GEOGRAPHIC SUSCEPTIBILITY OF *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE) TO INSECTICIDAL PROTEINS IN Bt-COTTON IN SOUTH AFRICA

THESIS

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by

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ABSTRACT

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) (African bollworm) is a typical noctuid with a very catholic taste in food plants and whose larvae feed on a wide range of cultivated and wild plants. It has been identified as the most polyphagous and injurious pest in South Africa. *Helicoverpa armigera* is also a key pest of cotton in many parts of the world. This key pest requires extensive control as it adversely effects yield and has built up resistance to synthetic pyrethroid insecticides.

Cotton is an important crop produced by commercial and small-scale farmers in South Africa. The local demand for cotton has not been exceeded yet, but to satisfy a demanding market, pest control costs play an important role in cotton production. The threat of an insect pest that has already shown resistance prompted the present study to investigate the possibility of resistance to Bt-cotton.

Genetically engineered or Bt-cotton was introduced commercially in 1996 in South Africa. All Bt-cotton plants contain one or more foreign genes derived from the soil-dwelling bacterium, *Bacillus thuringiensis* (Berliner), which produces protein crystals. These crystals were isolated and transferred into the genome of a cotton plant resulting in the plant producing it's own protein insecticide. In 1998, Monsanto (Pty) Ltd requested research into the geographic susceptibility of *H. armigera* to the insecticidal proteins in Bt-cotton in SA.

Laboratory reared and field sampled populations of *H. armigera* were exposed to a diet mixed with various baseline concentrations of the Bt-gene Cry1Ac freeze dried protein. This study also

determined the performance of *H. armigera* and *Spodoptera littoralis* (Boisduval) on different Bt-cotton field cultivars containing different Cry-protein genes.

Results obtained indicated a significant difference in susceptibility in two field populations of *H. armigera* to the Bt-protein Cry1Ac, even though the LD_{50},s in the 2003 season did not indicate resistance. Bt-cotton cultivar 15985 BX controlled *H. armigera* and *S. littoralis* larvae, the best followed in descending order by cultivar 15985 X, 15985 B and DP50 B. Results on *H. armigera* also indicated that the Cry-proteins in the plant parts of the different cultivars did not diminish as the season progressed. The Bt-cotton cultivars induced retarded growth of larvae, due to either a repellent effect or lack of feeding by larvae.

Widespread adoption of Bt-cotton by South African farmers led to regional declines in bollworm populations, reduced insecticide use, and increased yields. Genetically modified crops therefore contribute to a cost effective, sustainable, productive and efficient form of agriculture, with a resultant positive impact on the environment. As the market for commercial Bt-cotton in South Africa expands, it is recommended that a monitoring programme for potential resistant genes in *H. armigera* should be implemented at least every 2 - 3 years. This will ensure that effective resistance management strategies are utilised. Coupled with this are the Biosafety Risks regarding the effect of new proteins expressed in transgenic plants, which require further studies.

FRONTISPIECE

Top: *Helicoverpa armigera* Hübner, (Lepidoptera: Noctuidae), 5th instar larva African bollworm.
Bottom Left: Healthy cotton plant.
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PUBLICATIONS ARISING FROM THIS STUDY

Parts of the research presented in this thesis, already accepted for publication are the following:

M.J. VAN JAARSVELD AND L.M. PRETORIUS. 1999. Transgenic cotton? A lethal weapon against Lepidopteran pests. **Proceedings of the Twelfth Entomological Congress. 12-15 July 1999, Potchefstroom.**

M.J. VAN JAARSVELD AND L.M. PRETORIUS. 2001. Transgenic cotton - Progress in South Africa. **Proceedings of the Thirteenth Entomological Congress. 2-6 July 2001, Pietermaritzburg.**

M.J. VAN JAARSVELD. 2003. Geographic management of susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to insecticidal proteins in Bt-cotton in South Africa. **Proceedings of the Fourteenth Entomological Congress. 6-9 July 2003, Pretoria.**

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

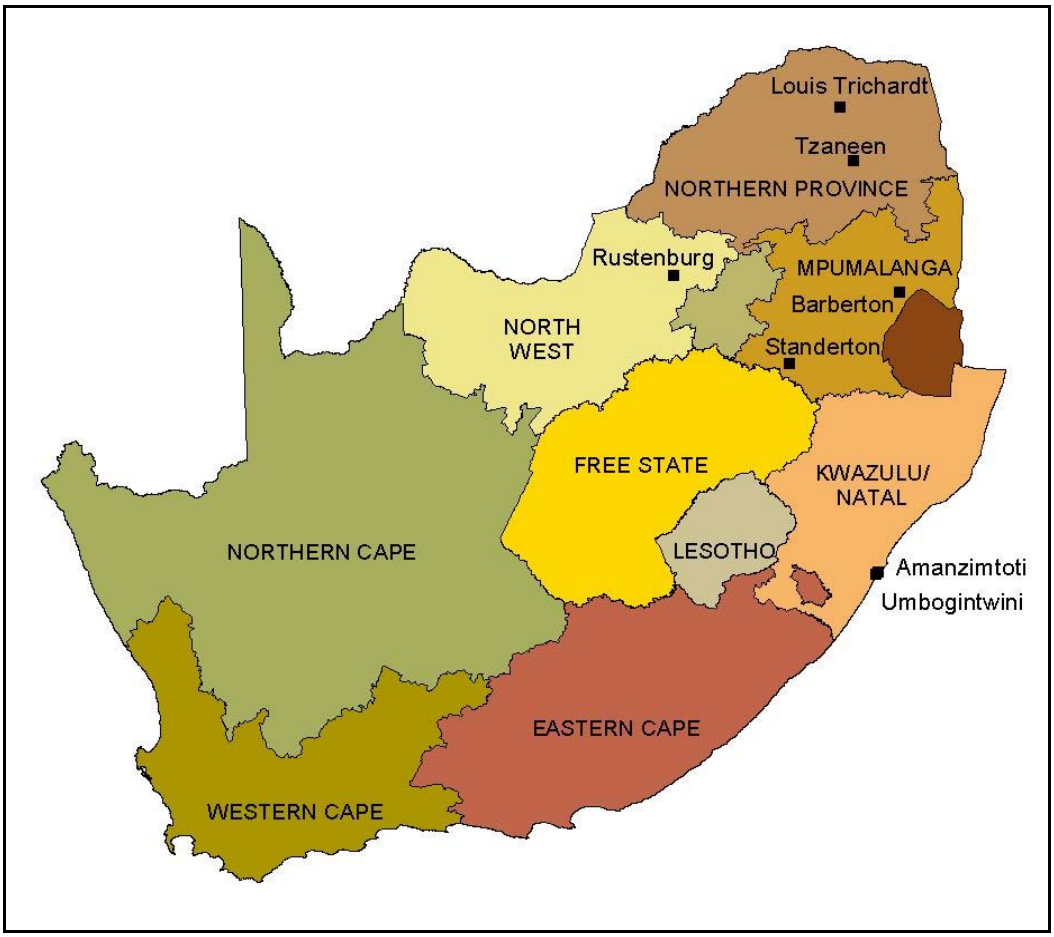
In 1516, according to historical documents a certain Barbosa (a Portuguese trader), met the indigenous people in South Africa who grew cotton and wore cotton clothing. This was a type of wild cotton, species which still exist today. The first cotton seed was planted in 1690 in the Western Cape, more or less 40 years after the arrival of Jan van Riebeeck. Cotton, however, prefers a warm climate and requires a substantial amount of moisture for the seed to germinate.

In 1846 Dr Adams brought seed from America and started growing cotton in the Amanzimtoti district in Kwa-Zulu Natal Province. Between 1860 and 1870 cotton was planted on a relatively large scale in both Kwa-Zulu Natal and the Cape Colony (Western Cape Province) due to the demand for this fibre which had arisen as a result of the American Civil War. After 1870 the large scale production of cotton in South Africa came to a virtual standstill and was only to be continued at the start of the twentieth century.

In 1904 about 12 to 14 hectares were planted in the Tzaneen area (Fig. 1.1) and in 1905 a cotton gin was erected in the area where cotton could be ginned and baled mechanically. In 1913 an experimental station which was to provide farmers with advice was established at Rustenburg under the direction of a Mr Taylor. Between 1913 and 1922 cotton was cultivated mainly in the Transvaal Lowveld, what is now called Mpumalanga Province.

The co-operative movement with regard to cotton had its origin in 1922 when a co-operative and a ginnery were established at Barberton. Already in the early stages of its cultivation, cotton played an important role in the manufacture of explosives. In 1924 African Explosives erected a ginnery at Umbogintwini in Kwa-Zulu Natal. In the same year a ginnery was also erected at Magut (Kwa-Zulu Natal) by a Mr. Rouxliard and another in 1935 at Louis Trichardt by the Lancashire Cotton Corporation Spinners of the United Kingdom.

At this stage in South Africa there were as yet no facilities for either spinning or weaving and the fibre was exported to Liverpool. Cotton was grown under irrigation in the Lower Orange River area for the first time in 1927 and in the early thirties cotton production dropped with the result that the next ginnery was only erected in the late thirties by Amaro at Standerton. According to Section 102 of the Co-operative Societies Act (Act 29 of 1929) cotton was officially declared an agricultural crop in 1939 (Cotton SA 2003; Http://www.cottonsa.org.za



(March 2003)).

Figure 1.1 Map of South Africa showing Provinces and historically important towns related to cotton.

Cotton is a herbaceous plant which grows to about 1.4 m with a characteristic arrangement of dimorphic branches (Eaton 1955; Van Heerden 1978). Growth of the monopodial main stem and

sympodial vegetative branches is indeterminate (Gillham 1972). The sympodia or fruiting branches grow outwards and produce several nodes, each node producing a flower. The succession of fruiting branches, arising from the nodes of the main stem, follows a set spiral course of three-eights of the circumference of the main stem. Leaves are formed on the sympodial branches with two buds at the base of each leaf. The axillary bud produces a vegetative branch and if the extra-axillary bud develops, it produces a further fruiting branch. The bolls first ripen near the bases of the branches (Van Heerden 1978; Van der Walt 1988). Growth is irregular, depending largely on the availability of soil moisture, so that fruiting forms are produced for the greater part of the season, with peak flowering period occurring about 90 - 100 days after plant emergence. Predictably at the beginning of the season, buds are the most abundant on the plants, but as the season progresses, flowers and bolls become relatively more abundant and towards the end of the season the bolls are the most abundant fruiting form. All stages of fruiting forms are present from about the fourth week after planting, but in varying proportions (Van der Walt 1988). Cotton is a perennial plant and can be ratooned.

Van der Walt (1988) divided the fruiting forms of a cotton plant into twelve classes or categories namely: (1) Growth tip, (2) Bud - bud barely visible, (3) Bud - calyx closed, epicalyx fully formed, (4) Bud - yellow corolla protruding from the calyx, shorter or equal to the calyx, (5) Bud - corolla prominent , unopened, longer than the calyx, (6) Flower - yellow, (7) Flower - purple, (8) Boll - corolla lost, boll smaller than calyx, (9) Boll - boll fills calyx, (10) Boll - boll larger than calyx, calyx split, boll shorter than epicalyx (structure can be suspended by holding tips of epicalyx together), (11) Boll - boll as long as epicalyx (structure cannot be held by tips of epicalyx), (12) Boll - boll longer than epicalyx, last category before boll burst. The maximum boll size, contains the unripe seeds and fibers originating from the seed coats. The contents of the boll are covered with a thick layer of tissue that harden during the last few categories before ripening. The ripe fruit (referred as “boll burst”) is a dry dehiscent schizocarp of three to five loculi, each containing eight to nine lint-bearing seeds (Van Heerden 1978). The lint-bearing seeds eventually ripen and are harvested, mainly for the textile industry.

In South Africa cotton is one of the few agricultural commodities where local demand exceeds production, thereby creating opportunities for new producers without the risk of causing a

surplus. As a result of increasing exposure to international competition, producers have to become innovative and productive. However, without a well-developed research service, the cotton industry will not be able to survive growing international competition.

There is a growing need to increase the output of world agriculture if the demands of a rising world population are to be met. The basis for this increase must be improved harvest yields of major crops from existing cultivated land. One practical means of increasing yield is to protect more of what is grown from loss to pests, especially insect pests. Insects are not only responsible for massive direct losses of productivity as a result of their herbivory, but also cause massive indirect losses due to their role as vectors for various plant pathogens. Currently crop protection relies primarily on synthetic chemical pesticides, the basis of a ca. US$ 10 billion per annum global pesticide market (Hilder and Boulter 1999). However, this chemical approach to crop protection is coming under increasing pressure. A good deal of the criticism of the agrochemical industry has an emotive rather than a scientific basis (Taylor 1994), nevertheless the view is now widely held that such agricultural systems are unsustainable. This view is based on huge costs in terms of non-renewable resources; efficiency in terms of the proportion of these resources which actually reach the intended target; the environmentally unacceptable consequences of the preceding criticisms, such as contamination of food chains and water sources and the growing consumer dissatisfaction with the publicly perceived consequences of high input agricultural practices. Total pesticide usage is in the decline worldwide, largely due to major reductions in usage in the European Union (EU) as a result of regulatory and public opinion pressure (Hilder and Boulter 1999).

The industries' preferred solutions to this situation tend to be based on risk reduction, rather than use reduction, e.g. the development of more target-specific compounds with less persistence in the environment and the extension of integrated crop management systems (IPM). The benefits however which have accrued to agriculture from the use of synthetic pesticides should not be belittled, but there is clearly an urgent need to develop partial substitution technologies which would allow a much more limited use of synthetic pesticides and yet provide adequate protection of crops within the sustainable agricultural framework.

Genetically engineering inherent crop resistance to insect pests offers the potential of a user-friendly, environment-friendly and consumer-friendly method of crop protection to meet the demands of sustainable agriculture in the 21st century. Genetic engineering of crops offers the prospect of many advantages; not just widening the potential pool of useful genes but also permitting the introduction of a number of different desirable genes at a single event and reducing the time needed to introgress introduced characters into an elite genetic background. Transgenic crop technology may soon prove to be the most important development in crop protection since the discovery of chemical insecticides. In cotton this technology is on the verge of being widely used. For over 50 years the bacteria, *Bacillus thuringiensis* (Berliner) that contains a gene that produces a toxin against certain insects, has been formulated and is applied as any other insecticide.

The development of insecticides based on the delta-endotoxin protein of *B. thuringiensis* has increased in response to the need for efficacious, environmentally safe and selective pesticides with unique modes of action. Although it is highly selective in that a certain Bt-strain produces a protein that is only toxic to a specific group of insects, advances in formulation and genetic engineering and the discovery of Bt-strains with a broader spectrum of activity have resulted in new microbial products with increased potency and greater stability.

Bt-cotton is one of the first crop protection products arising from this biotechnology. All Bt-cotton plants contain one or more foreign genes derived from the soil-dwelling bacterium, *Bacillus thuringiensis* and are thus transgenic plants. The insertion of the genes from *B. thuringiensis* cause cotton plant cells to produce crystal insecticidal proteins, often referred to as Cry-proteins (Hardee *et al.* 2001). The Cry1A transgene was determined to be present as a single copy by probing a Southern blot of C312/531 genomic DNA (Southern 1975) with a Cry1A DNA probe. The Cry1A chimeric protein which expressed, has been reported as virtually identical to native Cry1Ac, sharing its pest activity spectrum as well as 99% of its amino acid sequence identity (MacIntosh *et al.* 1990; Perlak *et al.* 1990). These insecticidal proteins are effective in killing some of the most injurious caterpillar pests of cotton, such as larvae of the African bollworm (*Helicoverpa armigera* Hübner) in South Africa. When the insect eats these Cry proteins, its own digestive enzymes activate the toxic form of the protein. Cry proteins bind

to specific receptors on the intestinal walls and rupture midget cells (Hardee *et al.* 2001). Susceptible insects stop feeding within a few hours after taking their first bite, and if they have eaten enough toxin, die within 2 or 3 days, without ingesting further food. The Bt-gene, modified for improved expression in cotton, enables the cotton plant to produce this Cry protein. The first varieties of Bt-cotton produced in the United States contained one Cry protein gene - Cry1Ac (Chapters 2 & 3) (Hardee *et al.* 2001).

Bt-cotton allows the producer to use less insecticides during pest management, which in turn decreases the potential damage to non-target organisms. Furthermore, it increases the useful life of pyrethroids and other synthetic toxins, increases the potential of biological pest control agents, reduces pesticide run-off into aquatic ecosystems, reduces adverse effects on humans, reduces labour costs and increases profits (Sachs *et al.* 1996; Matthews 1997; International Cotton Advisory Committee 2000).

Bt or transgenic-cotton is protected from lepidopteran pests until late in the season when the plants cease vegetative growth and begin fruit-set. At this stage cotton plants do not have enough insecticidal protein to protect them against these pests and one or two chemical sprays may be needed. Consequently the complex of other insect pests on cotton still has to be controlled chemically where needed. This however would increase the overall production costs of Bt-cotton. Pests that would need additional chemical control are e.g. cotton aphids, jassids, red spider mites, cotton stainers, thrips and white flies (Hardee *et al.* 2001)

Generally the biggest disadvantage in the production of Bt-cotton is the cost of the seed and technology fee (which is the cost of the patent). This disadvantage is nevertheless outweighed by the benefits listed above (Kirsten & Gouse 2002; (Cotton SA 2003; Http://www.cottonsa.org.za (March 2004)).

However, as with all registered insecticides, there is the threat of the development of resistance. Development of insect resistance to the endotoxin proteins of Bt is an issue of intense contemporary concern among farmers and agricultural policy makers. In South Africa synthetic

pyrethroid resistance occurs in the African bollworm and was investigated during the period of 1992 to 1995 by researchers at the Agricultural Research Council, Plant Protection Research Institute, Pretoria, South Africa, (Van Jaarsveld *et al.* 1998). These results underscore the need for a management programme to prevent resistance development against Bt. Regardless of any management strategy, base-line data are essential to monitor possible changes in population sensitivity to Bt-based products.

1.1 ECOLOGY OF *HELICOVERPA ARMIGERA* AND GENERAL FEEDING BEHAVIOUR PATTERNS ON COTTON

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a typical noctuid whose larvae feed on a wide range of plants and has been identified as the most polyphagous and injurious pest in South Africa (Annecke & Moran 1982; Van der Walt 1988). In many parts of the world *H. armigera* is a key pest of cotton and is no less so in South Africa. Consequently, it has been the subject of considerable study and significant progress has been made in the development of integrated pest management strategies for cotton in South Africa (Mumford & Van Hamburg 1985). The vernacular name "American bollworm" for *H. armigera* is an unfortunate misnomer which became entrenched because it was initially misidentified as a conspecific of the North American *Heliothis zea* (Boddie). *Helicoverpa armigera* occurs in the Old World, especially in Africa, Australia and India, but not in the new World, while the reverse is true for *H. zea* and the other important North American species *Heliothis virescens* (Fabricus) (Pearson & Maxwell Darling 1958; Annecke & Moran 1982; Van der Walt 1988).

For a long time spray thresholds for *H. armigera* were based on surveys of the eggs in the field, but egg counts were found to be an unreliable criterion, due to high mortality rates of egg populations and larval counts were shown to be more reliable indicators of the pest (Kfir & Van Hamburg 1983). The current spray threshold is set at 5 larvae/ 24 plants and 12 eggs/ 24 plants, with larvae from about the third instar counted as two (Basson 1986; Nel *et al.* 2002). The shift to *H. armigera* larvae as the key stage for the implementation of control programmes dictated a need for a better understanding of the biology of the larvae, particularly a description of the feeding habits of the larvae and the damage they cause to cotton under field conditions.

The adults are typical noctuid moths with nocturnal habits of flight, mating and oviposition. The female moth is stout bodied. A reddish-brown colour and somewhat bigger than the more greenish males (Jayaraj 1982; Van der Walt 1988). The number of eggs laid per female in the laboratory culture averaged about 1400 (Van der Walt *et al.* 1993). In the field, eggs are laid singly on plants, mainly during the early evening (Taylor 1982). On cotton, eggs are laid mainly on leaves, with preference for young leaves when the plants are still young, but favouring the

fruiting forms in older plants (Mabbett & Nachapong 1984). Most eggs are laid on the top third of the plants (Basson 1987). The oviposition sites bear no obvious relationship to the plant parts which are fed on by the first instar larvae. The larvae are full-grown after about 14 - 18 days and burrow into the soil where they overwinter or pupate, the pupal stage lasting about 11 - 14 days (Van der Walt 1988). Mature *H. armigera* larvae vary widely in colour from shades of yellow-green, pink to brown to almost black (Taylor 1982). They are recognised by a fine white mid-dorsal line, edged with black, as well as lateral markings, including a creamy white stigmatal line. Five larval instars were found by rearing *H. armigera* in the laboratory, on an artificial diet, with the larval instars lasted about 3.5, 2.5, 2.1, 2.7 and 2.5 days respectively (Van der Walt 1988; Van der Walt *et al.* 1993).

