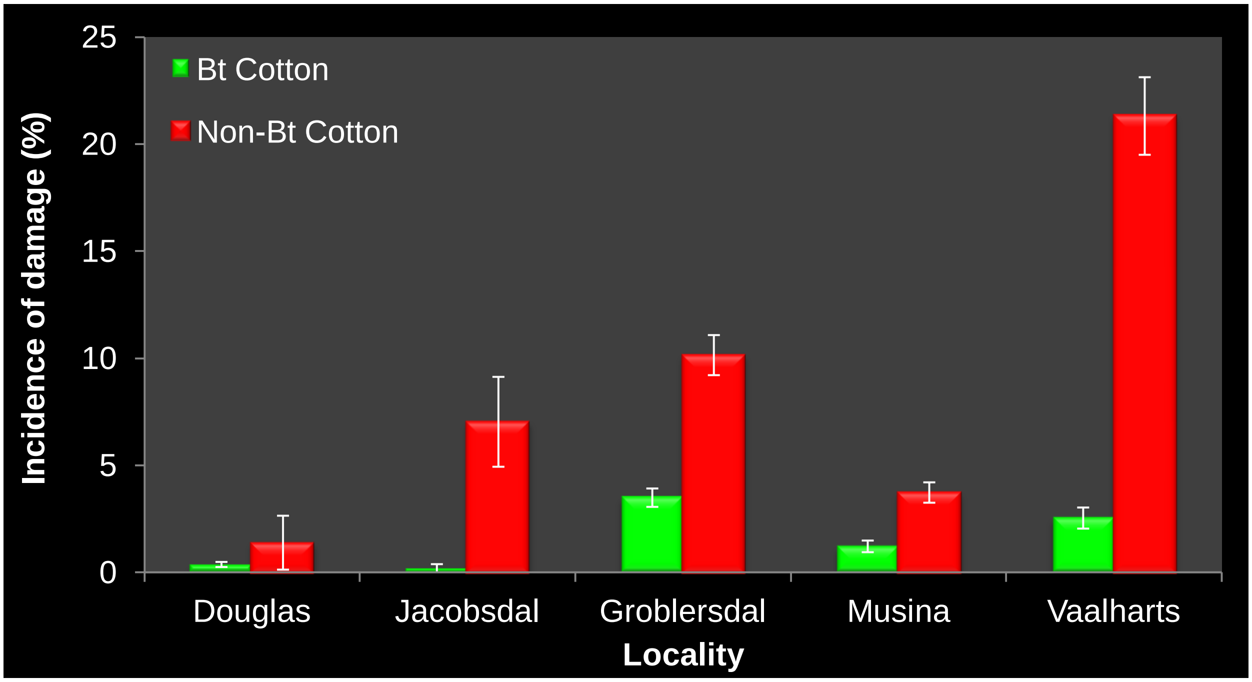

Figure 4.7: The incidence of bollworm damaged bolls on Bt- (Bollgard II®) and non-Bt cotton plants at different localities in South Africa during the 2010/11 growing season (Bars indicate standard errors).

One of the concerns about Bt cotton is that there is a decline in the efficacy of the toxicity as the plants mature occurring mainly during peak flowering (Fitt *et al*., 2004) The decline in the plants is sufficient to allow susceptible larvae to survive and develop on Bt cotton expressing Cry1Ac toxins. A 5–20 % survival of susceptible larvae towards the end of the growing season was found by Wu & Guo (2005). Larvae, however, also develop slower than on non-Bt cotton. Similar observations were made in this study regarding development time of *H. armigera* (chapter 2).

It is important not to rely only on field scouting, but feeding studies must also be done to assess the level of resistance in different populations of the target pest. As part of companies’ IRM requirements an annual resistance monitoring program should be implemented, the goal of which is to detect changes in resistance levels in pest populations.

4.5. Conclusion

Initial levels of refuge compliance were very low, especially at Groblersdal. This could have contributed to the development of the Bt-resistant *H. armigera* populations at this site. Insecticide applications on Bt cotton throughout the cotton producing regions were mainly done to control cotton stainers which reach economically important infestation levels towards the end of the growing season. It is clearly visible from scouting data that there is a big difference between Bollgard® and Bollgard II® cotton performance against bollworm damage. Bollgard II® cotton was effective in controlling bollworms in the different areas and will most likely contribute in controlling resistant bollworm populations in the foreseeable future.

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Chapter 5: CONCLUSIONS

The objectives of the study were to evaluate if *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *Diparopsis castanea* (Hampson) (Lepidoptera: Noctuidae) populations with resistance to Bt cotton existed in South Africa and to determine farmers' perceptions of the cultivation of Bt cotton as well as bollworm resistance development.

Cotton have been cultivated in South Africa since 1846 (Annecke & Moran, 1982) and transgenic Bt cotton (Bollgard®) expressing Cry1Ac proteins have been released during 1997 (Cotton SA, 2006), to control the bollworm complex. The bollworm complex, which consists of *H. armigera*, *D. castanea* and *Earias* spp. (Hill, 1983), attacks the flowering parts of cotton plants and causes significant qualitative and quantitative losses. The advantages associated with the adoption of transgenic crops include higher yields, reduced labour inputs and a reduction in the use of insecticides which result in higher profit and reduced impact on the environment (Fernandez-Cornejo & Klotz-Ingram, 1998; Gianessi & Carpenter, 1999; Fernandez-Cornejo *et al*., 1999; Bennett *et al*., 2004; Shankar & Thirtle, 2005). However, one of the biggest concerns regarding Bt crops is possible resistance development of the target pests (Ismael *et al*., 2001).