The distribution of *H. armigera* on cotton plants is influenced by the distribution of fruiting forms and the distances moved by the larvae (Van der Walt 1988; Wilson *et al.* 1982). Plant density and the time of season are the two key factors which not only determine the state of plant growth, but also influence the feeding pattern within-plant distribution. The larvae mainly occur in the upper third or at least in the upper two-thirds of cotton plants (Van der Walt 1988). Younger larvae up to the third instar, feed mainly in the upper third of the plant and the older larvae, (fourth and fifth instars), feed between the upper two thirds of the cotton plants. Only a very small proportion of larvae fed in the lower third. For most of the season the larvae remain in the upper two-thirds of the plants. Van der Walt (1988) also investigated the movement of larvae within a cotton plant and found that as most of the eggs are laid in the upper third of cotton plants and up to 90 % on the younger leaves, the majority of newly-hatched larvae move to the stem terminal bud, thereby increasing their chances of finding suitable food. Apart from the obvious fact that it would take small larva much longer to move a given distance than it would take a larger one, the limited movement clearly indicates that the smaller larvae tended to stay on the fruiting forms which they encountered initially. The first two larval instars moved between closely grouped feeding sites at the branch tips, whereas the later instars also fed on fruiting forms more widely separated on the branches, even moving between branches. Movement of *H. armigera* larvae between adjacent plants is also known to occur (Mabbett *et al.* 1980). Annecke & Moran (1982) stated that *H. armigera* larvae have a "...catholic taste in food plants, preferring flower buds, flowers and developing fruits...". Van der Walt (1988) also found that all *H. armigera* larval instars preferred fruiting forms over leaves and that the number of leaves fed on

was negligible and seldom amounted to more than “nibbling”. Apparently the larvae used the leaves mainly to move between fruiting forms.

Once thought to be identical with *Heliothis zea* (Boddie), the corn-ear worm or cotton bollworm of the new world - thus, the firmly established misnomer, American bollworm - *Heliothis armigera* (now *Helicoverpa armigera*) 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. However, it is probably not indigenous to southern Africa (Annecke & Moran 1982).

Because of its wide distribution and very catholic taste in food plants, preferring flower buds, flowers and developing fruits, the African bollworm is probably the most polyphagous and injurious pest of agriculture and home gardens in South Africa.

1.2 CONTROL STRATEGIES OF *HELICOVERPA ARMIGERA* OVER THE PAST TWENTY YEARS

There has been a noticeable tendency in South Africa since 1982 to reduce the number of insecticidal applications each season, following a research programme initiated by S.W. Broodryk during 1982 (ARC-PPRI, Annual Report). Instead of applying pesticides by rote according to the calender, attempts were made to spray only when populations of the key pest, African bollworm, were sufficiently high to pose a real threat to cotton and only when the plants had reached a state of growth at which the injury sustained would be reflected in the yield. To achieve this, scouts were trained to search for and count bollworm eggs on plants according to a carefully worked out sampling technique. The decision to use an insecticide was made when a certain threshold number of eggs per plant was exceeded. By this method it proved possible to reduce the number of insecticidal applications in the Groblersdal area over a few years from as many as 16 to as few as six to eight, with no loss in yield (Cotton SA 2003; Http://www.cottonsa.org.za (March 2003)).

During the period that the research was done, it was noticed that many bollworm eggs produced parasitoids instead of larvae, and that many young bollworm larvae perished from various causes, suggesting that a still greater number of eggs per plant could be safely tolerated. But the rate of parasitism and the mortality rate of the larvae varied greatly between 0 and 50 % and even higher, so that no simple correction factor could be applied. Instead, research was based on scouting for bollworm larvae instead of eggs, and new threshold injury levels were tested and set. The yield from two seasons when this technique was used strongly suggest that three or four insecticidal applications per season may often be sufficient to produce a satisfactory cotton crop (Annecke & Moran 1982).

The chemical registrations for the control of *Helicoverpa armigera* on cotton in South Africa are revealed in Table 1.1 (Bot *et al.* 1988; Vermeulen *et al.* 1990; Vermeulen *et al.* 1992; Nel *et al.* 1993; Krause *et al.* 1996; Nel *et al.* 1999; Nel *et al.* 2002). The application of the registered products are based on: (1) the checking/scouting of 24 plants at random in a 1 - 15 ha cotton field, weekly from 6 weeks after plant emergence. Apply a registered product when economic threshold is reached or exceeded (threshold value of 5 larvae/24 plants), (2) checking/scouting of eggs per plant, apply a registered product if 12 eggs/24 plants are exceeded.

Table 1.1 Chemical products registered during 1988, 1990, 1992, 1993, 1996, 1999 and 2002 for the control of *Helicoverpa armigera* on cotton in South Africa.

PESTICIDE / ACTIVE INGREDIENT	FORMULATION		CHEMICAL CLASS	REGISTRATION YEAR	APPLICATION DIRECTIONS Unless otherwise indicated - directions are for high volume application
	TYPE	GRAMS ACTIVE INGREDIENT			
alphamethrin	EC SC	100 g/ℓ 100 g/ℓ	pyrethroid	1988, 1990 *	Apply from flowering peak (10 - 12 weeks after plant emergence) to first boll split. Weekly applications are desirable up to the 18th week. Preventative treatment: weekly programme and fortnightly programme. Ground application: apply 200 ℓ spray mix/ha. Aerial application: apply in 30 ℓ water. Corrective treatment: when egg threshold is exceeded. Ground application: apply 200 ℓ spray mix/ha. Aerial application: apply in 30 ℓ water.
alphacypermethrin	EC SC UL	100 g/ℓ 100 g/ℓ 2.5 g/ℓ	pyrethroid	1992, 1993, 1996, 1999, 2002 1992, 1993, 1996, 1999, 2002	Apply from flowering peak (10 - 12 weeks after plant emergence) to first boll split. Weekly applications are desirable up to the 18th week. Preventative treatment: weekly programme and fortnightly programme. Ground application: apply 200 ℓ spray mix/ha. Aerial application: apply in 30 ℓ water. Corrective treatment: when egg threshold is exceeded. Ground application: apply 200 ℓ spray mix/ha. Aerial application: apply in 30 ℓ water. Preventive treatment Ground application: apply by means of Micronair model AU 5000 atomisers only.
Bacillus thuringiensis var *kurstaki*	SC	17 500 IU/ mℓ	bacterium	2002	Preventative treatment: apply when egg and larvae thresholds are reached and before larvae reach a length of 7 mm. To be applied only before square formation.

PESTICIDE / ACTIVE INGREDIENT	FORMULATION		CHEMICAL CLASS	REGISTRATION YEAR	APPLICATION DIRECTIONS Unless otherwise indicated - directions are for high volume application
	TYPE	GRAMS ACTIVE INGREDIENT			
beta-cyfluthrin	EC EC	25 g/ℓ 50 g/ℓ	pyrethroid	1992, 1993 * 1996, 1999, 2002	Apply as for alphamethrin. Use lower spray volumes on plants smaller than 60 cm.
beta-cypermethrin	EC	100 g/ℓ	pyrethroid	2002	Apply as for alphacypermethrin.
bifenthrin	EC	100 g/ℓ	pyrethroid	1988, 1990, 1992, 1993, 1996, 1999, 2002	Apply as for alphamethrin.
cyfluthrin	EC	50 g/ℓ	pyrethroid	1988, 1990, 1992, 1993, 1996 *	Apply as for alphamethrin. Use lower spray volumes on plants smaller than 60 cm.
cypermethrin	EC	200 g/ℓ	pyrethroid	1988, 1990, 1992, 1993, 1996, 1999, 2002	Apply as for alphamethrin.
cypermethrin-high cis	EC	200 g/ℓ	pyrethroid	1988, 1990, 1992, 1993, 1996, 1999 *	Apply as for alphamethrin.
deltametrin	EC TB	25 g/ℓ 0.5 g/tablet	pyrethroid	1988, 1990, 1992, 1993, 1996, 1999,2002 1996, 1999, 2002	Apply as for alphamethrin. Apply as for alphamethrin. Preventative treatment: weekly programme. Ground: dosage rate depends on infestation level.
deltamethrin/endosulfan	SC	2.5/475 g/ℓ	pyrethroid/organochlorine	1999 *	Preventative treatment: Ground and Aerial: dosage rate depends on plant height. Apply only in the period 1 January - 28 February. Ensure good coverage of upper third of the plants.

PESTICIDE / ACTIVE INGREDIENT	FORMULATION		CHEMICAL CLASS	REGISTRATION YEAR	APPLICATION DIRECTIONS Unless otherwise indicated - directions are for high volume application
	TYPE	GRAMS ACTIVE INGREDIENT			
endosulfan	WP	475 g/kg	organochlorine	1988, 1990, 1992, 1993, 1996, 1999, 2002	LV. Ground application. Aerial: in 30 ℓ of water.
	EC	350 g/ℓ		1988, 1990, 1992, 1993, 1996, 1999, 2002 1988, 1990, 1992, 1993, 1996, 1999, 2002	LV. Ground application. Aerial: in 30 ℓ of water.
	ULV / UL	250 g/ℓ		1988, 1990, 1992, 1993, 1996, 1999, 2002	**Aerial application only:** Dosage depends on plant size.
	SC	475 g/ℓ			Apply as for alphamethrin.
esfenvalerate	EC	50 g/ℓ	pyrethroid	1988, 1990, 1992, 1993, 1996, 1999, 2002	Apply as for alphamethrin.
	EC	200 g/ℓ		2002	Apply as for alphamethrin.
fenvalerate	EC	200 g/ℓ	pyrethroid	1988, 1992, 1996, 1999, 2002	Apply as for alphamethrin.
fluvalinate	EC	240 g/ℓ	pyrethroid	1988, 1990 *	Apply as for alphamethrin.
indoxacarb	SC	150 g/ℓ	oxadiazine	2002	Preventative treatment: weekly spray programme. Do not apply more than 5 times/season. Addition of wetter/sticker is recommended.
permethrin	EC	500 g/ℓ	pyrethroid	1988, 1990, 1992, 1993, 1996, 1999, 2002	Apply as for alphamethrin.

PESTICIDE / ACTIVE INGREDIENT	FORMULATION		CLASS	REGISTRATION YEAR	APPLICATION DIRECTIONS Unless otherwise indicated - directions are for high volume application
	TYPE	GRAMS ACTIVE INGREDIENT			
profenofos during 2002 referred to also as profenofos (premium grade)	EC	500 g/ℓ	organo-phosphorus	1988, 1990, 1992, 1993, 1996, 1999, 2002	Apply appending on plant size in 100-200 ℓ water/ha on a weekly basis or according to scouting information. Apply as for alphamethrin.
tau-fluvalinate	EC	240 g/ℓ	pyrethroid	1992, 1993, 1996 *	Apply as for alphamethrin.
tau-fluvalinate/thiometon	EC	72/200 g/ℓ	pyrethroid/organophosphorus	1993, 1996 *	Ground application in at least 200 ℓ water.
thiodicarb	SC	375 g/ℓ	oxime carbamate	1988, 1990, 1992, 1993, 1996, 1999, 2002	Apply as for alphamethrin.
tralomethrin	EC	36 g/ℓ	pyrethroid	1992, 1993, 1996, 1999, 2002	Apply as for alphamethrin.
zeta-cypermethrin	EW	100 g/ℓ	pyrethroid	2002	Apply as for alphacypermethrin.

***** This product is no longer registered for the control of *H. armigera* on cotton in South Africa.

Basson (1987) showed that valid agricultural practices reduced pest control costs and prevented the development of pesticide resistance. This programme was based on integrated control strategies which exploited the natural enemies of the bollworm to the full, but did not pretend to be the ultimate solution to cotton pest control. Depending on further experience and the introduction of new pesticides, further adjustments and modifications need to be made. This programme deviate slightly from the recommendations on pesticide labels. Effective pest control in cotton is based on the following practices: (1) scouting for the number of bollworm present in the field; (2) choice of the correct pesticide; (3) the planning and implementation of an effective spraying strategy. It is usually not necessary to apply insecticides within the first 8 weeks of emergence of the cotton seedlings. Scouting should commence at seedling emergence and intensify from 6 weeks after emergence. Scouting should be done at least once a week, although twice-weekly inspections would be the ideal, until the end of the growing season - about 20 weeks after seedling emergence. The economic threshold level set during this time was an average of 5 larvae per 24 plants (larvae smaller than 5 mm ignored). From plant emergence to 7 weeks (i.e. to first flowering stage); no control for African bollworm is necessary. Seven weeks to 11 weeks after plant emergence (first flowering stage to peak flowering stage); chemical control usually commences during this stage and it is especially important not to use synthetic pyrethroids or pesticides that will stimulate red spider mite populations. Twelve weeks to 20 weeks after plant emergence (peak flowering to start of boll burst stage); is the important phase in the development of cotton. Synthetic pyrethroid sprays should be limited to a maximum of 2 - 3 sprays during this period. Twenty one weeks to harvesting (after first boll burst to harvesting), usually requires no pest control.

Van Jaarsveld *et al.* (1998), published results of, *H. armigera* resistance, to different synthetic pyrethroids, of different field populations in South Africa. Following these results the Resistance Working Group of the Agricultural and Veterinary Chemical Association of South Africa (AVCASA) asked the Registrar of Act 36 of 1947 to stipulate an additional clause on the labels of all synthetic pyrethroid pesticides registered for controlling *H. armigera* in South Africa (Farmer's Weekly 1994). The following modifications/additions were implemented on all synthetic pyrethroid labels with effect 1995: (1) synthetic pyrethroids may only be applied to cotton during the period January 1 to February 28, (2) they may not be applied to other crops more than twice in a growing season, (3) if pyrethroids gives poor control, the crop may not be

resprayed with another pyrethroid, not even at a correct dosage. Instead, a product from another chemical group must be sprayed (Table 1.1). By following this regulations, the selection pressure favouring the onset of resistance development, will be reduced - not only in *H. armigera* but in other pests as well.

1.3 CONVENTIONAL *BACILLUS THURINGIENSIS* PESTICIDES

The biological insecticide *B. thuringiensis* has been used for more than 50 years to control lepidopteran pests on a variety of crops. Strains of the bacteria are also used for control of certain Diptera (Burges 1982) and Coleoptera (Zehnder & Gelernter 1989). The development of insecticides based on the delta-endotoxin protein of *B. thuringiensis* has increased in response to the need for efficacious, environmentally safe and selective pesticides with unique modes of action. Advances in formulation and genetic engineering, and the discovery of strains with a broader spectrum of activity, have resulted in new microbial products with increased potency and greater stability. In addition, crops such as cotton (Perlak *et al.* 1990), tomato (Fischhoff *et al.* 1987), and tobacco (Stone & Sims 1993) was genetically modified to express many types of *B. thuringiensis* delta-endotoxins.

Bio-insecticides like Bt that are sprayed on crops may perform as well as synthetic insecticides in very limited situations, but the performance of Bt-insecticides has been inconsistent in many instances. The erratic performance in cotton is attributed to four reasons, the toxin is rapidly degraded by ultraviolet light, heat, high leaf pH, or desiccation; larvae must eat enough treated plant tissue to get a lethal dose of toxin; since the toxin has no contact effect, the sites where bollworms feed are difficult to cover with the foliar-applied sprays; and Bt Cry-proteins are less toxic to older larvae.

1.4 TRANSGENIC COTTON: AN HISTORICAL OVERVIEW

Since the first reports of transgenic plants appeared in 1984 (Horst *et al.* 1984), there has been very rapid progress directed at using this new technology for the practical ends of crop protection. Genetically engineered cotton was introduced commercially in 1996 and since then has been widely adopted in the cotton growing industry, e.g. by 1999/2000 12 % of global cotton fields consisted of transgenic cotton (International Cotton Advisory Committee, 2000). The first commercially available Bt-cotton, INGARD (Australia) and BOLLGARD (United States), were released in 1996 (Olsen & Daly 2000).

The gene that produces these protein crystals was isolated in the 1980's by MONSANTO (Pty) Ltd. and was transferred into the genome of a cotton plant with the result that all the cells of that plant produce its own protein insecticide. The CryIA gene was found to be the most appropriate for use in cotton. It will adequately kill the vast majority of the major lepidopteran pests on cotton during the major part of a season. The pests include the African bollworm, the Spiny bollworm (*Earias biplaga* (Walker)), the Red bollworm (*Diparopsis castanea* (Hampson)), Plusia looper (*Anomis flava* (Fabricius)), Lesser armyworm (*Spodoptera exigua* (Hübner)) and the Cluster caterpillar (*Spodoptera littura* (Fabricius)). These are all pests on cotton in South Africa. Importantly non-target organisms are not influenced when they fed on transgenic-cotton (Sachs *et al.* 1996).

A cotton plant modified to produce Cry-protein within the plant tissues that larvae feed on, overcomes most of the limitations, mentioned above. The plant-produced Bt-proteins are protected from rapid environmental degradation since they are not directly exposed to the environment and hence ultra violet light.

Different Bt-strains produce different Cry-proteins and there are hundreds of known strains. Most Cry-proteins are active against specific groups of insects, like the lepidopteran species *S. littoralis* attacking cotton in South Africa . However, resistance remains a problem and a variety of factors may influence the rate at which bollworms become resistant to Cry-toxin in Bt-cotton.

These factors include: (1) the number of generations of bollworms exposed each year to Bt-plants containing the same or similar toxins; (2) the percentage of each generation exposed to Bt-plants containing the same or similar Cry-toxins, the mortality level that Cry-toxin causes among bollworms carrying one copy of a resistance allele and one copy of a susceptible allele (the mortality level is determined by the Cry-toxin concentration in the plant, which in turn may determine the functional dominance of the allele affecting resistance); (3) the frequence with which Cry-resistance alleles are expressed in the bollworm population before exposure to Cry-toxins and the dominant or recessive nature of the resistance alleles; (4) the migration patterns of bollworm moths; (5) the survival advantage or disadvantage that resistance allele(s) offer bollworms in the presence and absence of Cry-toxins; (6) the number of susceptible moths available for mating with moths carrying resistance gene(s).

Regardless of any resistance management strategy, resistance monitoring, or susceptibility and effective monitoring is an activity generally accepted as an integral tool to measure successful, Bt-cotton cultivation.

1.5 TRANSGENIC COTTON IN SOUTH AFRICA

Cotton contributes around US$ 50 million annually to the national income of South Africa. Approximately 100,000 hectares are planted to cotton, mostly in hot, dry areas of the country. These farms are planted by about 1,500 commercial scale and 3,600 smallholder farmers (Cotton SA 2003) (Table 1.2).

Cotton has been a major crop for resource poor farmers in the semi-arid Makhatini Flats, for over 40 years. Since passing the South African GMO Act (Genetic Modified Organisms Act) of 1997, and while the rest of Africa and other developing countries continued debating about biotechnology, these smallholder farmers began planting genetically enhanced Bt-cotton.

As part of the South African government strategy for an export-driven economy, small-scale farmers are now strongly encouraged to produce cash crops such as cotton. Nearly 98% of the

smallholder cotton production in South Africa is from approximately 3,000 small-scale farmers in Makhatini Flats, KwaZulu-Natal Province and 500 in Tonga, Mpumalanga Province.

Since 1998, smallholder farmers in Makhatini Flats have been farming a genetically modified cottonseed variety, NuCOTN 37-B with Bollgard. This variety contains the Bt-gene that confers resistance to bollworm and reduces the need for insecticides to control this pest complex. Adoption of the Bt-variety has been rapid. In 1998/99 there were 75 farmers, growing less than 200 ha of Bt-cotton. In 1999/00, this rose to 411 farmers with little under 700 ha, and in 2000/01, to 1 184 farmers with about 1 900 ha. Thus, in only three years, 60% of the cotton producers in Makhatini, representing almost two thirds of the area, have adopted Bt (Bennett *et al.* 2003). In three seasons (1998/99, 1999/00 and 2000/01), there has been a reduction in the average number of sprays per season (from 11 to four sprays) for Bt-adopters. Cotton farmers on the Makhatini Flats who switched to Bt cotton in 2002 increased their yield from 640 kg/ha to 980 kg/ha, 53 % more cotton (Buthelezi 2003). This earned them an additional income of R 1,190/ha plus a saving of R 430 on spraying cost/ha. Their total income from dryland cotton was R 1,620/ha (US$ 1=ZAR6.39 (2003)).

Managing crops is easier and the ongoing risk of bollworm attacks, the major pest of cotton, has been drastically reduced. This will inevitably affect costs. Bt-cotton farmers have benefited from lower production cost due to less pesticide and labour costs, as well as a significant yield increase, between 27 - 48 %. Although the seeds cost more, the lower production cost, combined with higher yield, provides the farmer with higher gross margins of an average US$ 50 per hectare (Cotton SA 2003; Http://www.cottonsa.org.za (March 2003)).

Rapid adoption of Bt-cotton by 92 % of small holders for the 2002/2003 season has resulted from the sharing of positive communication by word of mouth. A reduction in labour and pesticide inputs coupled with higher yields and gross margins, have already assisted in improving farmers' lifestyles in the especially the Makhatini Flats. These emerging observations, along with first-hand experiences, provides potential users of this technology, especially in developing countries, with useful information to enable a rational choice of adopting or not adopting this new technology.

Table 1.2 Cotton production and seed production (yield) in South Africa between 1992/93 and 2002/03.

MARKETING YEAR	UNDER IRRIGATION (ha)	DRYLAND (ha)	AVERAGE YIELD (kg seed per ha)
1992/93	19 048	28 711	1 065
1993/94	7 240	27 886	862
1994/95	11 258	55 941	974
1995/96	19 038	35 096	1 037
1996/97	17 609	72 809	1 160
1997/98	15 954	67 017	746
1998/99	20 361	69 578	1 065
1999/00	31 263	67 356	1 222
2000/01	10 486	40 282	1 258
2001/02	18 539	38 153	1 529
2002/03	9 791	28 897	1 280

The number of hectares cotton planted each year in South Africa, depends largely on the availability of financing and the cotton price. The main reason for the decrease in number of hectares cotton planted during the 2002/2003 cotton season, is mainly due to the poor estimated prospects during planting time. Many cotton hectares (irrigation and dry land) have been planted to maize, wheat and sunflower due to more attractive returns from these crops in relation to cotton. The cotton industry has re-affirmed it's objective for 30 % of the domestic crop to be derived from emerging farmers by the year 2005, as cotton growing lends itself ideally to cultivation by small-holders and could play an important role to settle and enlarge the developing sector in rural areas.

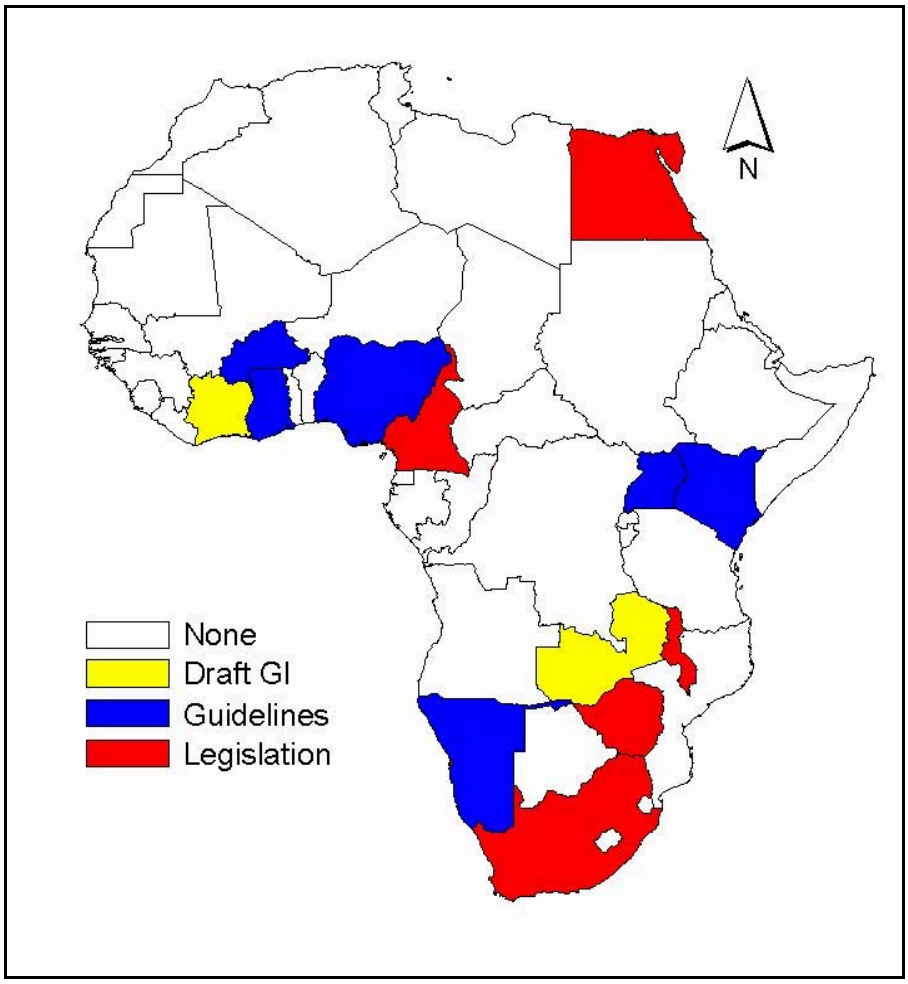


Figure 1.2 Legislation, guidelines and drafts with reference to the cultivation of Bt-cotton in Africa.

The “Cartagena Protocol on Biosafety to the Convention on Biological Diversity”, which aims to regulate trade in genetically modified organisms (GMO’s), came into force on 11 September 2003 after five-year-long negotiations over trade advantages and disadvantages. In accordance with the precautionary approach contained in Principle 15 of the Rio Declaration on Environment and Development, the objective of the Protocol is to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focussing on transboundary movements. At the moment, there is no international law

other than the Cartagena Protocol to govern the environmental impacts of GMO's (Science in Africa 2003, Http://www.scienceinafrica.co.za (November 2003)).

When it comes to implementing and regulating the Protocol, however, developing nations are faced with all kinds of handicaps - for a variety of reasons. For instance, the Protocol depends on full information for its effective implementation - it requires a labelling and traceability regime too be negotiated once it comes into force. The poverty of developing countries, especially the least developed among them, mostly in Africa, remains a crucial handicap: they are simply to poor to allocate adequate resources for biosafety. Even more worrying is the fact that, should a risk occur, these countries will find it hard to muster the financial and technical capacity needed to combat it. Risk assessment in the South also becomes complicated because of the complex tropical and subtropical environments. A micro-organism under contained use functions optimally at high temperatures. If it escapes into the open environment in the North, it is unlikely to survive the winter cold. But in the hot tropical and subtropical environments of the South, it may survive and flourish indefinitely. The South should, therefore, put in place biosafety systems that restrict contained use only to laboratory conditions from which escape of GMO's is impossible (Fig. 1.2).

A more intractable issue, of course, is trade and environment. Trade rules favour the North, and the international agreement on Trade-related Aspects of Intellectual Property Rights - or TRIP's- makes GMO's especially problematic for the South. TRIP's puts the burden of proof of innocence on the person accused of the infringement of a process patent. This could spell trouble when a GMO cross-pollinates with the unmodified crop of a smallholder farmer and his crop becomes contaminated by patented genes. Absurdly, the farmer is assumed to be a process patent infringer. The culprits - the wind and insects - cannot be summoned to court as witnesses. However, at the insistence of the South, there is now a commitment to negotiate a liability and redness regime under the Protocol in case of damages caused by GMO's (Biosafety 2003, Http://www.biodiv.org/biosafety/faqs.asp (November 2003)).

According to Green *et al.* (2003), a purchaser of Bollgard™ (Bt cotton) in South Africa is under obligation to sign a license agreement, stating that for every 100 ha of Bollgard cotton planted,

a refuge of either 20 ha sprayed non-transgenic cotton, or 5 ha unsprayed non-transgenic cotton will be planted. The small-scale farmers on the Makhatini Flats, however, may neglect planting this refuge and a possible 'alternative' that could act as a refuge area. However the possibility that genes introduced by genetic engineering may "escape" (be transferred via pollen) to wild or weedy related species growing nearby becomes now cited as one of the major risks of GMO's. Gene flow between crops and wild species from which they derived, however, is a well-documented natural phenomenon (Traynor 2002).Through this long-established mechanism for gene transfer, any gene in a cultivated crop of plant, irrespective of how it got there, can be transferred to it's wild or semi-domesticated relatives. Some other concerns regarding the safety of new proteins expressed in transgenic plants are the unintended, deleterious effect on other organisms including birds, insects, browsing animals, and soil organisms in the local environment. This however is a Biosafety Risk Assessment and inherently a critical component for future studies.

1.6 RESISTANCE MANAGEMENT IN AUSTRALIA

Conventional Bt has been used in Australian cotton for the control of *Helicoverpa* spp. since 1989 (Forrester 1994). It started with the use of less than 10,000 litres and increased to 200,000 litres by 1994, but still represents only about 0.5 of a spray in cotton which normally receives 6 to 8 conventional insecticide sprays for *Helicoverpa* control each season.

The differential resistance risk of *H. armigera* and *H. punctigera,* in Australia has clearly indicated the critical importance of ecology in resistance management. These two sibling species have similar biochemical capabilities for metabolic detoxification of xenobiotics (Forrester *et al.* 1993) but there has been no recorded resistance to any insecticide in *H. punctigera* despite *H. armigera* having developed resistance to virtually every insecticide used against it in any quantity. Forrester *et al.* (1993) indicated that this difference is probably due to the highly migratory, polyphagous nature of *H. punctigera* compared to the relatively oligophagous and facultatively migratory *H. armigera*. It is suggested that the large pool of unsprayed susceptible *H. punctigera* is so vast that it effectively swamps any resistance which develops in the intensively sprayed cropping areas. In other words; *H. punctigera* effectively 'manages its own

resistance'. A possible resistance management strategy for *H. armigera* on transgenic or Bt-cotton, would rely on continual dilution of rare resistant mutants by large numbers of unsprayed susceptibles allowed to breed in designated refugia areas. This high dose/high immigration resistance management approach would essentially maintain resistant *H. armigera* individuals as rare, functionally recessive heterozygotes. Two other factors were critical for the success of this strategy: (1) strains of conventional Bt with different toxin profiles (e.g. *Bt* subsp. *aizawai*) were used on non-transgenic alternative crop hosts of *H. armigera* (e.g. sorghum, sunflowers, grain legumes, maize, tomatoes, oilseeds) and (2) development of transgenic alternative host crops of *H. armigera* (especially sorghum and maize) should concentrate on toxins other than those from Bt. The successful Australian IRM (Insect Resistance Management) strategy for summer field crops has become a model for the resistance management of *Helicoverpa* spp. (Forrester *et al.* 1993). The success of the strategy for conventional insecticides has left a legacy of confidence and credibility which can be tapped for any future resistance management efforts centred on Bt and/or transgenic cotton (Forrester 1994).

According to Benson's (1971) hypothesis for the management of resistance through the large scale release of susceptible insects into the pest population, even if it meant 'sacrificing some of our food to the right insects, those with susceptible genotypes', is the only long-term solution to IRM as it 'controlled the evolution of pest species'. It is important to note that Benson's hypothesis and the example of nature's own highly successful IRM strategy for *H. punctigera* have remarkable parallels with Roush (1994b) suggestion for a resistance management strategy for transgenic-cotton. Roush (1994a) suggests a high dose/refugia resistance management approach which essentially maintains resistant *H. armigera* individuals as rare, recessive heterozygotes and that the small refugia area would act as a dilution source for any resistant individuals which survive on the transgenic cotton. For this strategy to work the concentration of toxin in the plant should be high enough to kill most heterozygotes and the refugia should be both temporally and spatially contiguous with the transgenic-crop. Cotton is the ideal refugium crop as it remains an attractive host for the same period as the transgenic-crop. Therefore, growers should leave a small refugium area on each farm. The viability of this technique depends on the high level of toxin expression in the plant and is critical for the long-term viability (Forrester 1994).

The current recommended resistance management strategy for Bt-crops is a high expression (dose)-refuge strategy (Gould 1998; Shelton *et al.* 2000). This strategy is designed to work best where resistance is due to a single recessive gene and expression of toxin is high enough to kill all or nearly all heterozygotes. The refugia are composed of non-transgenic plants that will support sufficient homozygote susceptible insects to outnumber and breed with resistant insects.

1.7 AIMS OF STUDY

As the agronomic use of *B. thuringiensis* products increases, appropriate management programmes are important to ensure their efficacious long-term use. The type of resistance that evolves to Bt can be quite distinct, depending upon the species, selection regime, or geographical origin of the founder colony (Heckel 1994, Tang *et al.* 1997). It is therefore important to take initial surveys of the insect susceptibility to Bt to establish a baseline for monitoring possible changes in population sensitivity to *Bacillus thuringiensis* based products.

MONSANTO (Pty) Ltd., one of the world leaders in the development of transgenic plants, approached the Plant Protection Research Institute of the Agricultural Research Council, to assess the natural variability of the African bollworm in susceptible response to Bt in different cotton production areas in South Africa. Therefore, the objectives of this study were to sample field populations of *H. armigera* in different geographical areas in South Africa, the mass rearing of the different field populations, the evaluation of the field strains together with a susceptible laboratory strain to determine baseline concentrations of the purified endotoxin Cry1Ac (Chapter 2), incorporated in the artificial diet and the determination of a candidate diagnostic concentration (Chapter 2). The above mentioned objectives were performed during the 1998 cotton season to determine baseline dose-concentrations where a mortality range of between 20 - 90 % was obtained. This dose-range formed the background for repeated studies during the 2000 and 2003 cotton growing seasons in South Africa. With this information a background for the implementing of appropriate resistance management and product use strategies is discussed in Chapter 4.

An extension of the study involves the evaluation of different transgenic-cotton strains for the control of *H. armigera* and *Spodoptera littoralis* when exposed not only to artificial diet with incorporated *Bacillus thuringiensis* protein, but to different Bollgard cotton cultivars which contain different strains of *B. thuringiensis*, cultivated in the field and the expression of Bt-gene's in different plant parts (Chapter 3). Future management strategies are also discussed in Chapter 4.

CHAPTER 2

GEOGRAPHIC VARIABILITY IN THE SENSITIVITY OF *HELICOVERPA ARMIGERA* TO BT INSECTICIDAL PROTEIN CRY1Ac

2.1 INTRODUCTION

During the 1998 - 1999 cotton season, the Plant Protection Research Institute of the Agricultural Research Council was contracted by Monsanto to perform baseline laboratory studies to determine the geographic variability in the sensitivity of *H. armigera* to Bt-insecticidal protein Cry1Ac. This was performed to establish a geographic baseline for comparison with future population responses to the increased Bt-cotton cultivation in South Africa. As any kind of chemical insecticides, the value of Bt could be seriously diminished by widespread development of insect resistance to Bt-toxins. Recently, several common species of pest insects have evolved resistance to Bt, indicating that biological pesticides can suffer the same fate as chemical pesticides (Gould *et al.* 1995). The type of resistance that evolves to Bt can be quite distinct, depending upon the species, selection regime, or geographical origin of the founder colony (Heckel 1994). Therefore, it is important to take an initial survey of the insect susceptibility to Bt to establish a baseline for monitoring possible changes in the future. Similar studies were conducted by (Wu & Guo 1997) in China, (Stone & Sims 1993) in different states in the USA, (Luttrell & Knighten 1999) in the southern and western states of the U.S. Cotton Belt, (Wu *et al.* 2002) in northern China and (Akhurst *et al.* 2003) in Australia.

This study form the background for implementing appropriate resistance management and product use strategies in agriculture, especially in cotton cultivation in South Africa. Reported here are results of the dynamics of *H. armigera* susceptibilities to Cry1Ac during the 1998, 2000 and 2003 seasons.

2.2 MATERIALS AND METHODS

2.2.1 GEOGRAPHIC SAMPLING

Five different field populations of *H. armigera*, were sampled in different geographical areas in South Africa, during the 1998, 2000 and 2003 cotton seasons (Table 2.1). Larvae (instar 4 - 5) were collected in glass vials (10 cm x 1.5 cm) containing an artificial diet (Shorey & Hale 1965), which were sealed by an absorbent cotton plug. Vials were kept in cooler boxes, within a temperature range of 9 - 13 ^{0}C, during transport to the ARC-PPRI, Rietondale Campus.

No sampling took place in Weipe during the 1997/1998 cotton season, no sampling of Makhatini population in 1999/2000 cotton season and no sampling of the Roedtan population during 2002/2003 cotton season. The reason for the sampling actions not performed were due to lack of a suitable number of specimens in the natural populations available in the field.

The identity of randomly selected adults from the different field populations, were verified by the Biosystematics Division of ARC-PPRI and accessioned in the National Collection of Insects in Pretoria, South Africa. Approximately 360 specimens of *H. armigera* larvae were collected in each area, with most specimens being in the fourth or fifth instar of developmental stage.

Table 2.1 Field populations of *Helicoverpa armigera* sampled (±360 in each area) in different geographical areas in South Africa and tested for susceptibility to the purified endotoxin MVP II Cry1Ac. *Helicoverpa armigera* specimens were also sampled on different host plants during the study period.

LOCALITY	GPS COORDINATES	SAMPLING DATES					
		1998	Host Plant	2000	Host Plant	2003	Host Plant
Hartswater*	-27.7500 E 24.8000 S	19-03-1998	Maize•	09-02-2000	Lucern•	14-15 January	Cotton•$_1$
Weipe**	-22.3561 E 29.9866 S	Not sampled	-	01-02-2000	Cotton•$_1$	14-01-2003	Cotton•$_1$
Roedtan***	-24.4900 E 29.0800 S	04-02-1998	Cotton•$_1$	24-03-2000	Grain sorghum◦	Not sampled	-
Groblersdal****	-24.9891 E 29.2832 S	25-02-1998	Cotton•$_1$	22-03-2000	Cotton•$_1$	08-01-2003	Cotton•$_1$
Makhatini Flats*****	-27.3589 E 31.6144 S	25-26 March	Cotton◦$_1$	Not sampled	-	17-02-2003	Cotton◦$_1$

* - Northern Cape Province

** - Limpopo Province

*** - Limpopo Province

**** - Mpumalanga Province

***** - Kwa-Zulu Natal Province

◦ - Dryland

• - Irrigated

$_1$ - Conventional = Non Bt-cotton

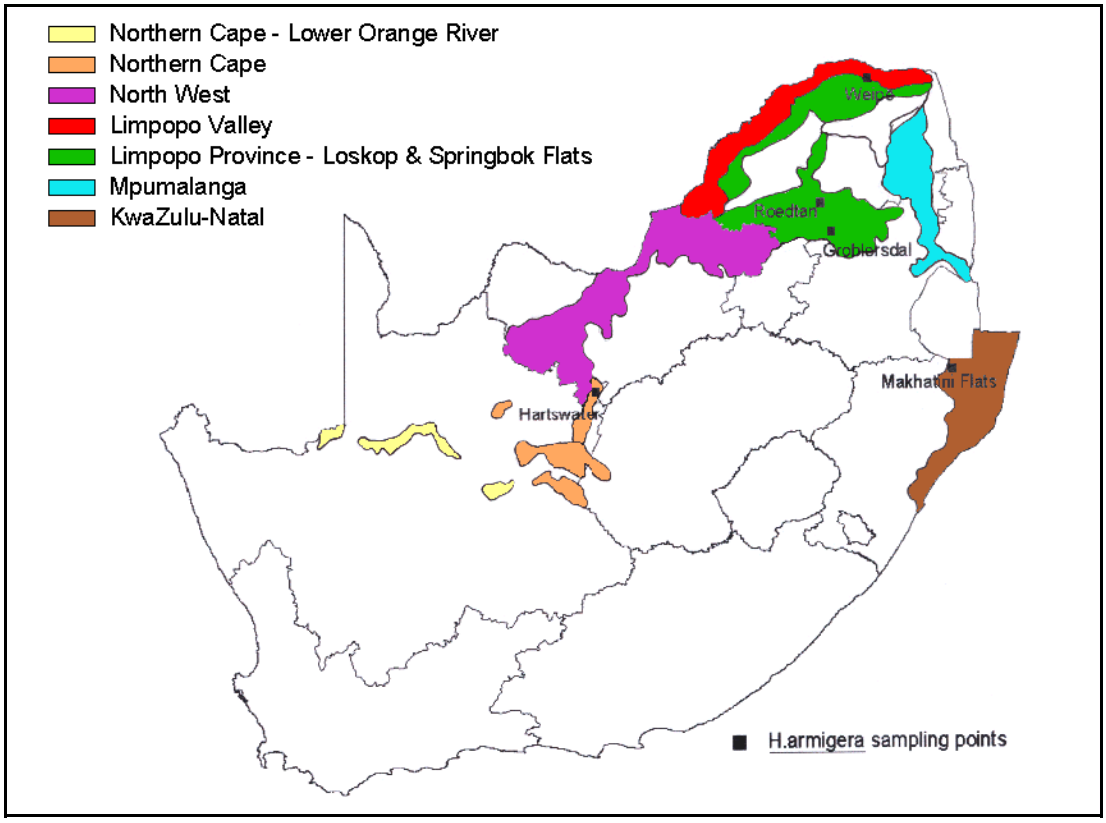


Figure 2.1 Cotton production areas in South Africa and sampling localities of *Helicoverpa armigera* field populations.

2.2.2 SUSCEPTIBILITY TESTING

Larvae of the ARC-PPRI susceptible laboratory strain were used as a control. The *H. armigera* neonate larvae were selected from a 25-year-old laboratory culture, reared at the insectary at Rietondale Research Station. This culture has never been exposed to any insecticides, but field sampled specimens were incorporated during 1991.

The susceptible strain and all field sampled populations were reared by ARC-PPRI at the Insectary, at Rietondale Campus, Pretoria, South Africa. All populations were reared under optimal conditions, and monitored by means of a thermohygrograph, at 25 +/- 2 °C, RH 65-70%

and exposed to a photo-period 16:8 (Light: Dark). The insects were reared on a modified meridic larval diet (Shorey & Hale 1965) in which dry, powdered kidney beans were substituted for soaked pinto beans. (Table 2.2). The eggs were deposited by the females on cloth netting. The netting with eggs was disinfected by soaking for 10 minutes in 2 % formaldehyde, washed under running tap water for 10 - 20 minutes, drip-dried and placed in lidded glass jars for the eggs to hatch. First-instar larvae were transferred (singly or in groups of two or three) to the larval diet. About 10 g of the diet was forced into 100 x 25 mm glass tubes with a domestic cake syringe (nozzle diameter 13 mm) and the tubes were stoppered with absorbent cotton wool plugs. Because the larvae are cannibalistic, only one larva per tube normally survived to pupation.

Table 2.2 Composition of the modified artificial diet as described by Shorey & Hale (1965) for rearing *Helicoverpa armigera* larvae in the laboratory.

INGREDIENTS	QUANTITY
White kidney beans (powdered)*	360 g
Brewer's Yeast	48 g
Methyl-4-hydroxy benzoate	3 g
Agar, Commercial Gel**	20 g
Ascorbic Acid	4.8 g
Sorbic Acid	2.1 g
Formaldehyde	1.1 mℓ
Distilled water	1 000 mℓ

The water was boiled with the ingredients stirred in and left to cool for 30 minutes. After cooling, the gelled medium was used as explained in the text.

* - *Phaseolus vulgaris* L.

** - The agar is superfluous and can be omitted from the diet.

The pupae were disinfected in a 0.2 % sodium hypochlorite solution. As adults about 15 male and 15 female moths (in which the greenish males are distinct from the brownish females) were placed in oviposition cages. The cages were 5 litre Perspex jars containing a layer of vermiculite and a Petri dish with cotton wool pieces soaked in a 7 % sucrose solution to feed the moths. The

females deposited their eggs on the cloth-netting lids of the cages and these were collected every second day.