The concerns about the development of resistance to Bt cotton is a result of the use of a single gene to control the target pest. There is a possibility that resistance to specific *cry* proteins can develop in the same way that an insect develop resistance to chemical insecticides (Mellet *et al*., 2003). *Helicoverpa armigera* is a key pest of cotton in South Africa and is a species that showed great capacity to develop resistance to chemical insecticides (Forrester *et al*., 1993). It is therefore expected that if the target pest have the ability to develop resistance to chemical insecticides they have the potential to develop resistance to Bt cotton if they are over-exposed to the insecticidal protein. It is expected that *H. armigera* will develop resistance to Bt cotton expressing Cry1Ac proteins in South Africa if insect resistance management strategies are not followed.

There are four Lepidoptera species that already developed resistance to Bt crops. Two of these species developed resistance to Bt cotton, namely *Heliothis zea* (Lepidoptera: Noctuidae) in southeastern United States (Luttrell *et al*., 2004) and *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) in India (Bagla, 2010). The other species are *Spodoptera frugiperda* (Lepidoptera: Noctuidae) that developed resistance to Bt maize in Puerto Rico (Matten *et al*., 2008) and *Busseola fusca* (Lepidoptera: Noctuidae) to Bt maize in South Africa (Van Rensburg, 2007). Although resistance of *H. armigera* to Bollgard® cotton have not been reported by farmers or industry in South Africa, this study showed some level of resistance at some localities. Significant damage to Bt cotton was observed under field conditions and in the laboratory, *H. armigera* collected from several sites in South Africa completed its life cycle on Bt cotton. This study reported that *H. armigera* populations from Groblersdal, Vaalharts, Potchefstroom and Parys populations were resistant to Bt cotton (chapter 2).

This study, in which resistance was reported for the first time, was done 14 years after release of Bt cotton in South Africa. It is not known when the first significant damage to Bt cotton started to appear under field conditions. It can however be assumed that the 2010/11 growing season was not the first season in which resistance became evident. The time period after release of Bt cotton and this report of resistance was 14 years. This was much longer than the period observed for *B. fusca*, which was eight years. This slow rate of resistance development in bollworms could probably be ascribed to several reasons such as poor refuge compliance, amongst others. However, the possibility of reduced selection pressure on bollworm populations in Bt cotton, resulting from a significant decrease in cotton production in the country should not be excluded. The area planted to cotton in South Africa decreased with 87 % from 100 000 ha to 13145 ha between the 1999/00 and 2010/11 growing season.

Several studies indicate that second-generation Bt crops such as Bollgard II® that express more than one Bt toxin can delay the development of resistance (Tabashnik *et al*., 2002; Zhao *et al*., 2003; Bird & Akhurst, 2004). Bollgard II® cotton expresses

Cry1Ac and Cry2Ab2 proteins and was planted for the first time in South Africa during the 2010/11 growing season. A study conducted by Luo *et al*. (2008) indicated that no cross resistance in *H. armigera* between Cry1Ac and Cry2Ab proteins. For this reason Bollgard II® cotton was released to manage potentially Cry1Ac-resistant populations of *H. armigera* in China. Bollgard II® cotton will therefore contribute to improved management of the resistant *H. armigera* populations in South Africa. If increased crop value results in increased cotton production and the South African cotton industry grow in the foreseeable future, more Bollgard II® cotton will be planted in the country. It can therefore be expected that selection pressure on resistance development will also increase. It will therefore be important to monitor for resistance development in the bollworm complex in South Africa.

Diparopsis castanea (Lepidoptera: Noctuidae) is one of the major pests of cotton in the Makhathini Flats area in KwaZulu-Natal, South Africa (Green *et al*., 2003). Cultivation of genetically modified Bt cotton expressing the Cry1Ac protein commenced in this area during the 1998/99 growing season. Cotton in this area is mainly planted by small-scale farmers that plant on average between one and three hectares annually (Bennett, 2002). No conclusions can be made from this study on resistance of *D. castanea* to Bt cotton but the relatively long time to 100 % larval mortality could indicate development of tolerance to Cry1Ac proteins. Farmers that plant Bt cotton are obligated to sign a licensing agreement that they will plant a non-Bt refuge area as a resistance management strategy (Monsanto, 2010). Small-scale farmers in the Makhathini Flats area, however, may neglect planting refugia. It is therefore necessary to also monitor levels of resistance of *D. castanea* to Bt cotton. The absence or low level of resistance observed in the *D. castanea* population collected in the Makhathini Flats area could possibly be ascribed to the availability of unstructured refugia in the form of wild host plants of this pest. A study by Green et al. (2003) indicated that the natural vegetation served as a natural refuge that could contribute to delaying development of resistance (Green *et al*., 2003).

Field surveys are generally accepted as an integral tool to measure the success of Bt crops. However, monitoring should also be conducted to follow changes in the susceptibility of pest populations (Wu *et al*., 2002). Another useful tool is the use of surveys to assess farmers' perceptions about transgenic crops (Grieshop *et al*., 1988). Surveys conducted during this study showed that compliance to refuge requirements was low. Resistance reported in this study could therefore pertly be ascribed to low refuge compliance among farmers in the different cotton production regions. The adoption of refugia plantings over the first 7-9 years after release of Bt cotton was low and the signing of contracts to ensure that farmers plant refugia followed a similar trend. Survey results indicated that the level of compliance to refuge requirements during the 2009/10 and 2010/11 seasons were high and it is therefore expected that development of resistance of *H. armigera* to new-generation Bt cotton varieties will take significantly longer to develop.

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