Larvae of all field sampled populations were reared through to the F_1-generation. By then sufficient test organisms were available. Neonate larvae from each population were used in the evaluations.

2.3 LABORATORY BIOASSAYS WITH THE FREEZE DRIED PRODUCT MVP II FOR THE DETERMINATION OF BASE-LINE CONCENTRATIONS

The freeze-dried product MVP II with the 20 % active ingredient Delta endotoxin Cry1Ac, *Bacillus thuringiensis* were supplied by the client (Monsanto SA (Pty) Ltd), for incorporation into the artificial diet to determine the susceptibility of *H. armigera* to this product. The PPRI susceptible laboratory reared population was used to determine base-line concentrations, ranging from approximately 20 - 90 % mortality. Different amounts of the endotoxin were weighed and incorporated into the artificial diet (Table 2.3). Approximately 3 g of the diet was put into a glass polytop vial with a single neonate larva and closed with an absorbent cotton wool plug. Mortality was recorded 6 days post-exposure to determine a base-line concentration range.

Table 2.3 Base-line concentrations of Cry1Ac used in an artificial diet to determine susceptibility of the laboratory population of *Helicoverpa armigera*.

Amount of Freeze dried product MVP II Cry1Ac	**Amount of Active ingredient in diet µg/g**
1. 0.001 g/100g diet	2
2. 0.004 g/100g diet	8
3. 0.016 g/100g diet	32
4. 0.064 g/100g diet	128
5. 0.256 g/100g diet	512
6. Untreated	Control

The base-line concentrations were evaluated for each population sampled in the different areas with 6 replicates of 15 larvae per replicate per concentration/dose. Exposed larvae were kept in a culture room under the same controlled conditions as the susceptible laboratory population (see Chapter 2.2.2).

The susceptibility of the different field populations and the susceptible laboratory population were evaluated by incorporating the purified endotoxin into the diet. This was performed by mixing the product in different concentrations (Table 2.3) into the slightly heated artificial diet (45 - 50 ^{o}C). Three grams (3g) of the treated medium were placed in a Petri-dish.

Untreated diet was used as a control. After the diet had cooled, a single neonate was transferred onto the diet, with a fine camelhair brush, and closed by the Petri-dish lid. With the predetermined dose-range (during 1999), exposures were repeated during 2000 and 2003, with field populations sampled during the applicable cotton season (6 x replicates with 15 larvae/replicate for each dose/concentration = 90). Mortalities and developmental stages were recorded 6 days post-exposure (1998 & 2000) and 5 days post-exposure during 2003. Although the temperature, humidity and photo-period cycles were kept the same throughout the study period (1999 - 2003), it was found that the artificial medium became too dry for larval consumption on day 6, therefore mortality assessments were performed 5 days post-exposure.

2.3.1 GROWTH INHIBITION RESPONSE EVALUATION

Larval weight on day 5 post-exposure in response to the Cry1Ac protein was evaluated for all *H. armigera* populations, over the study period (1998, 2000 and 2003). A **candidate diagnostic concentration** was also determined and refers to a concentration of Cry1Ac which can prevent at least 50 % of a population developing to **the second instar** within five (5) days, (Wu *et al.* 2002). In a similar study the diagnostic concentration was also calculated for *H. armigera*, but was determined as the development time to **the third instar** over a period of 5 - 7 days (Wu & Guo 1997). Due to the difference in development of *H. armigera* susceptible laboratory

population in South Africa, the criteria for a diagnostic concentration differs from the study conducted in China.

2.4 STATISTICAL ANALYSIS

Multiple comparisons between the populations were performed using Probit analysis (Van Ark 1983). Tests for parallelism, LC_{50} and LC_{90} concentrations and comparison of slopes were performed. Abbot's formula (Abbot 1925) was used to calculate percentage control. In computing the effectiveness of insecticides, when an actual count of the living and dead insects in both the treated and untreated plots, or checks, is available, it is obvious that the insects which die from natural causes must be considered (Abbot 1925).

2.5 RESULTS

Data regarding mortality obtained when *H. armigera* neonate larvae exposed to different concentrations of the purified Cry1Ac Bt protein, incorporated into the artificial diet of *H. armigera*, are shown in Table 2.4. Natural mortality totals reflected in Table 2.4, when neonate *H. armigera* larvae were exposed to the untreated diet during 1998, 2000 and 2003 varied between totals of 1 - 14 in all populations. The Roedtan population during 1998 reflected the highest (14/90 = 15.51 %), and natural mortality from the Makhatini Flats population was (9/90 = 10 %). According to Van Ark (1981), natural mortalities of up to 15 % is acceptable in entomology studies.

Mortalities obtained with the susceptible laboratory population during 1998, with increased concentrations, ranged from 27 (2 µg/g), 78 (8 µg/g), 80 (32 µg/g) , 90 (128 µg/g) and 90 (512 µg/g) respectively (a total mortality of 90 reflects 100 % mortality). When Cry1Ac was incorporated during the 2000 season mortalities ranged from 51 (2 µg/g), 88 (8 µg/g), 89 (32 µg/g), 89 (128 µg/g) and 90 (512 µg/g). The trend continued during the 2003 season, with mortalities ranging from 56 (2 µg/g), 81 (8 µg/g), 90 (32 µg/g), 90 (128 µg/g) and 90 (512 µg/g). These base-line concentrations 2 µg/g, 8 µg/g, 32 µg/g, 128 µg/g and 512 µg/g, used during the

1998 season, were sufficient to control the susceptible laboratory strain with mortalities ranging from 30 - 100 %. No significant differences were evident (Table 2.5).

The Weipe field population was only evaluated during 2000 and 2003 due to lack of a number of specimens available in the field. Mortality results obtained during 2000 were 35 (2 µg/g), 72 (8 µg/g), 84 (32 µg/g), 86 (128 µg/g) and 89 (512 µg/g), followed by the 2003 season with mortalities ranging from 58 (2 µg/g), 69 (8 µg/g), 81 (32 µg/g), 83 (128 µg/g) and 83 (512 µg/g) respectively. The susceptibility of this population decreased slightly over the two seasons, but although statistically different (Table 2.5), a lower LD_{50} concentration (0.35 in 2003 and 2.56 in 2000) was determined for the 2003 season, therefore no positive tolerance level could be detected. Mortality results obtained with the Hartswater, Roedtan and Groblersdal field populations did not differ significantly over the study period, when Cry1Ac was added to the artificial medium.

Mortality results obtained with the Makhatini Flats population, during 1998 and 2003 resulted in a significant difference (Table 2.5), but a lower LD_{50} was also determined during the 2003 season (1.20 in 2003 and 8.74 in 1998), therefore no tolerance levels could be determined.

Table 2.4 Mortalities (six replicates x 15 = 90) with Standard Deviations (SD), obtained 6 day post-exposure (1998 & 2000) and 5 days post exposure (2003) on different dates with different populations against different concentrations of Cry1Ac Bt protein.

CONCEN-TRATION OF CRY1Ac	POPULATIONS																	
	SUSCEPTIBLE						WEIPE						HARTSWATER					
	1998		2000		2003		1998		2000		2003		1998		2000		2003	
	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD
Untreated	1	0.37	1	0.37	8	1.11	-	-	2	0.75	5	0.69	4	0.75	1	0.37	2	0.47
2 µg/ g	27	0.96	51	1.99	56	1.25	-	-	35	2.79	58	2.9	37	1.34	28	2.43	52	0.75
8 µg/ g	78	1.29	88	0.47	81	1.38	-	-	72	0.58	69	1.5	57	1.26	63	1.89	74	0.94
32 µg/ g	80	0.75	89	0.37	90	0	-	-	84	1	81	0.95	80	1.25	85	0.69	77	1.46
128 µg/g	90	0	89	0.37	90	0	-	-	86	0.75	83	1.07	80	1.11	86	0.75	82	0.94
521 µg/g	90	0	90	0	90	0	-	-	89	0.37	83	1.07	90	0	89	0.37	88	0.47

Table 2.4 Continued

CONCEN-TRATION OF CRY1Ac	POPULATIONS																	
	ROEDTAN						GROBLERSDAL						MAKHATINI FLATS					
	1998		2000		2003		1998		2000		2003		1998		2000		2003	
	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD	NO	SD
Untreated	14	0.47	1	0.37	-	-	3	0.5	1	0.37	6	0	9	0.5	-	-	4	0.74
2 µg/ g	34	1.25	21	1.26	-	-	38	1.6	45	2.22	60	2.65	25	1.07	-	-	46	2.75
8 µg/ g	70	1.37	68	0.94	-	-	68	1.97	85	0.69	83	1.07	54	0.82	-	-	67	1.77
32 µg/ g	79	0.68	89	0.37	-	-	62	1.7	90	0	83	1.07	70	0.75	-	-	70	0.94
128 µg/g	88	0.47	90	0	-	-	78	1.53	90	0	87	0.5	76	0.75	-	-	66	0.82
521 µg/g	87	0.47	90	0	-	-	90	0	90	0	90	0	88	0.47	-	-	84	0.82

Table 2.5 Susceptibility (LD_{50}) of different populations neonate *Helicoverpa armigera* larvae to *Bacillus thuringiensis* (freeze dried protein), incorporated into an artificial medium, during 1998, 2000 and 2003.

POPULATION	1998		2000		2003	
	LC_{50} (µg/g)	Slope = SE	LC_{50} (µg/g)	Slope = SE	LC_{50} (µg/g)	Slope = SE
Susceptible (Lab)	3.14	± 1.51	1	± 1.38	1	± 0.77
Makhatini Flats	**8.74**	**± 1.68**	**Not evaluated**		**1.2**	**± 0.74**
Roedtan	4	± 0.91	4.08	± 4.33	Not evaluated	
Groblersdal	3.2	± 0.93	1.46	± 1.46	0.43	± 0.25
Weipe	**Not evaluated**		**2.56**	**± 0.95**	**0.35**	**± 0.28**
Hartswater	3.65	± 0.84	3.83	± 1.11	0.77	± 0.39

The susceptible population during the three years 1998, 2000 and 2003 showed LD_{50}'s of 3.14 µg/g, 1.00 µg/g and 1.00 µg/g and slopes of ±1.51, ±1.38 and ±0.77, respectively. This represents a 3.1-fold difference in susceptibility but is not significant different (Table 2.5). The LC_{50},s in the Makhatini Flats ranged from 8.74 µg/g (1998) to 1.20 µg/g (2003); this represented a 7.3-fold significant difference in susceptibility. The Weipe field populations also represents a significant 7.3-fold difference in susceptibility with a LC_{50} of 2.56 µg/g during 2000 and a LC_{50} of 0.77 µg/g during 2003. This however does not result in a positive resistance level because the LD_{50} concentration during 2003 were lower than in 1998 and 2000 respectively. The susceptibility of the Roedtan field population ranged from 4.00 µg/g (1998) to 4.08 (2000) with no significant difference in susceptibility. The LC_{50},s in the Groblersdal field populations ranged from 3.20 µg/g (1998); 1.46 µg/g (2000) and 0.43 µg/g (2003); this represents a 2.2-fold and 3.4-fold difference in susceptibility, with the most susceptible population in 2003. The Hartswater field populations showed LC_{50}, s of 3.65 µg/g (1998); 3.83 µg/g (2000) and 0.77 µg/g (2003); this represents no difference in susceptibility over the study period.

The results of the laboratory trials over the study period indicated that the purified *B. thuringiensis* endotoxin protein incorporated into the diet, provided excellent control even at very low concentrations. No significant differences in susceptibility were detected in most populations against the purified Cry1Ac protein ($p \leq 0.01$, Bonferoni ranked t-test). These results confirm that no sign of resistance build-up or positive tolerance levels could be detected in the sampled field populations, when exposed in the laboratory to the purified endotoxin Cry1Ac.

Figures 2.2 - 2.7 depict the percentage control obtained on day 6 with *H. armigera* neonate larvae, with different populations, over the study period, when exposed to the base-line concentration range of Cry1Ac, incorporated into an artificial diet. These corrected mortalities (% control) were determined using Abbott's formula (Abbot 1925). The Standard Deviations (SD) are also reflected in the figures and were very low throughout the study period. These results confirmed the results on mortality analysed in Tables 2.4 and 2.5, but Fig. 2.2 - 2.7 reflect the percentage control.

Fig 2.2 illustrates the results for the susceptible laboratory population. Percentage control over the three test seasons 1998, 2000 and 2003, increased from 30-58-60 (2 µg/g), 85-98 and decreased to 90 (8 µg/g), at 32 µg/g it was 90 % for 1998 and 100 % during 2000 and 2003. At 128 and 512 µg/g, the control was 100 % during all three seasons. Figure 2.3 (Makhatini Flats) population depicts mortalities of 18-58 % with 2 µg/g, 1998 and 2003 and 57-75 % with 8 µg/g. With 32 µg/g 78-80 % were obtained, followed by 84-75 % with 128 µg/g during 1998 and 2003. With the highest concentration 512 µg/g 98 and 95 % were obtained during 1998 and 2003. Results in Fig. 2.4 - 2.7 followed the same trend in all the populations in the respective test seasons. The determined base-line concentrations (1998); 2 µg/g, 8 µg/g, 32 µg/g, 128 µg/g and 512 µg/g gave mortality responses ranging from ± 20 - 90 %. This are clearly graphically represented in all figures. Therefore to achieve successful control of *H. armigera* larvae in the field a concentration of between 8 µg/g and 32 µg/g would be sufficient when expressed in Bt-cotton plant parts.

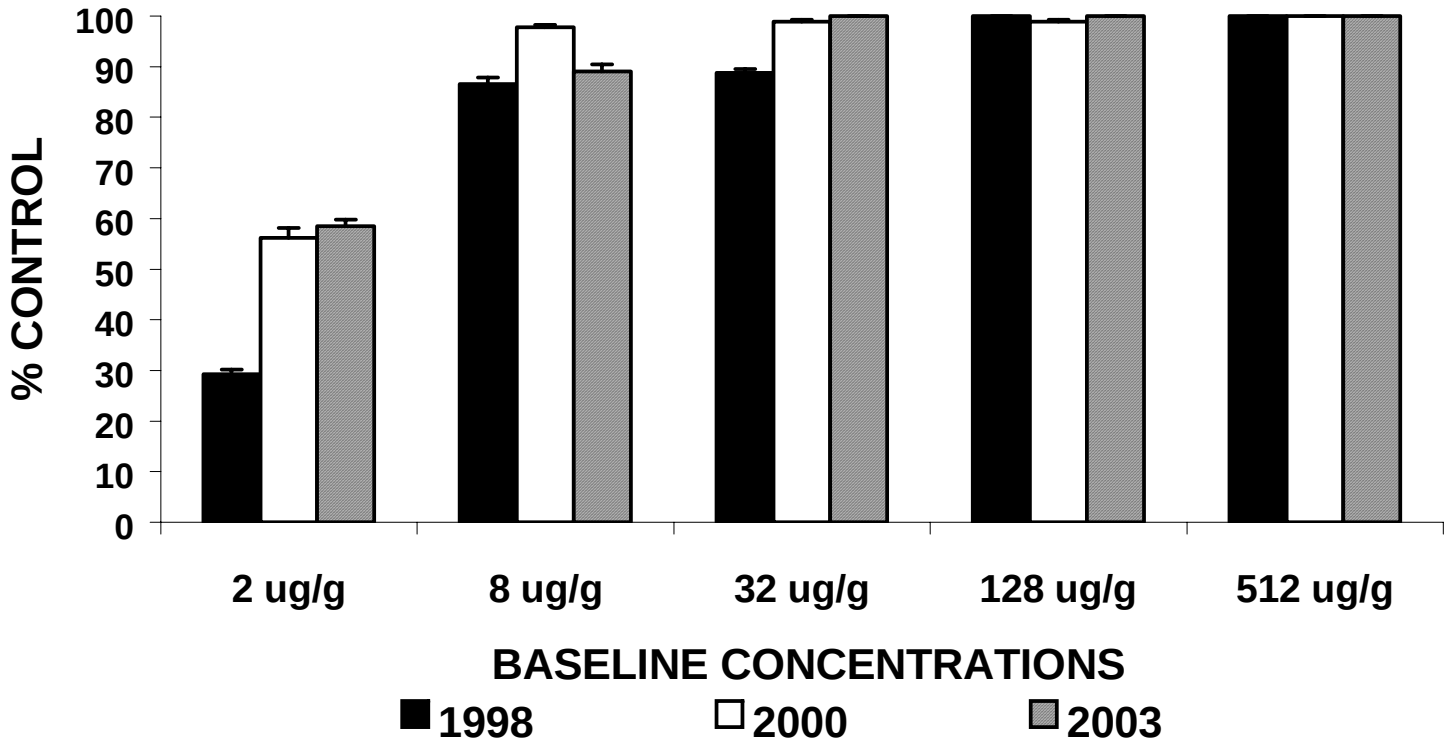


Figure 2.2 The percentage control obtained in *Helicoverpa armigera* neonate larvae (susceptible laboratory population), during 1998, 2000 and 2003 cotton seasons, when exposed to different concentrations of the purified endotoxin protein Cry1Ac, which was incorporated into the artificial diet. Correction factor: Abbot's formula (Abbot 1925).

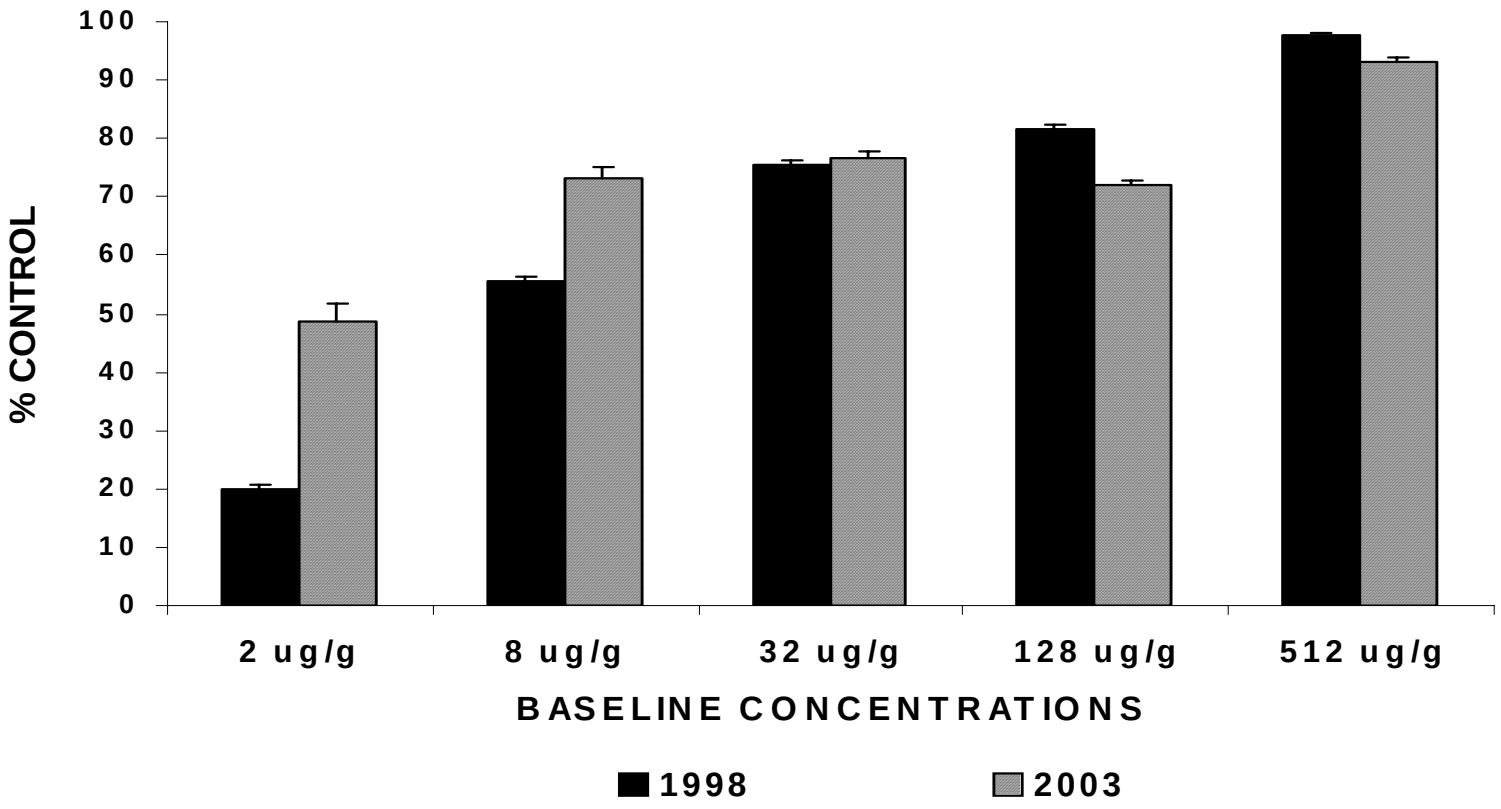


Figure 2.3 The percentage control obtained in *Helicoverpa armigera* neonate larvae sampled in the Makhatini Flats, during 1998 and 2003 cotton seasons, when exposed to different concentrations of the purified endotoxin protein Cry1Ac, which was incorporated into the artificial diet. Correction factor: Abbot's formula (Abbot 1925).

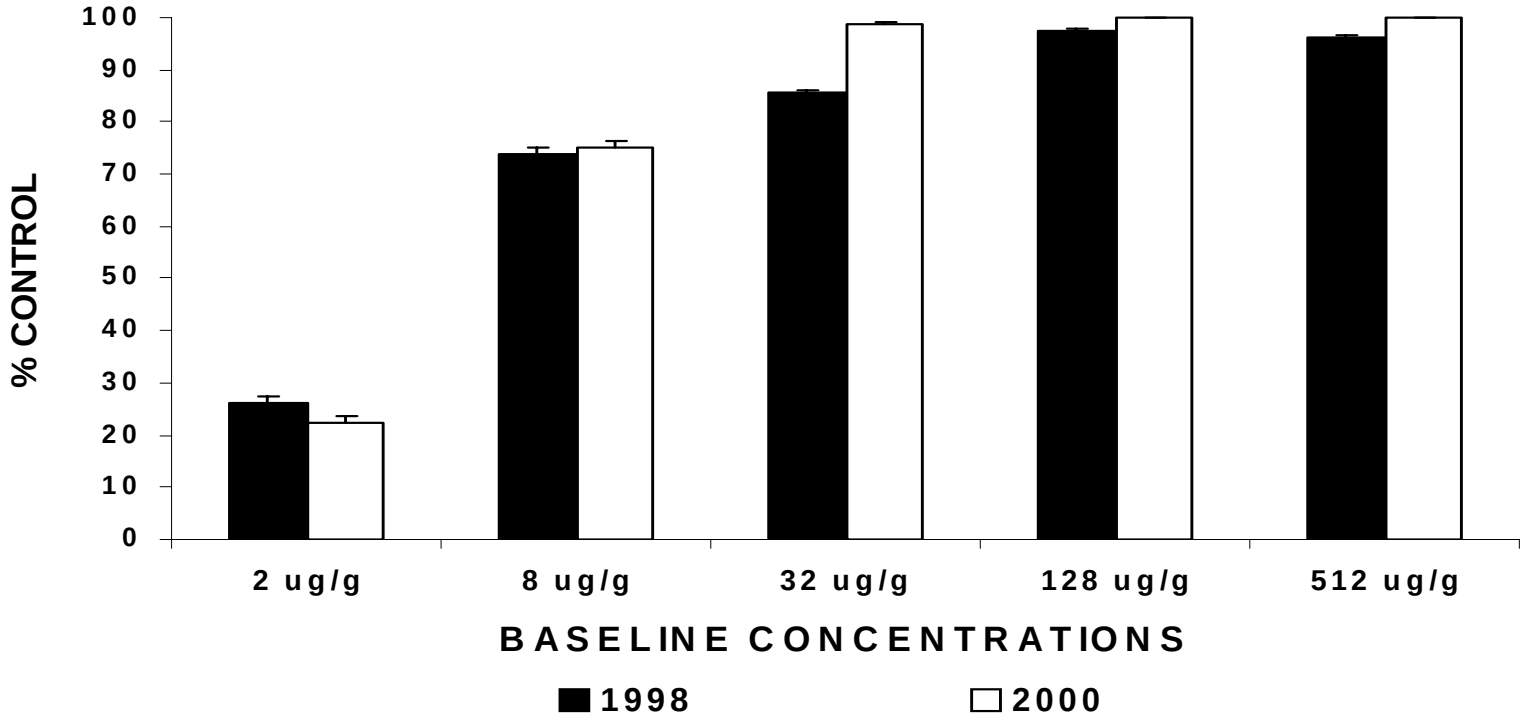


Figure 2.4 The percentage control obtained in *Helicoverpa armigera* neonate larvae sampled in the Roedtan area, during 1998 and 2000 cotton seasons, when exposed to different concentrations of the purified endotoxin protein Cry1Ac, which was incorporated into the artificial diet. Correction factor: Abbot's formula (Abbot 1925).

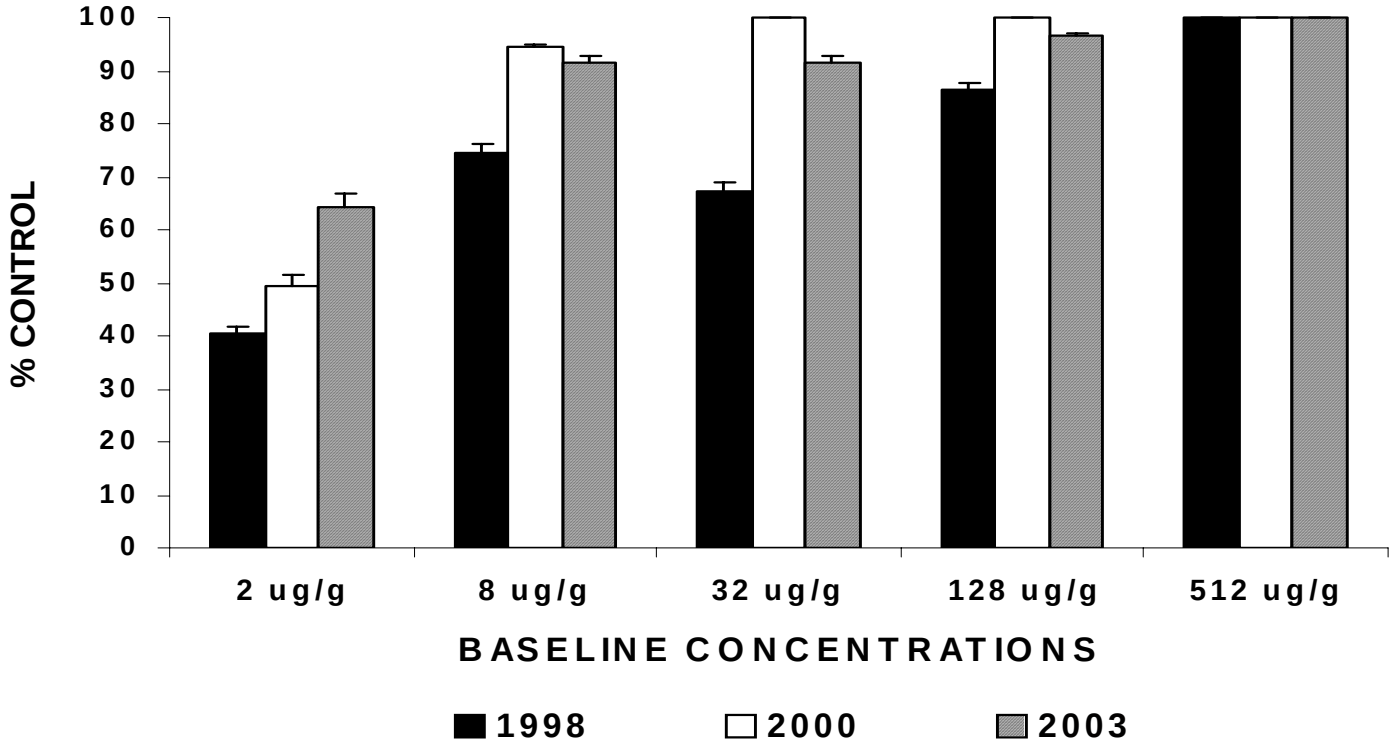


Figure 2.5 The percentage control obtained in *Helicoverpa armigera* neonate larvae sampled in the Groblersdal areas, during 1998, 2000 and 2003 cotton seasons , when exposed to different concentrations of the purified endotoxin protein Cry1Ac, which was incorporated into the artificial diet. Correction factor: Abbot's formula (Abbot 1925).

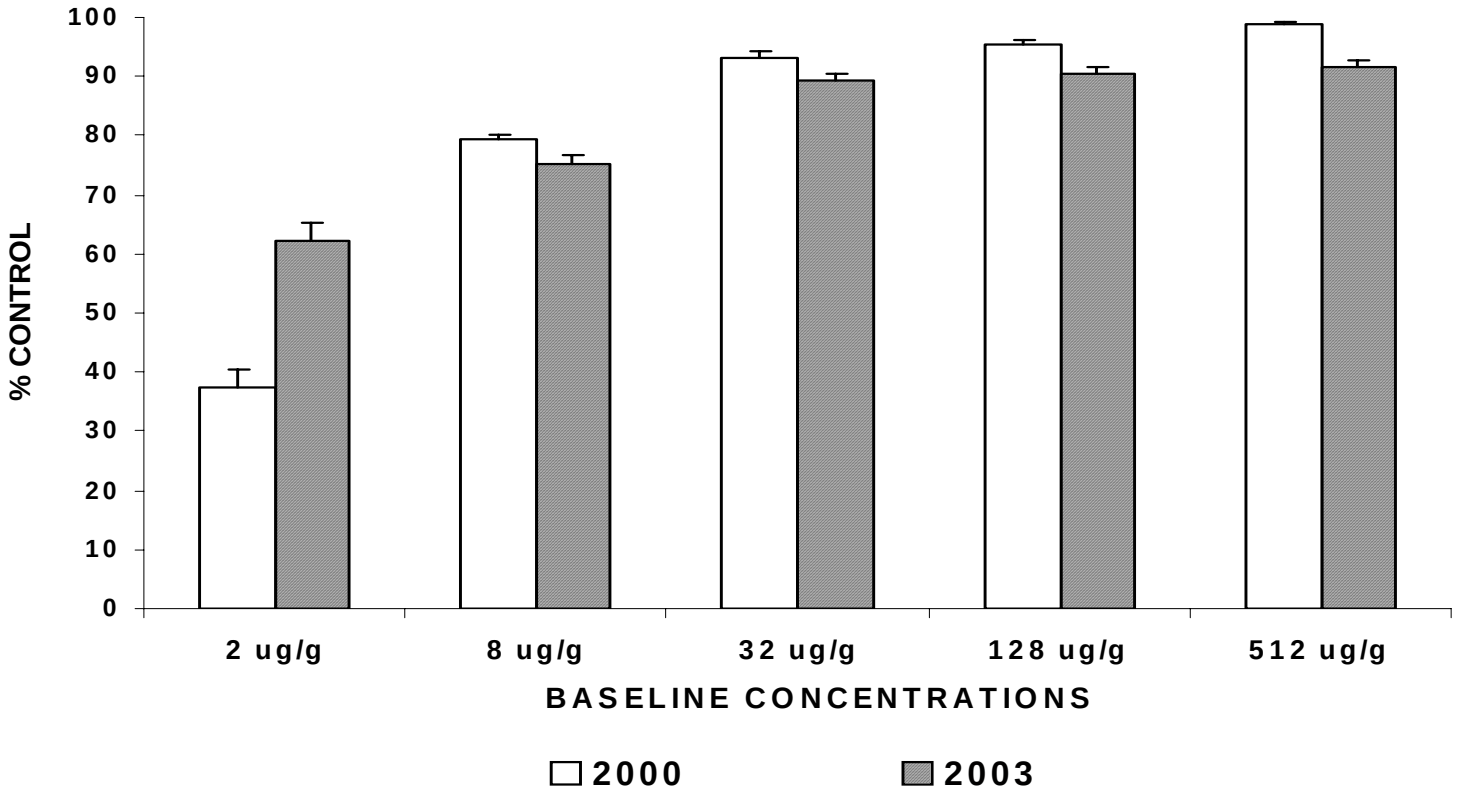


Figure 2.6 The percentage control obtained in *Helicoverpa armigera* neonate larvae sampled in the Weipe area, during 2000 and 2003 cotton seasons, when exposed to different concentrations of the purified endotoxin protein Cry1Ac, which was incorporated into the artificial diet. Correction factor: Abbot's formula (Abbot 1925).

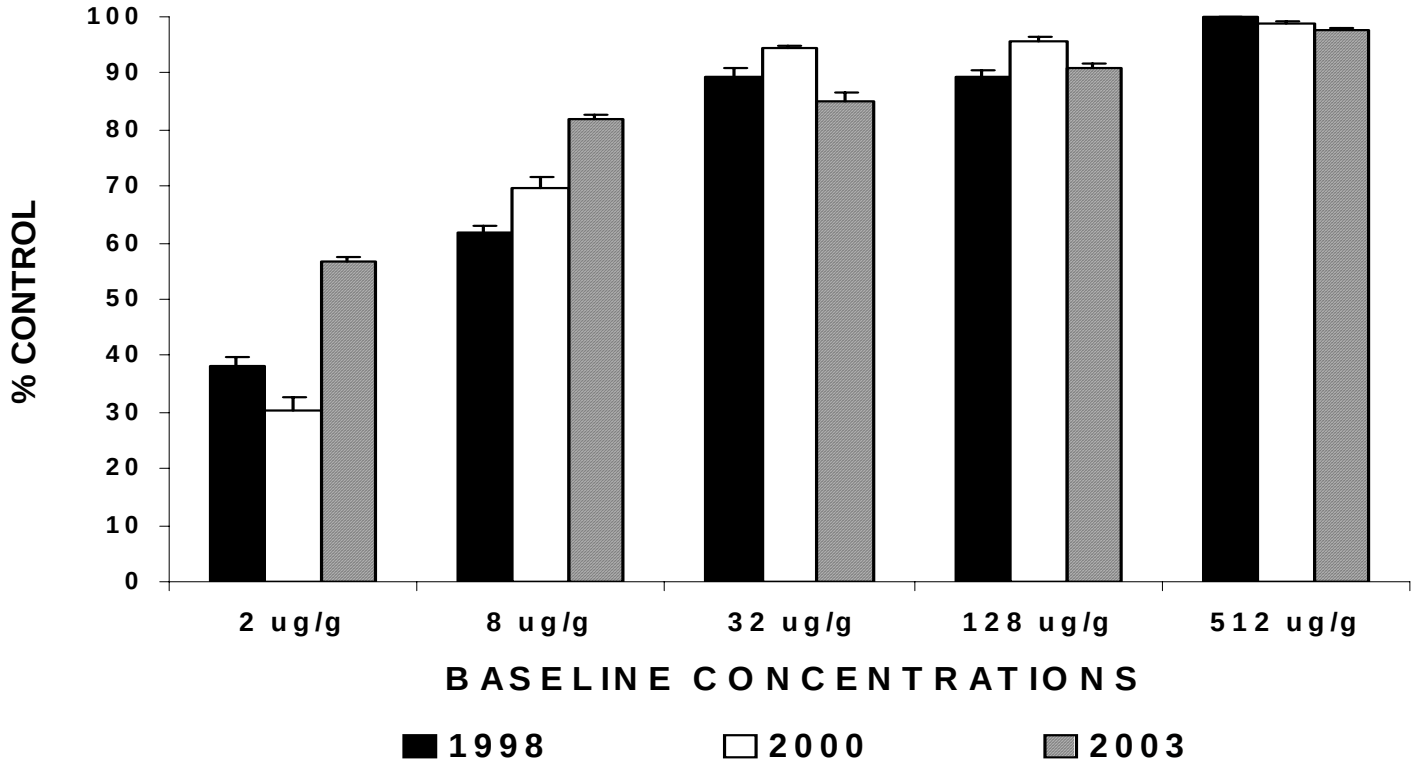


Figure 2.7 The percentage control obtained in *Helicoverpa armigera* neonate larvae sampled in the Hartswater area, during 1998, 2000 and 2003 cotton seasons , when exposed to different concentrations of the purified endotoxin protein Cry1Ac, which was incorporated into the artificial diet. Correction factor: Abbot's formula (Abbot 1925).

2.6 GROWTH INHIBITION PROPERTIES OF CRY1Ac

Normal development of *H. armigera* larval stages, reared in the laboratory, were defined as: egg stage (3.4 days), neonate larva to 1st instar (3.5 days), 1st larval instar to 2nd instar (2.5 days) and 2nd to 3rd instar (2.1 days) (Van der Walt *et al.* 1993). Various concentrations of Cry1Ac (Table 2.6), added to the artificial diet, indicated that larval development on untreated diet (control) versus treated diet differ.

Table 2.6 Larval development stages (%) recorded on day 5, when exposed to different concentrations of Cry1Ac (incorporated in an artificial diet) in the laboratory. The results are reflected as the totals of 1998, 2000 and 2003 seasons (n = 450, 5 x populations).

CONCEN TRATION	DEVELOPMENTAL STAGE					
	DEAD	NEONATE	1ST INSTAR	2ND INSTAR	3RD INSTAR	4th INSTAR
Untreated	1.3	0.2	0.2	6.4	53.8	38
2 µg/ g	40	27.6	31.8	0.7	0	0
8 µg/ g	83.8	13.1	3.1	0	0	0
32 µg/ g	97.1	2.9	0	0	0	0
128 µg/ g	98	2	0	0	0	0
512 µg/ g	99.6	0.4	0	0	0	0

Results in Table 2.6 indicated that neonate *H. armigera* larvae exposed to the untreated (control) diet, showed normal development. In this study 53.8 % larvae developed to the 3rd instar and 38 % to the 4th instar within five days. When neonate larvae were exposed to 2 µg/g Cry1Ac, only 0.7 % reached 2nd instar. A 1.3 % natural mortality in the untreated control diet was recorded, whereas 40 - 99.6 % mortality was recorded with increased concentrations of 2 µg/g, 8 µg/g, 32 µg/g, 128 µg/g and 512 µg/g. At 8 µg/g Cry1Ac added to the diet, development of *H. armigera*, stopped at the first instar, when only 3.1 % of exposed larvae, were able to develop followed by

no further development. When 32 µg/g, 128 µg/g and 512 µg/g Cry1Ac were added to the diet no larval development was recorded.

At a concentration of 2 µg/g added to the diet, only 0.7 % of the total number of larvae (450 , 5 x populations x 3 seasons), could survive and develop to the 2nd instar within 5 days. According to these results, an estimated diagnostic concentration/dose would be recommended at ≤2 µg/g Cry1Ac protein (Table 2.6). Wu *et al.* (2002) reported a diagnostic concentration of 1.0 µg/ml diet (liquid artificial diet). Results in Table 2.6 clearly indicated that increased concentrations of Cr1Ac, led to growth retardation and lack of development. Results further show that Cry1Ac, incorporated into the diet, at the lower concentrations (2 µg/g and 8 µg/g), exhibited some anti-feeding properties and a repellant effect at the higher concentrations (32 µg/g, 128 µg/g and 512 µg/g), where no development was recorded.

2.7 DISCUSSION

Having conducted monitoring in the laboratory of field sampled *H. armigera* populations, sampled in the different cotton production areas in South Africa, (Table 2.4), results indicated a significant difference in the susceptibility to the Bt-protein Cry1Ac, over the study period (1998, 2000 and 2003), in the Makhatini Flats and Weipe field populations. This however does not result in a positive resistance level due to lower LD_{50} concentrations determined during the 2003 season. In the field populations sampled in Roedtan, Groblersdal and Hartswater no significant differences over the study period were indicated, therefore no negative susceptibility levels could be found. In China, when different field populations were evaluated, a slight increase in LD_{50} concentrations was found, during the study period 1998, 1999 and 2000 (Wu *et al.* 2002). They therefore concluded no general tolerance or susceptibility movement among Chinese *H. armigera* populations, from 1998 and 2000.

Results during the study period in South Africa did not confirm the presence of resistance, but demonstrated that the field populations evaluated were susceptible to the Cry1Ac protein and that a movement towards resistance in the *H. armigeara* field populations was not found. Due to the anti-feeding properties exhibited one can also assume that cotton containing a Bt-gene would not

be the preferred food source in the field. Tabashnik *et al.* (2003) reported that after seven years of large-scale planting of Bt-crops, resistance of *H. armigera* in the field can not be reported. In particular monitoring *H. armigera* in northern China for three years, revealed no increases in the frequency of resistance despite widespread adoption of Bt-cotton.

Results from 2000 and 2003 in balance with the initial base-line concentrations determined in 1998, provided important information on the susceptibility of *H. armigera*, field populations in South Africa. This was the first study performed in this country, since the commercialization of Bt cotton in 1996 in South Africa. To ensure the continued effectiveness in years to come, of Cry1Ac Bt cotton (Bollgard™), continued monitoring for potential resistant genes in *H. armigera* should be a long term objective. This is of great importance as the market for the commercial Bollgard™ cotton expands. Continued collection and analysis of such data at least every 2 - 3 years are critical to the development and continual assessment of resistance management strategies.

CHAPTER 3

EVALUATION OF DIFFERENT TRANSGENIC COTTON STRAINS

3.1 INTRODUCTION

In South Africa cotton is one of the few agricultural commodities where local demand exceeds production, thereby creating opportunities for new producers without the risk of causing a surplus. As a result of increasing exposure to international competition, producers have to become innovative and productive to maintain profit margins. There is a growing need to increase the output of world agriculture if the demands of a rising world population are to be met. The basis to meet these demands is to increase and improve harvest yields of major crops on existing cultivated land. One practical means of increasing yield would be to protect more of what is grown from loss to pests, especially insect pests. Genetically engineering inherent crop resistance to insect pests offers the potential of a user-friendly, environment-friendly and consumer-friendly method of crop protection to meet the demands of sustainable agriculture in the 21st century.

Genetic engineering of crops also offers the prospect of many other advantages; not just widening the potential pool of useful genes but also permitting the introduction of a number of different desirable genes at a single event and reducing the time needed to introgress introduced characters into an elite genetic background. Bt-cotton allows the producer to use less insecticides during pest management, which in turn decreases the potential damage to non-target organisms. Bt or transgenic-cotton is protected from lepidopteran pests until late in the season when the plants cease vegetative growth and begin fruit-set. After fruit set the cotton plants do not have enough insecticidal protein to protect them against lepidopteran pests and one or two chemical sprays may be needed. The aim of this part of the study was to determine performance curves of *Helicoverpa armigera* (Hübner) and *Spodoptera littoralis* (Boisduval), when exposed not only to artificial diet with incorporated *Bacillus thuringiensis* protein, but to different Bollgard cotton cultivars which contain different strains of *B. thuringiensis,* cultivated in the field.

3.2 MATERIALS AND METHODS

During this study five cotton cultivars (4 x Bt-cotton cultivars) + (1 x standard cotton cultivar) were evaluated. During the first sampling interval performed on 18 January 2000, only leaves and squares were sampled and during the final sampling action on 18 May 2000, no leaves were available for sampling and evaluations were performed only on squares and small bolls. Three different plant parts (leaves, squares and small bolls) of each cotton cultivar were evaluated.

3.2.1 TEST PLANTS

- DP50 - a non-transgenic recurrent parent, a commercially standard cotton cultivar
- DP50 B - the original transformant of 15985 with Bollgard (Cry2Ab)
- 15985 B - a segregant containing only Bollgard (Cry1Ac)
- 15985 X - a segregant containing only Cry X
- 15985 BX - containing Bollgard (Cry1Ac) and Cry X were used during this study.

The cotton cultivars 15985 X and 15985 BX (expressing two different *Bacillus thuringiensis* ∂-endotoxins), have not yet been granted regulatory approval for movement into commerce during 2000. A modified Cry1A gene encoding a chimeric Cry1Ab/1Ac toxin (Hofte & Whiteley 1989, Perlak et al. 1990), and a modified Cry2Ab gene encoding a Cry2Ab toxin (Widner & Whiteley 1989, Dankocsik *et al.* 1990) are expressed in these cotton plants.

Terminal leaves, squares and young bolls of each cultivar were tested against both lepidopteran species. All test material were collected either prior to or on the day of exposure, to ensure freshness and thus attractiveness for neonate larvae to feed on. In the field the different plant parts of each cultivar were placed separately in clearly labelled paper bags, which were placed into a plastic bag and sealed. The bags were placed in insulated cooler boxes for transport to a monitored cold room (0-4 ^{0}C) until fed to neonate larvae. This was the best way to ensure that the plant parts stayed fresh and palatable when fed to neonate larvae. Similar methods were used by Greenplate (1999); Greenplate *et al.* (2002a) and Chitkowski *et al.* (2003).

3.2.2 TEST INSECTS

HELICOVERPA ARMIGERA NEONATE LARVAE (AFRICAN BOLLWORM)

Neonate larvae of *H. armigera* (Hübner), were selected from the same susceptible laboratory culture as described in Chapter 2.2.2.

SPODOPTERA LITTORALIS NEONATE LARVAE (COTTON LEAFWORM)

Spodoptera littoralis larvae were field collected (± 200, in different instars). Sampling were performed on green beans as host plants and took place in the Marble Hall district (Mpumalanga Province). Larvae were transported to the laboratory on fresh green bean leaves as food source and were transferred immediately onto the artificial medium for further development. Larvae were not reared on a *S. littoralis* artificial diet as described by Navon (1985) but reared on the same diet as *H. armigera* in the same culture room. This modified artificial diet described by Shorey & Hale (1965) proved to be suitable for mass rearing of different noctuid species, including *S. littoralis*.

3.2.3 BIOASSAYS

Cotton plant parts were placed singly in clean plastic containers (75ml) of which the bottom was covered by approximately 3 mm of a 2% agar/water solution in order to prevent dessication of plant parts. A single neonate larva was transferred onto the plant part with a fine camelhair brush and checked for movement before the container was sealed with a ventilated plastic lid. All containers and brushes used, were either rinsed with or dipped in 90% alcohol before use. Each exposure consisted of six replicates of 15 larvae each for each plant part for each cultivar. In this part of the study five cotton cultivars (4 x Bt-cotton cultivars) + (1 x standard cotton cultivar) have been evaluated. All plant parts were fresh and in good condition when used during exposures. Due to the difficulty in finding a natural population of *S. littoralis* in the field, sampling was done late in the season. This resulted in only one evaluation with the F_1-generation reared in the laboratory.

3.2.4 OBSERVATIONS AND EXAMINATIONS

Three day post-exposure larval mortality counts and development on leaves were recorded and fresh leaves were supplied as required. The squares and small bolls were inspected for freshness and were replaced when necessary. To avoid unnecessary handling and thus damage to larvae, no readings were taken on squares and small bolls on the third day. Six days post-exposure larval-mortalities, larval-development and larval-mass were recorded on all plant parts. Surviving larvae were weighed as a group per replicate on each plant part of each cultivar. Where larvae were too small to weigh mass average was taken (Van der Walt *et al*. 1993). All test material was placed into plastic bags, sealed and taken to ARC-Plant Protection Research Institute, Roodeplaat Campus and incinerated.

3.2.5 STATISTICAL ANALYSIS

Statistical analysis were performed using the statistical program GENSTAT 5 (GENSTAT 5 Committee 1993). The data was analysed by Analysis of Variance (ANOVA), testing for differences between cultivars. Abbot's formula were used (Abbot 1925) to calculate percentage control.

3.3 RESULTS

3.3.1 BIOLOGICAL EFFICACY DATA

HELICOVERPA ARMIGERA

During the 2000 cotton growing season leaves, squares and bolls were collected from five different cotton cultivars. Data obtained on the total number exposed and mortality counts (6 days post-exposure) when *H. armigera* neonate larvae were exposed to different plant parts of five cotton cultivars are shown in Table 3.1.

Table 3.1 The exposure dates, plant parts exposed, number of *Helicoverpa armigera* larvae exposed and the mortalities obtained 6 days post-exposure, on five different cotton cultivars.

Exposure Date	Plant Parts	Total Number Exposed	MORTALITY OF LARVAE				
			DP50 Standard/Control	DP50 B Bt-cotton	15985 B Bt-cotton	15985 X Bt-cotton	15985 BX Bt-cotton
			Total	Total	Total	Total	Total
18-01-2000	Leaves	90	24	44	56	41	62
	Squares	90	19	70	79	82	85
	Total	180	43	114	135	123	147
18-02-2000	Leaves	90	14	26	29	46	37
	Squares	90	24	68	66	81	84
	Bolls	90	61	83	88	88	88
	Total	270	99	177	183	215	209
07-03-2000	Leaves	90	14	37	27	47	51
	Squares	90	41	67	73	87	85
	Bolls	90	61	83	90	87	88
	Total	270	116	187	190	221	224
24-03-2000	Leaves	90	16	54	42	46	51
	Squares	90	24	79	63	80	85
	Bolls	90	76	84	86	88	90
	Total	270	116	217	188	214	226
11-04-2000	Leaves	90	25	34	39	53	61
	Squares	90	40	81	65	86	83
	Bolls	90	73	88	85	90	89
	Total	270	138	203	189	229	233
03-05-2000	Leaves	90	4	32	38	33	60
	Squares	90	33	65	72	71	80
	Bolls	90	67	83	88	86	90
	Total	270	104	180	198	190	230
19-05-2000	Squares	90	56	86	72	88	90
	Bolls	90	74	90	89	90	90
	Total	180	130	176	161	178	180

Mortalities recorded on the standard/control cotton cultivar (DP50) were 24 on leaves and 19 on squares (43 total) for total plant parts. These results were recorded on 18 January 2000 and no bolls were sampled at the time on any of the cotton cultivars, due to the young age of the plants, no bolls were yet produced. During the last exposure 19 May 2000, no leaves were sampled, for exposure, on any of the cotton cultivars, because the available leaves were hard and no longer palatable to larvae (Table 3.1).

Mortality on DP50 B (Bt-cotton cultivar) was recorded on 18 January 2000 as 44 on leaves, and 70 on squares. This already showed a big difference in the mortality rates between DP50 (standard/control cultivar) and the Bt-cotton cultivars (Table 3.1).

On 11 April 2000, the mortality on leaves, squares and bolls were 34, 81 and 88 respectively and on 3 May 2000 32, 65 and 83. On 19 May 2000, in the absence of leaves 86 and 90 on squares and bolls respectively. Mortality results obtained with the Bt-cotton cultivar 15985 B followed the same trend as the cultivar DP50 B with only significant differences during the last 3 events.

The Bt-cotton cultivars 15985 X and 15985 BX resulted in much higher mortality rates throughout the season. From Table 3.1 it could be seen that there were no differences between the mortalities on 15985 X and 15985 BX by May 2003. The larval mortality on 15985 X and 15985 BX was between 98 and 100% for all observations. To bear these results out, further statistical analyses were performed.

Results obtained throughout the season after exposing neonate *H. armigera* larvae to different plant parts of different cultivars were analysed by ANOVA for differences between cultivars and sampling dates and the cultivar-versus-sampling date interaction (Table 3.2).

Table 3.2 Analysis of Variance of *Helicoverpa armigera* mortality counts.

SOURCE OF VARIATION	DEGREES OF FREEDOM	
CULTIVAR	*C-1 ∴ 5-1	= 4
DATE	**D-1 ∴ 7-1	= 6
CULTIVAR/DATE	(C-1)(D-1)	= 24
RESIDUAL	N-(C-1)-(D-1)-(C-1)(D-1)	= 175
TOTAL	***N-1 ∴ 210-1	= 209

***C** = 5 Cultivars

****D** = 7 Dates

*****N** = 5 x 7 x 6 replicates = 210 experimental units

The cultivar-versus-date interaction was highly significant ($P<0.001$) (Table 3.2). This is interpreted that different mortalities of neonate *H. armigera* larvae were exhibited during the season on the different cultivars. This did not indicate that the Cry-protein diminished in the plant parts during the season. The mortalities varied slightly and in some cases increases as the season progressed (Tables 3.1 and 3.2).

The mean separation was also performed using Fisher's Protected T-test LSD (Least Significant Differences) at the 5% significance level on the interaction table of means (Table 3.3). This means that the mortalities of neonate *H. armigera* larvae on the different plant parts (leaves, squares and bolls) differed significantly during the season, but did not decrease. This is in contrast to Greenplate (1999) and Greenplate *et al.* (2000a), who reported that plants of different Bollgard varieties express the Cry1Ac toxin less in fruiting structures than in terminal growth or newly expanded leaves, and the level of the toxin decreases as the plant mature. The results obtained in South Africa support results reported by Chitkowski *et al.* (2003), that they observed no differences in mortality attributable to plant age.

Table 3.3 The mean mortality of neonate larvae of *Helicoverpa armigera* exposed to the different plant parts of different cotton cultivars sampled on seven intervals throughout the season. Means are based on the sixth day total plant assessments (n = 270/cultivar).

COTTON CULTIVAR	EXPOSURE DATES							CULTIVAR MEAN
	18-01-2000	18-02-2000	07-03-2000	24-03-2000	11-04-2000	03-05-2000	19-05-2000	
DP50 (control)	7.17 **d***	16.50 **d***	19.33 **c***	19.33 **d***	23.00 **d***	17.33 **e***	21.67 **c***	17.76
DP50 B	19.00 **d**	29.50 **c**	31.17 **b**	36.50 **b**	33.83 **c**	30.00 **d**	29.33 **a**	29.9
15985 B	22.50 **c**	30.50 **c**	31.67 **b**	31.83 **c**	31.50 **b**	33.00 **c**	26.83 **b**	29.69
15985 X	20.50 **b**	36.00 **b**	36.83 **a**	35.67 **b**	38.17 **a**	31.67 **b**	29.67 **a**	32.64
15985 BX	24.50 **a**	34.83 **a**	37.33 **a**	37.67 **a**	38.83 **a**	38.33 **a**	30.00 **a**	34.5
Date Mean	18.73	29.47	31.27	32.2	33.07	30.07	27.5	
SEM Cultivar	**42**							
SEM Date	**30**							
SEM Cultivar/Date	**6**							
F probability	**P<0.001**							
LSD (5%) Cultivar	**1.01**							
LSD (5%) Date	**1.19**							
LSD (5%) Cultivar/Date	**2.67**							

***** - (within a column) means with the same letter do not differ significantly at 5% level.

The highest mortality of *H. armigera* was recorded on cultivar 15985 BX (cultivar effect, 5% LSD = 1.01) (Table 3.3), when the total plant observations were analysed. No significant differences between cultivars 15985 X and 15985 BX on 07/03/2000, 11/04/2000 and 19/05/2000 were detected when evaluated within the cultivar-versus-date interaction. Interpretation based on performance of the cultivars in controlling *H. armigera* neonate larvae, based on total plant results, indicated that cultivar 15985 BX caused the highest mortality, followed by cultivar 15985 X, 15985 B and DP50 B (Table 3.3). Results of similar studies conducted in South Carolina and

Louisiana (USA) also confirmed that the Bt-cotton cultivar 15985 BX performed best in controlling *H. armigera* (Chitkowski *et al.* 2003, Gore *et al.* 2003).

During this study (2000) on day 6 post-exposure, on leaves, squares and bolls, all surviving larvae were weighed and the average mass per larva was determined (Table 3.4).

Table 3.4 The exposure dates, plant parts exposed, number of *Helicoverpa armigera* larvae exposed and the average mass/larva noted 6 days post-exposure.

Exposure Date	Plant Parts	Total Number Exposed	AVERAGE LARVAL MASS				
			DP50 Standard/Control	DP50 B Bt-cotton	15985 B Bt-cotton	15985 X Bt-cotton	15985 BX Bt-cotton
			(g)	(g)	(g)	(g)	(g)
18-01-2000	Leaves	**90**	0.00394	0.00229	0.00048	0.00075	0.00097
	Squares	**90**	0.00729	0.0005	0.00033	0.00215	0.00017
	Total	**180**	0.00562	0.0014	0.00041	0.00145	0.00057
18-02-2000	Leaves	**90**	0.00655	0.00121	0.00153	0.00258	0.00174
	Squares	**90**	0.0035	0.0002	0.00067	0.00017	0.00221
	Bolls	**90**	0.00458	0.00017	0.00017	0.00017	0.00017
	Total	**270**	0.00488	0.00053	0.00079	0.00097	0.00137
07-03-2000	Leaves	**90**	0.0064	0.00153	0.00256	0.00104	0.0014
	Squares	**90**	0.00384	0.00051	0.00054	0.00017	0.00046
	Bolls	**90**	0.00464	0.0017	-	0.00017	0.00017
	Total	**270**	0.00496	0.00074	0.00283	0.00046	0.00068
24-03-2000	Leaves	**90**	1007	0.0016	0.0023	0.00401	0.00134
	Squares	**90**	0.00452	0.0004	0.0004	0.0016	0.00017
	Bolls	**90**	0.00286	0.00017	0.00061	0.00017	-
	Total	**270**	0.00582	0.00072	0.0011	0.00193	0.00076
11-04-2000	Leaves	**90**	0.0061	0.00206	0.00189	0.00102	0.001
	Squares	**90**	0.01057	0.00051	0.0014	0.00172	0.00222
	Bolls	**90**	0.00471	0.00017	0.00046	-	0.00017
	Total	**270**	0.00713	0.00091	0.00125	0.00137	0.00113
03-05-2000	Leaves	**90**	0.00399	0.00103	0.00168	0.00082	0.00017
	Squares	**90**	0.00203	0.00034	0.00023	0.00025	0.00017
	Bolls	**90**	0.01756	0.00017	0.00456	0.00022	-*
	Total	**270**	0.00786	0.00051	0.00216	0.00043	0.00044
19-05-2000	Squares	**90**	0.0032	0.0025	0.00212	0.0025	-
	Bolls	**90**	0.01537	-	0.00017	-	-
	Total	**180**	0.00929	0.0025	0.00115	0.0025	-

* - Larvae to small to weigh

Van der Walt *et al*. (1993) reported normal average mass of larvae on day six of development as 0.02 g. Results obtained with the standard/control cultivar DP50 reflects a much lower average mass/larva ranging from 0.00203 g obtained with surviving larvae on squares during 3 May 2000 to the highest 0.01756 g on bolls on the same date. All average weights obtained during the season were below the normal reported average, on the standard/control DP50 cultivar but, the normal weight reported was determined with larvae laboratory reared on an artificial medium (Van der Walt *et al*. 1993). With reference to this study laboratory reared larvae were exposed as neonates to different plant parts (normal field situation).

Larval average mass obtained with the Bt-cotton cultivars DP50 B and 15985 B ranging from 0.00017 g on different dates, to 0.0025 g on squares (DP50 B) on the last event and 0.00256 g on leaves (15985 B) on 7 March 2000. A decrease in average larval weight could clearly be seen between the standard/control cotton cultivar (DP50, 0.00786) and the two Bt-cotton cultivars DP50 B, 0.0025g and 15985 B, 0.00283g respectively.

Average larval mass obtained with the highly efficient two Bt-cotton cultivars 15985 X and 15985 BX were recorded as 0.00017 g on various dates with the highest average mass 0.00401 g on leaves (15985 X) on 24 March 2000 and 0.0022 g on squares (15985 BX) on 11 April 2000.

Although mortalities between the Bt-cotton cultivars 15985 X and 15985 BX differed significantly from the other cultivars DP50 B and 15985 B, a difference on the larval weight could not be recorded.

Results however showed retarded larval growth on all Bt-cotton cultivars. This explicit characteristic showed clearly that Bt-cotton plant parts also had a repellent effect, because no or minimal feeding occurred in most instances.

SPODOPTERA LITTORALIS

An opportunity was created by Monsanto (Pty) Ltd, to test the efficacy of the different Bt-cotton cultivars in controlling *S. littoralis*. On one occasion only (Table 3.5), the same evaluations with the five different cotton cultivars as described in (Table 3.1), were performed with *S. littoralis*, neonate larvae from a laboratory reared population. Specimens were sampled on green beans as

host plants and took place in the Marble Hall district (Mpumalanga Province, South Africa) and reared through to the F_1- generation, which was used during this study.

The same plant parts as used in the *H. armigera* studies (leaves, squares, bolls), were used during this exposure performed on 02 May 2002. Although results obtained with the standard/control cultivar DP50 and the Bt-cotton cultivar DP50 B were comparable, within the exposures to leaves and squares; leaves (DP50 -3), (DP50 B - 7) and squares (DP50 - 20), (DP50 B - 8), no significant differences could be found (Table 3.6). This results also showed that no differences were obtained between DP50 (standard/control) and the two Bt-cotton cultivars DP50 B and 15985 B. Total number of specimens controlled with (DP50 (standard/control) - 49); (DP50 B - 58) and (15985 B - 63), showed therefore that this Bt-cotton cultivars cannot successfully control *S. littoralis*. Results obtained with the Bt-cotton cultivars 15985 X and 15985 BX showed much higher mortalities of (158) and (182) respectively. This numbers resulted in % mortalities of (15985 X - 58.5 %) and (15985 BX - 67 %). Compared to results of *H. armigera,* this Bt-cotton cultivars cannot control *S. littoralis* to the same extent as *H. armigera*.

Due to the single event evaluation only, it is impossible to predict what would happen in a full growing season. The results Table 3.5, however reported here should be regarded as a reference study for further evaluations in South Africa. Chitkowski *et al.* (2003) reported positive results from field studies of different Bollgard cultivars in controlling *Spodoptera frugiperda* (J.E. Smith) and *Spodoptera exigua* (Hübner).

Table 3.5 The exposure dates, plant parts exposed, number of *Spodoptera littoralis* larvae exposed and the average mass/larva noted 6 days post exposure.

Exposure Date	Plant Parts	Total Number exposed	MORTALITY OBSERVATIONS				
			DP50 Standard/Control	DP50B Bt-cotton	15985 B Bt-cotton	15985 X Bt-cotton	15985 BX Bt-cotton
			Total	Total	Total	Total	Total
02-05-2000	Leaves	90	3	7	19	39	41
	Squares	90	20	8	11	56	58
	Bolls	90	26	43	33	63	83
	Total	270	49	58	63	158	182

The data was analysed by Analysis of Variance (ANOVA) testing for differences between cultivars (Table 3.6). The ANOVA F-test indicated highly significant differences (P<0.001) between cultivars exposed on 2 May 2000, for each plant part as well as on the total plant. Mean separation of cultivars was analysed using Fisher's Protected T-test LSD (Least Significant Differences) of means at the 5% significance level.

Table 3.6 Analysis of Variance of *Spodoptera littoralis* mortality counts.

SOURCE OF VARIATION	DEGREES OF FREEDOM	
CULTIVAR	*C-1 ∴ 5-1	= 4
RESIDUAL	**N-C ∴ 30-5	= 25
TOTAL	N-1 ∴ 30-1	=29

***C** = 5 Cultivars
****N** = 5 x 6 replicates = 30 experimental units

Table 3.7 shows the mortality means and significant differences between the cotton cultivars when *S. littoralis* neonate larvae were exposed to the different plant parts (leaves, squares and small bolls). This is interpreted that the different cultivars controlled *S. littoralis* significant differently on the once off evaluation.

Table 3.7 Mortality means and significant differences between the cotton cultivars when neonate larvae of *Spodoptera littoralis* were exposed to the different plant parts. The exposure took place on 2 May 2000 and the totals are based on the sixth day assessments.

COTTON CULTIVARS	TOTAL/PLANT	LEAVES	SQUARES	SMALL BOLLS
DP50 (control)	8.17 **b***	0.50 **c***	3.33 **c***	4.33 **e***
DP50 B	9.67 **b**	1.17 **cb**	1.33 **b**	7.17 **d**
15985 B	10.50 **b**	3.17 **b**	1.83 **b**	5.50 **c**
15985 X	26.33 **a**	6.50 **a**	9.33 **a**	10.50 **b**
15985 BX	30.33 **a**	6.83 **a**	9.67 **a**	13.83 **a**
SEM	1.42	0.79	0.684	0.462
F probability	P<0.001	P<0.001	P<0.001	P<0.001
LSD (5%)	4.14	2.3	2	1.35

***** - (within a column) means with the same letter do not differ significantly at 5%.

Generally cultivar 15985 BX exhibited the highest rate of mortality, although not significantly different from 15985 X, except when analysis was performed on small bolls. Although cultivar DP50 (control) generally had the lowest mortality counts, it did not differ significantly from cultivar DP50 B and 15985 B when analysis were performed on the total plant mortality counts.

Although the results expressed in Table 3.7 shows significant differences between the Bt-cultivars 15985 X and 15985 BX to all the other cultivars, this is not definite proof that these two cultivars are adequate in controlling *S. littoralis* neonate larvae due to the fact that evaluations were performed on one day only. To be able to prove the efficiency of the Bt- cultivars more replicates throughout the season are required.

Table 3.8 The exposure dates, plant parts exposed, number of *Spodoptera littoralis* larvae exposed and the average larval mass determined 6 day post-exposure.

Exposure Date	**Plant Parts**	**Total Number exposed**	**AVERAGE LARVAL MASS**				
			DP50 Standard/Control	**DP50 B Bt-cotton**	**15985 B Bt-cotton**	**15985 X Bt-cotton**	**15985 BX Bt-cotton**
			(g)	**(g)**	**(g)**	**(g)**	**(g)**
02-05-2000	Leaves	**90**	0.00403	0.00276	0.00342	0.00134	0.00126
	Squares	**90**	0.0015	0.00155	0.0016	0.00201	0.00064
	Bolls	**90**	0.00085	0.0009	0.0008	0.00201	-*
	Total	**270**	0.00213	0.00171	0.00193	0.00179	0.00095

* - Larvae too small to weigh

Figure 3.1 depicts the percentage mortality obtained when *S. littoralis* was exposed to the different cotton cultivars.

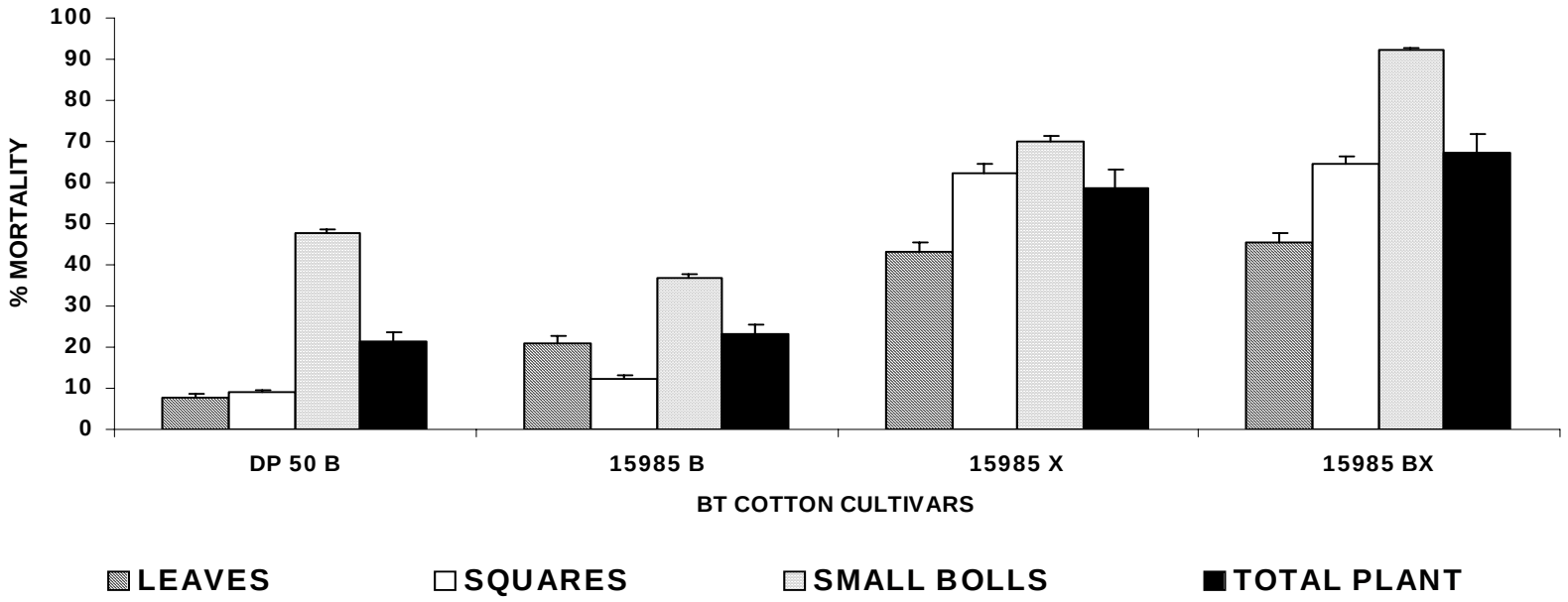


Figure 3.1 The percentage mortality obtained on 2 May 2000, after exposure of *Spodoptera littoralis* neonate larvae to different plant parts of the different cotton cultivars. Results are based on total mortality assessed 6 days post-exposure.

3.3.2 PERCENTAGE CONTROL (ABBOT'S FORMULA)

The percentage control of larvae on the cotton cultivars, were also determined using Abbot's formula based on total counts (6 x replicates) on day 6. Mortality of *H. armigera* were shown depicted in Figures 3.2 - 3.4 for leaves, squares and small bolls respectively, throughout the cotton growing season. Figure 3.5 depicts the percentage mortality on the plant parts lumped together (total) throughout the cotton growing season for *H. armigera* neonate larvae. In computing the effectiveness of insecticides, when an actual count of the living and dead insects in both the treated and untreated plots, or checks, is available, it is obvious that the insects which die from natural causes must be considered (Abbot 1925).

In Fig 3.2 it was immediately apparent that the percentage larval mortality on leaves of the different cultivars varied. On three of six sampling dates the larval mortality on DP50 B cultivar was the lowest (15, 8, 25) on 18 February, 11 April and 3 May 2000 respectively. The highest larval mortalities were obtained on cultivar 15985 BX on 18 January, 7 March, 11 April and 3 May 2000 as 55, 50 55 and 60 were recorded.

In Fig 3.3 it is clear that no obvious difference between cultivars DP50 B and 15985 B were experienced in mortalities when *H. armigera* larvae were exposed to squares. Although differences occurred on two dates (24 March and 11 April) no other differences were apparent. It is clear that cultivars 15985 X and 15985 BX expressed the Bt-genes best, therefore resulted in the highest control of between 70 and 100 % control.

In Fig 3.4 higher % control was experienced with again cultivars 15985 X and 15985 BX, performing best. This trend in results were also experienced in Fig. 3.5 when totals of all plant parts were depicted.

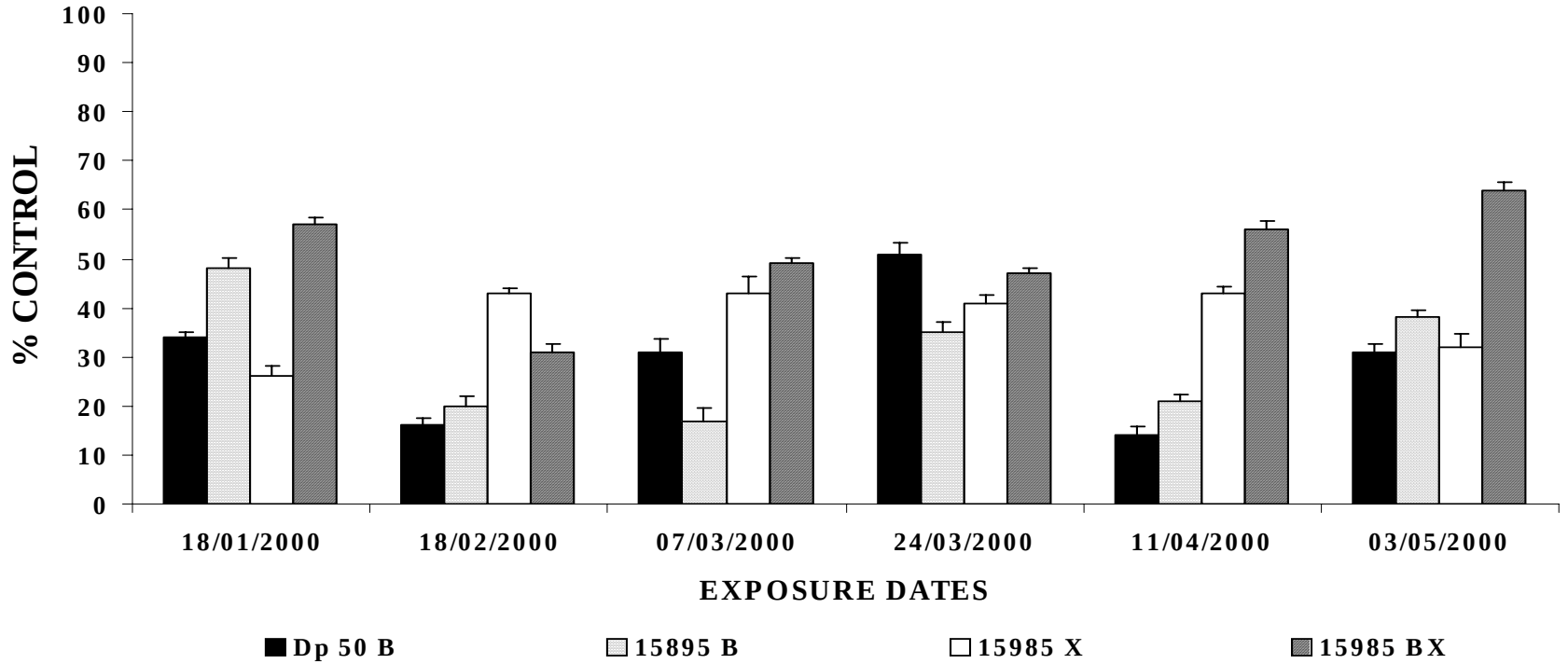


Figure 3.2 The percentage control obtained during the cotton growing season, after exposure of *Helicoverpa armigera* neonate larvae to leaves of the different cotton cultivars. Results are based on total mortality assessed 6 days post-exposure. Correction factor: Abbot's formula (Abbot 1925).

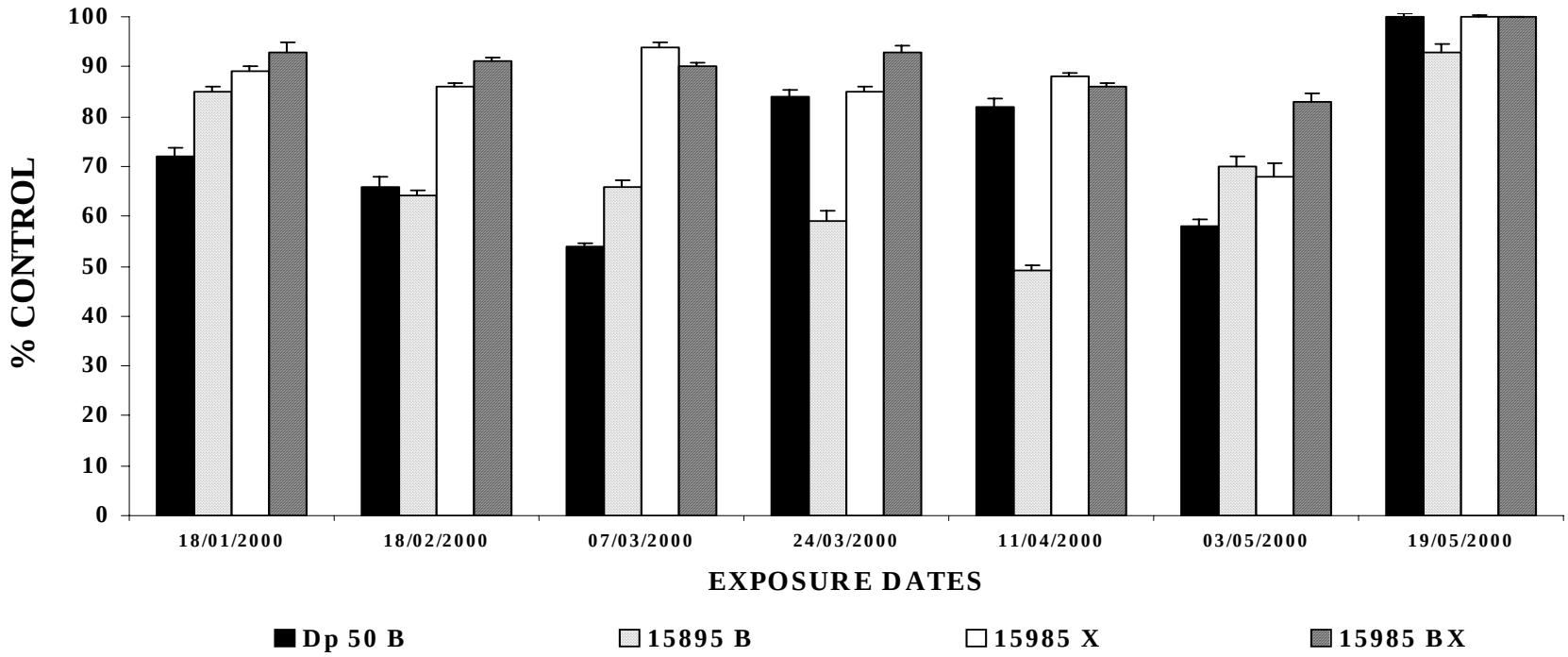


Figure 3.3 The percentage control obtained during the cotton growing season, after exposure of *Helicoverpa armigera* neonate larvae to squares of the different cotton cultivars. Results are based on total mortality assessed 6 days post-exposure. Correction factor: Abbot's formula (Abbot 1925).

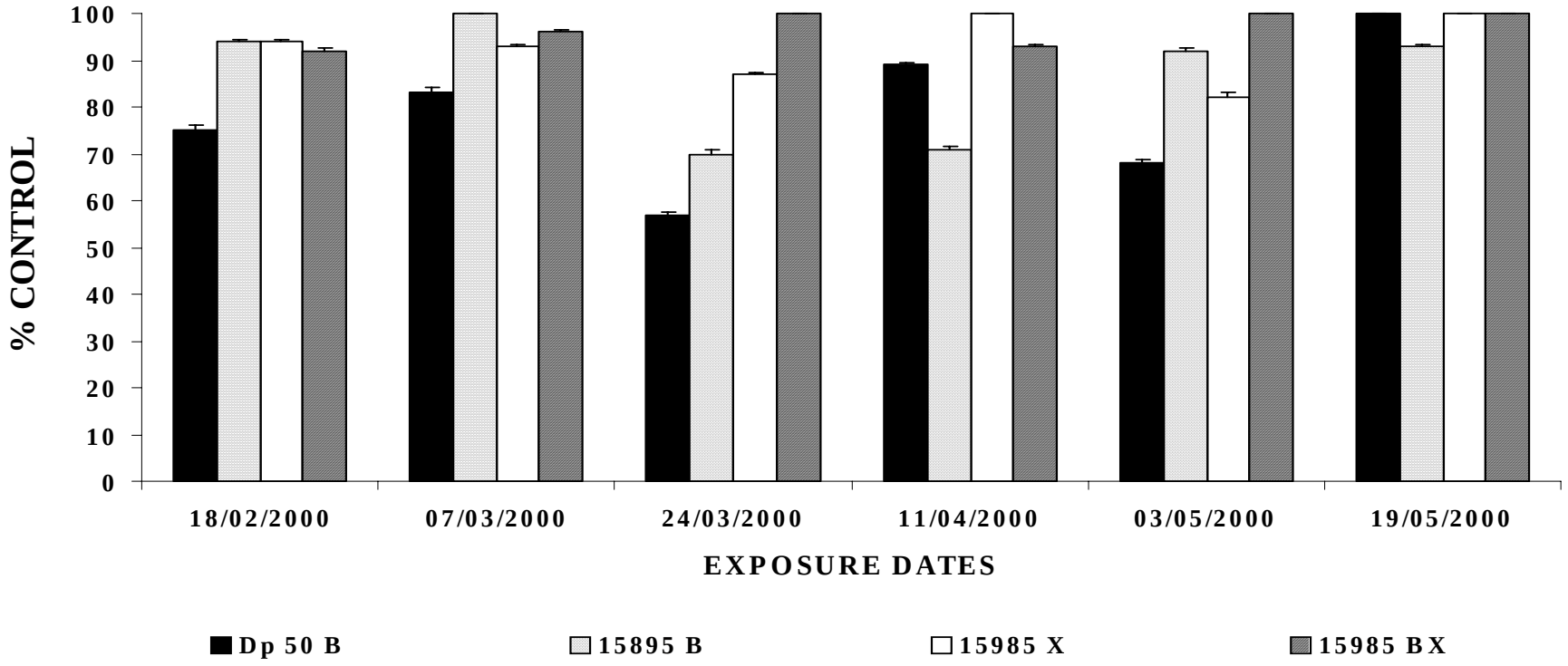


Figure 3.4 The percentage control obtained during the cotton growing season, after exposure of *Helicoverpa armigera* neonate larvae to bolls of the different cotton cultivars. Results are based on total mortality assessed 6 days post-exposure. Correction factor: Abbot's formula (Abbot 1925).

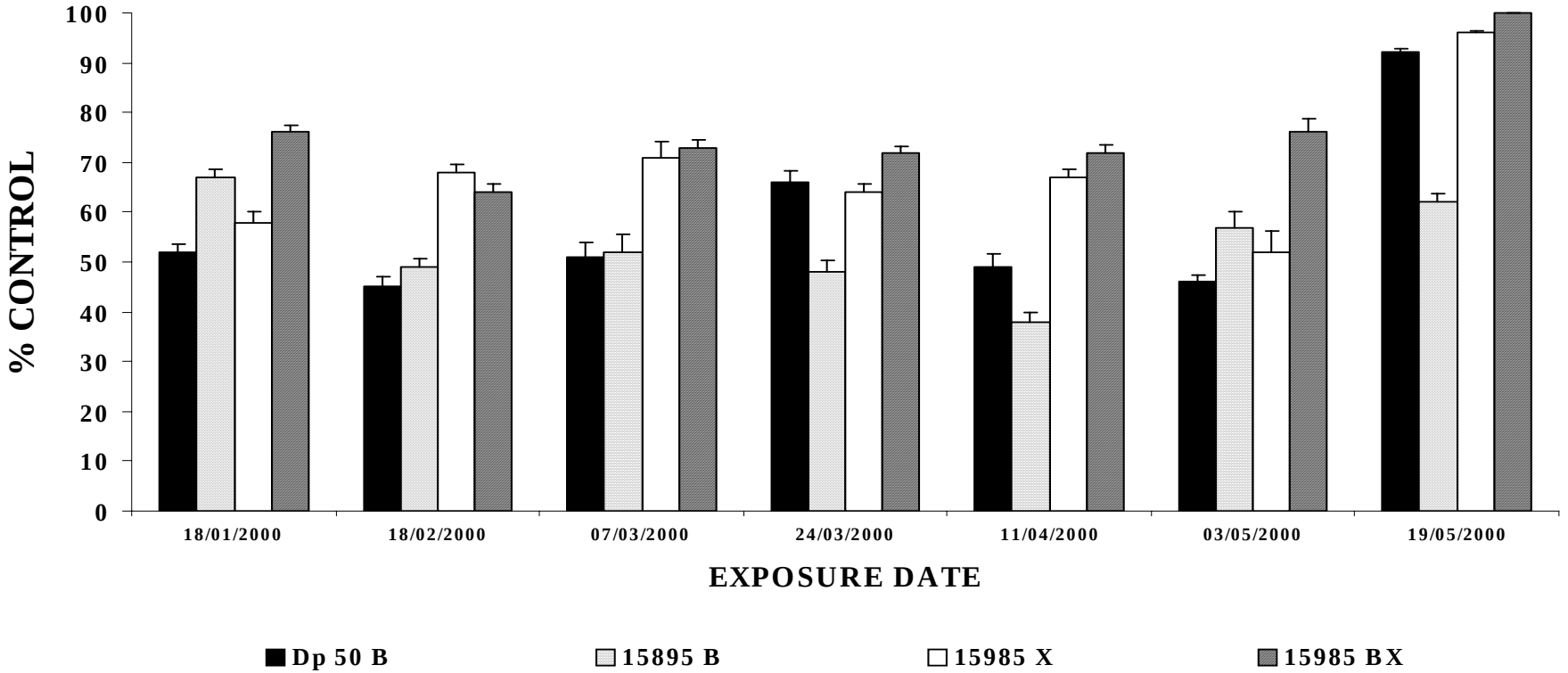


Figure 3.5 The percentage control obtained during the cotton growing season, after exposure of *Helicoverpa armigera* neonate larvae to all plant parts (leaves, squares and bolls) of the different cotton cultivars. Results are based on total mortality assessed 6 days post-exposure. Correction factor: Abbot's formula (Abbot 1925).

3.4 DISCUSSION

All Bt-cotton cultivars cultivated during the cotton season provided good control of *H. armigera* bollworm larvae. Mortality on terminal leaves was low, because they did not feed. Despite the fact that *H. armigera* larvae preferred squares as their optimal food source (Van der Walt 1988, Annecke & Moran 1982), mortality on squares was also high. The high mortality which occurred on the small bolls was related to the fact that small bolls are also not an optimal food source for neonate larvae and thus natural mortality could occur as a result of starvation (Table 3.1).

Although results showed significant differences between the Bt-cultivars 15985 X and 15985 BX to all the other cultivars, this is no definite proof that these two cultivars are adequately in controlling *S. littoralis* neonate larvae. Evaluations with *S. littoralis* were performed only once. To be able to prove the efficiency of the Bt-cultivars more replicates should be done throughout the season.

Greenplate *et al.* (2000b), Jackson *et al.* (2000), Stewart *et al.* (2001) all reported that the cotton cultivars containing more than one Bt-gene, enhanced levels of lepidopteran control when compared with the current single-gene transgenic varieties in laboratory and field studies.

In conclusion for this study it is clear that there is generally no difference in the expression of the Bt-gene throughout the cotton season and that the cotton cultivars 15985 X and 15985 BX were the most effective in controlling neonate larvae of *H. armigera.*

The efficacy of and the *B. thuringiensis* content in Bt-cotton decrease as cotton plants age (Olsen & Daly 2000). In a study conducted by Greenplate (1999), CryIAc values decreased from 57.1 µg/g dry weight 53 days after planting to 6.7 µg/g 116 days after planting. Mean terminal CryIAc values decreased from 163.4 µg/g to 34.5 µg/g dry weight over the same time period. Greenplate (1999) concluded that CryIAc levels in fruit and terminals of Bollgard cotton decline steadily over the growing season with higher CryIAc levels in the terminals than in the fruit throughout the season.

Olsen & Daly (2000), argued that it is not only a decrease in the concentration of CryIAc, but also physiological changes in cotton plants and environmental factors that alter the toxicity of Bt-cotton over time, e.g. an increase of tannin content in older cotton plants decreases the efficacy of CryIAc.

Concerns by the scientific community have been expressed on the use of only one Bt-gene. It is possible that the insects will become resistant to this gene in the same way as they do to insecticides (Chapter 4).

CHAPTER 4

RESISTANCE MANAGEMENT

4.1 BT-TOXIN INTERACTION AND RELATIVE CONTRIBUTION IN NEW COTTON STRAINS

Insect resistance management (IRM) regimes for Cry1Ac-expressing cotton included, mainly the implementation of non-Bt-cotton refuges to preserve susceptible individuals and dilute resistance alleles (Gould *et al.* 1994, Roush 1994a,b). The refuge was considered to be most effective if resistance was functionally recessive and if concentrations of toxin in plants were sufficient to kill all or nearly all heterozygous individuals for the resistance allele (Roush & McKenzie 1987, Roush & Daly 1990). While these so-called high-dose criteria were assumed for extremely sensitive species like *Heliothis virescens* and *Pectinophora gossypiella* (Saunders) (Bartlett *et al.* 1995, Gould 1998, Henneberry *et al.* 2000), species like *Helicoverpa zea* and *Heliothis armigera* (Hübner), (New- and Old-World bollworms, respectively) are inherently less susceptible to Cry1Ac and could not be included in the high-dose scenario (Greenplate *et al.* 1998) (Cotton Research and Development Corporation 2000). However, it was suggested that even if high-dose requirements were not fulfilled, a refuge will generally be beneficial in diluting resistance alleles (Roush 1994).

The utility of toxin mixtures has been considered as a strategy for resistance management (Gould 1986, Tabashnik 1989, Roush 1997, 1998, Greenplate *et al.* 2003) with the potential to delay resistance development, especially if the toxins in question independently demonstrated high-dose characteristics *in planta* and if target insects did not exhibit cross-resistance (Gould 1986, Roush 1997, 1998). Roush (1998) suggested that co-expressed, or pyramided, toxins provide value in delaying resistance even in circumstances where high-dose criteria for both toxins are not met. Gould *et al.* (1992) suggested that it may be, even in the face of a threat of cross-resistance, if the activities of two pyramided toxins are independent and additive in nature, and if the expression of both toxins leads to the expression of a high-dose effect for the combination, these pyramided toxins can also delay resistance, even with a possibility of cross-resistance, if combined with the

effective use of refuges, and if introduced prior to the development of significant resistance to either toxin.

Cotton varieties which simultaneously express two different *Bacillus thuringiensis* ∂-endotoxins, have been developed. Although not yet granted regulatory approval for movement into commerce, these cotton varieties have demonstrated enhanced levels of lepidopteran control when compared with the current single-gene transgenic varieties in laboratory and field studies (Greenplate *et al.* 2000, Jackson *et al.* 2000, Stewart *et al.* 2001). A modified Cry1A gene encoding a chimeric Cry1Ab/1Ac toxin (Hofte & Whiteley 1989, Perlak *et al*. 1990), and a modified Cry2Ab gene encoding a Cry2Ab toxin (Widner & Whiteley 1989, Dankocsik *et al.* 1990) are expressed in these plants.

A comparison of amino acid sequences revealed that Cry1Ac and Cry2Ab share <30 % identity (Crickmore *et al.* 1998). The relationship between Cry2Ab and Cry1Ac in terms of lepidopteran resistance management is being explored. Akhurst *et al.* (2002) and Akhurst *et al.* (2003) demonstrated no cross- resistance to plant-produced Cry2Ab in a Cry1Ac resistant *H. armigera* population. Although no studies to determine cross-resistance of *H. armigera* populations in South Africa, to the different Bt-cotton strains, were performed to date, results discussed in Chapter 3 revealed that the different Bt-cotton strains can adequately control *H. armigera* up to 2003.

4.2 ALTERNATIVE HOST PLANTS

For Bt-cotton technology to be preserved, build-up of resistance to the Bt-toxin or toxins expressed in the transgenic cotton plant has to be prevented. An important resistant management prevention/requirement includes the necessity, that every field of insect-resistant crops must have an associated refuge of non-GM crops in order for the insects to develop without selection to the insect-resistant varieties. According to Mallet & Porter (1992), the susceptible genes in the bollworm population can be conserved by planting ‘refugia’ or ‘toxic-free’ non-transgenic-cotton plants on the same cultivated land. Resistance in bollworm populations can occur if bollworms reach maturity in a field of Bollgard cotton because of the presence of a tolerant gene within their populations (Green *et al.* 2003a).

If only Bt-cotton is available to larvae and generation after generation fed on these host plants it can give rise to potentially resistant larvae and the survival of the resistant/tolerant gene in the population will be ensured. However if a refuge is planted or alternative natural hosts are available so that movement of moths occurs from transgenic-cotton to alternative non genetic host plants the build up of Bt-resistant lepidopteran pests can be prevented. As moths migrate, susceptible males and females from non Bt-plants could mate with males and females that fed and reached maturity on Bt-cotton. The moths maturing on the Bollgard cotton, are potentially more resistant or tolerant towards the gene, and they have a higher probability of mating with a susceptible individual from the larger population of moths originating from the refuge area, or with moths from any other host plant from the surrounding natural bush or field. The probability of establishing this tolerant gene pool therefore, becomes much smaller since this interaction could cause sufficient genetic dilution to counteract selection for resistance against the effect of the toxin expressed by transgenic cotton plants (Green *et al.* 2003b).

According to Green *et al.* (2003b), a purchaser of Bollgard™ (Bt-cotton) in South Africa is under obligation to sign a license agreement, stating that for every 100 ha of Bollgard cotton planted, a refuge of either 20 ha sprayed non-transgenic cotton, or 5 ha unsprayed non-transgenic cotton will be planted. The small-scale farmers on the Makhatini Flats, however, may neglect planting this refuge and a possible 'alternative' that could act as refuge hosts.

Annecke & Moran (1982), reported alternative host plants for *H. armigera* larvae, such as *Ricinus communis*, mainly in the northern parts of South Africa, *Chicorum intybus*, mainly in the Eastern Cape and *Gossypium hirsitum* (wild cotton) in the cotton growing areas of South Africa.

Green *et al.* (2003), reported evidence that on the Makhatini Flats in Kwa-Zulu Natal, South Africa, the four bollworm species making up the bollworm complex on cotton, African bollworm *Helicoverpa armigera* (Hübner) (also called American bollworm), spiny bollworms *Earias biplaga* (Walker) and *Earias insulana* (Boisduval) and the red bollworm *Diparopsis castanea* (Hampson), maintain natural populations on alternative host plants. Green *et al.* (2003), reported that African bollworms were found on eight plant species, namely, *Abutilon austr-africanum* (Hochr.), *Abutilon guineense* ((K.Schum.) Baker f. & Exell), *Acanthospermum hispidum* (DC), *Cienfuegosia hildebrandtii* (Gärcke), *Corchorus trilocularis* (L.), *Hibiscus vitifolius* (L.), *Justicia flava* ((Vahl) Vahl) and *Pavonia burchellii* ((DC.) R.A.Dyer). These plant species could be divided into the following groups: (1) pre-cotton alternative host plants: *C. hildebrandtii*,

Hibiscus calyphyllus (Cav.), *H. vitifolius, J. flava, Sida dregei* (Burt Davy) and *Sida cordifolia* (L.); (2) alternative host plants during the cotton-flowering phase: *A. austro-africanum, A. guineense, Sida rhombifolia* (L.) subsp. *rhombifolia, Malvastrum coromandelianum* ((L.) Garke) and *Melhania forbesii* (Planch. Ex Mast.); (3) post-cotton alternative host plants: *A. hispidum, Hibiscus cannabinus* (L.) var. *cannabinus, H. vitifolius, Hibiscus praeteritus* (R.A. Dyer) and *S. dregei*.

Most of the plant species identified as bollworm hosts were widespread across the Makhatini Flats, especially *Abutilon* spp., *Hibiscus* spp., *Sida* spp. and *C. trilocularis. C hildebrandtii*, were abundant in small areas or patches of natural veld (Green *et al.* 2003). The number of host plants found in the possible refuge area (veld or fallow fields) (5%) exceeded the number of host plants found in the Bt-fields for all species groups except *A. austro-africanum*, indicating that these areas could be considered as an alternative refuge to non-Bt cotton. Bollworms found on alternative host plants indicated that these plants could serve as an alternative to cultivated cotton plants later in the season. The constant presence of weeds and indigenous plants that act as hosts for the bollworm complex provided a year round source of food for the bollworm complex. The bollworm populations arising from non cotton host plants were thus likely to interbreed with bollworms exposed to the Bt-gene in Bollgard™ cotton (Green *et al.* 2003).

4.3 ALTERNATIVE METHODS AND IMPLEMENTATIONS FOR RESISTANCE MANAGEMENT

Various methods have been suggested and some implemented to slow the possibility of insect resistance to Bt-cotton:

- The use of non-transgenic-cotton within a transgenic-cotton field to provide refuge for susceptible moths (25 % non-Bt cotton). Peck *et al.* (1999), found that resistance to *B. thuringiensis* developed faster in areas where refuge fields were changed randomly than in areas where the same refuge fields were used from year to year. The success of using mixed stands of Bt- and non-Bt-cotton, depended on the size of the susceptible populations, adult mobility (Halcomb *et al.* 1996), amount of larval movement (Halcomb *et al.* 1996; Peck *et al.* 1999), feeding and the proportion of resistant to susceptible individuals (Halcomb *et al.* 1996).
- Preservation of crop refuges to allow homozygous susceptible insects to breed with resistant insects to produce heterozygous susceptible insects (Halcomb *et al.* 2000).

- Adding additional Bt-genes to increase the number of endotoxins (Benedict *et al.* 1993; Sachs *et al.* 1996; Matthews 1997; Hilder & Boulter 1999; Peck *et al.* 1999).
- Combining insecticidal proteins with insect resistant plant traits (pyramiding), e.g. Sachs *et al.* (1996) found that pyramiding of CryIA(b) with the high-terpenoid trait increased resistance to tobacco budworm *H. virescens* and improved the durability of CryIAb in cotton. Greenplate *et al.* (2003) reported that in a study, where transgenic-cotton expressing two toxins encoded by different modified Bt genes, Cry1Ac and Cry2Ab, results showed a substantial increase in toxicity to *H. virescens* and *H. zea*, over the Cry1Ac-only cotton. Comparison of mean responses showed Cry2Ab to be the major contributor to the total insect activity in the two-gene cotton plants.
- Using tissue-specific expression of *B. thuringiensis*, e.g. expression in the reproductive fruiting plant organs and not in the leaves (Benedict *et al.* 1993; Sachs *et al.* 1996).
- Low dose expression in protected plant parts, rotating or eliminating specific *B. thuringiensis* products through time (Benedict *et al.* 1993; Sachs *et al.* 1996).
- Production of low sub-lethal doses in some or all plants to promote interaction with natural enemies and sequential introduction of cultivars that produce different insecticidal proteins for which different adaptive strategies are needed by the target insects (Sachs *et al.* 1996).

If taking the following factors, that might influence the rate at which lepidopterans can become resistant to Bt-cotton, into consideration, the success of managing and/or preventing resistance development are pliant. The factors are: (1) the number of generations of bollworms exposed each year to Bt- plants containing the same or similar toxins; (2) the percentage of each generation exposed to Bt-plants containing the same or similar Cry-toxins, the mortality level that Cry-toxin causes among bollworms carrying one copy of a resistance allele and one copy of a susceptible allele (the mortality level is determined by the Cry-toxin concentration in the plant, which in turn may determine the functional dominance of the allele affecting resistance); (3) the frequence with which Cry-resistance alleles are expressed in the bollworm population before exposure to Cry-toxins and the dominant or recessive nature of the resistance alleles; (4) the migration patterns of bollworm moths; (5) the survival advantage or disadvantage that resistance allele(s) offer bollworms in the presence and absence of Cry-toxins; (6) the number of susceptible moths available for mating with moths carrying resistance gene(s).

4.4 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Bollgard™ cotton, or cotton containing the Bt-gene, has the ability to control the bollworm complex (Frederici 1993) and the introduction of Bollgard™ cotton varieties, (NaCotn37^{Ba}and NaCot^{35B}), to the South African cotton industry offers an effective means of controlling the bollworm complex *(Helicoverpa armigera, Diparopsis castanea* and *Earias insulana*). The reduced need of insecticides when planting Bollgard™ cotton created new management opportunities for commercial and the small scale farmer, in rural communities, where insecticides are costly and difficult to apply effectively. Consequently the complex of other insect pests on cotton still has to be controlled chemically where needed. This however would increase the overall production costs of Bt-cotton. Pests that would need additional chemical control are e.g. cotton aphids, jassids, red spider mites, cotton stainers, thrips and white flies (Hardee *et al.* 2001)

Before introducing Bt-cotton to the South African market the efficacy of such cotton against *H. armigera* larvae, was well-known in different countries in the world, but laboratory studies and field studies were also needed for South African environmental circumstances. These test were performed during 1998 in the laboratory on artificial diet, to which different concentrations (2 µg/g, 8 µg/g, 32 µg/g, 128 µg/g and 512 µg/g) of the Bt-protein Cry1Ac was added and larvae exposed too. *H. armigera* larvae were also allowed to feed on leaves, squares and bolls of different cotton cultivars (expressing different Bt-genes). In all tests neonate *H. armigera* larvae were used and mortalities and subsequent larval development were followed.

When using test results from exposing larvae of *H. armigera* to different plant parts, the results may be wrongly interpreted because the favourite larval food (neonate - 2nd instars), was squares (Van der Walt 1988) and not leaves or bolls thus the highest mortality was obtained on squares, indicating that the favourite food of neonate and subsequent larval stages was squares. Thus it does not mean that the Bt-gene was expressed the highest in squares only (Chapter 3).

Olsen & Daly (2000) and Greenplate (1999), indicated that Cry proteins decreased as cotton plants matured (Chapter 3). This could not be proofed during the part of this study (Chapter 3) as percentage control of larvae feeding on all plant parts remained high throughout the season (Chapter 3) (Fig. 3.1 - Fig. 3.5). Although cultivar-versus-date interactions were significant ($P < 0.001$) (Table 3.2) no decline in the mortalities of larvae were found, as plants matured throughout the season. Therefore no decline of the Cry protein over the cotton growing was

experienced. A study conducted by (Van Jaarsveld 2000) (E1999/008C) for Monsanto (Pty) Ltd, also concluded that even with ratoon (stand-over cotton) Bollgard cotton in the Limpopo Province, South Africa, no decrease in mortalities of *H. armigera* was shown.

Results of laboratory monitoring of the susceptibility of *H. armigera* field populations (Chapter 2) indicated no increased LD_{50} values of the tested larval populations over the study period (1998, 2000 and 2003). Results however during this study period in South Africa for the effectiveness of Cry1Ac, did not guarantee a total absence of resistance to the Bt-gene. The results also demonstrated that the field populations evaluated, were susceptible to the Cry1Ac protein and that a movement towards resistance in the *H. armigera* field populations was not indicated.

The key to managing resistance in *H. armigera* to insecticides were to vary the control practices so that the selection pressure would be insufficient for the insect to develop resistance to any one control measure. This could also be true when Bt-cotton varieties are used as a control measure against *H. armigera*. It is already recommended that within a Bt-cotton planting approximately 20 % of a field should be planted to non-Bt-cotton to allow for exchange of genes with bollworm from non-Bt-cotton. The role of alternative bollworm host plants in the vicinity of Bt-cotton could also play an important role. This should therefore be the basis for long term, sustainable pest control in cotton.

Current (GM) Genetically modified crops, in conjunction with conventional agricultural practices, can contribute to a cost effective, sustainable, productive and sufficiently safe form of agriculture. Reduced use of insecticides could lead to cost effectiveness and less impact on the environment. It also opens new crop production levels for both commercial and small scale farmers. Widespread adoption of Bt-cotton led to regional declines in bollworm populations, even after taking into account reduced insecticide use. Bt-cotton led to long term suppression of bollworms. Transgenic crops open up new avenues for pest control. One practical means of increasing yield would be to protect more of what is grown from loss to pests, especially insect pests. Genetically engineering inherent crop resistance to insect pests offers the potential of a user, environment and consumer-friendly method of crop protection to meet the demands of sustainable agriculture in the 21st century.

Cotton and thus Bt-cotton is only one of many host plants of *H. armigera* (Green *et al.* 2003a). On cotton the preferred food for neonate larvae are squares but older instars (2 - 5) feed on all

plant parts. If neonate larvae are killed throughout the season on Bt-cotton, this could lead to a decline in larval populations reaching maturity on cotton. When *H. armigera* is controlled, cotton yields increase, this was already shown under insecticidal control and were also apparent where Bt-cotton was grown (Broodryk, *pers comm*. 2003). If Bt-cotton represents an important part of a pest diet, the pest is not too sedentary, and its reproductive potential is not too high, then declines can be observed. Effective Bt-technology in cotton represents an effective and safe means of controlling the major pests. This results in increasing yields, more efficient land usage and reduces the environmental impact of pest control in cotton production. Since cotton is grown on approximately 2.5 million hectares in Africa, most of which comprises small plots of less than 10 hectares, the introduction of Bt- cotton across Africa has the potential to dramatically increase cotton yields among smallholder farmers, thereby improving the quality of life of a great number of people. The successful and rapid adoption of this more expensive technology in the Makhatini Flats provides a model for smallholder cotton farmers in Africa and across the world, and testifies to the incredible benefits that can be achieved through the responsible implementation of agricultural bio-technologies.

The significant reduction in time taken for crop management and water collection necessary to make up the pesticidal sprays means that the women and children (who would usually undertake this task) have more free time for other activities, including education. By freeing up time traditionally spent on farming, greater opportunities exist for the family. They can grow other crops and spend more time in school. Benefits to the community are expected to result from these gains. The public and the environment also win from the health perspective since pesticide reduction use means less production, shipment, storage and exposure to chemicals. This is said to be one of the major advances (Cotton SA 2003, Http://www.cottonsa.org.za (March 2003)). Commercial farmers likewise has to increase yield and cut costs to remain profitable farmers and meet market demand.

The commercial growth of genetically modified *Bacillus thuringiensis* cotton cultivars can be recommended and offers a practical alternative in an IPM programme and could lead to considerable potential benefit for the cotton industry of South Africa. Bt-cotton have come to stay and the benefits will only continue as long as they are managed wisely. However, how long Bt-cotton remains effective may depend upon how well growers and pest managers follow resistance management guidelines. Improper usage dramatically decreases the effective life of a product. If Bt-cotton are carefully managed, their effectiveness may be extended for many years. But if

the technology is abused, bollworms will quickly become resistant. Preserving the effectiveness of Bt-cotton is one way to keep pest management costs at the lowest level.

Biotechnology is a very new field, and much about the interaction of Living Modified Organisms (LMO's) with various ecosystems is not yet known. Some of the concerns about the new technology include its potential adverse effects on biological diversity, and potential risks to human health. Potential areas of concern might be unintended changes in the competitiveness, virulence, or other characteristics of the target species; the possibility of adverse impacts on non-target species (such as beneficial insects) and ecosystems; the potential for weediness in genetically modified crops (where a plant becomes more invasive than the original, perhaps by transferring it's genes to wild relatives); and the stability of inserted genes (the possibilities that a gene will lose it's effectiveness or will be re-transferred to another host). While advances in biotechnology have great potential for significant improvements in human well-being, they must be developed and used with adequate safety measures for the environment and human health.

To ensure the continued effectiveness, of Bt-cotton in years to come, continued monitoring for potential resistant genes in *H. armigera* should be a long term objective. This is of great importance as the market for the commercial Bt-cotton expands. Continued collection and analysis of such data at least every 2 - 3 years are critical to the development and continued assessment of resistance management strategies. Together with these concerns regarding the safety of new proteins expressed in transgenic plants are critical components for future studies in Biosafety Risk Assessment.

This present study on the effect of Bt-cotton cultivars on *H. armigera*, holds great promise for the control of this key insect pest on cotton. The knowledge and results obtained in the laboratory has to be transferred to the field and already supports field data where increased yields when using Bt-cotton was obtained in spite of using less insecticidal sprays (Broodryk, Olivier *pers comm*). The information of this new technology and the use of it, should also be extended to all farmers and the general public, in that way they should understand why genetic engineering is important and why legislation and thus the responsible use of Bt-cotton, and the maintenance and cross breeding of *H. armigera* gene pods are critical. The "once off" results with *Spodoptera littoralis* should be extended and other lepidopteran pests also tested.

As other Bt-cotton cultivars become available and efficacy and resistance monitoring needs to be done, this study will serve as a benchmark for South Africa.

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