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The expression of Bt *Cry1Ac* in transformed cotton under abiotic stress

By

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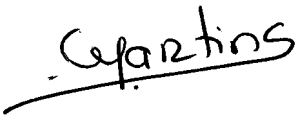
DECLARATION

I declare that the thesis/ dissertation, which I hereby submit for the Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Célia Marília Martins



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The expression of Bt *Cry1Ac* in transformed cotton Bt *Cry1Ac* under abiotic stress

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ABSTRACT

Bacillus thuringiensis (Bt) is a gram-positive common soil bacterium that produces crystals (*Cry*) containing proteins that are toxic to certain insects, in particular larvae of Lepidoptera and Diptera. The Bt toxin in the past has been widely used as a bioactive compound for the biological control of mainly lepidopteran pests. Most recently a variety of crops, including cotton and maize, have been genetically modified to express a Bt toxin to confer resistance to lepidopteran pests. However, the effect of abiotic environmental stress, such as drought and heat, which are typical for Africa, on Bt toxin expression in a genetically modified crop has so far not been fully evaluated. This study focuses on the expression and stability of the *Cry1Ac* insecticidal protein from *Bacillus thuringiensis* in genetically modified cotton plants under drought and heat stress. These include the physiological and biochemical characterization of the expressed Bt toxin gene under drought stress as well as the biological activity against first-instar larvae of the African cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae). Non-genetically modified cotton (*Gossypium hirsutum* cv. Opal), as well as genetically modified cotton (cv. Nuopal) expressing the Bt

toxin *Cry1Ac*, were exposed to drought and heat stress. Drought stress was induced by withholding watering plants until the soil moisture content reached 25-30 % of field capacity. Non-stressed control plants were watered and soil moisture content to 80-100 % of field capacity was maintained. For heat stress, plants were grown at 38 to 32 ºC during the day and night, respectively, whereas control plants were grown in a growth cabinet at a 28/25 ºC day/night cycle. For growth analysis plants were harvested every second week after planting. At each harvest, different parts of the plant were collected and their fresh and dry weight determined. For biochemical analysis and determining biological activity against first-instar larvae of *H. armigera*, two types of experiments were carried out, the first experiment four weeks after treatment induction and the second experiment eight weeks after treatment induction. Different plant material (leaves, flowers and immature green bolls) were used for Bt detection as well as for determining biological activity against first-instar larvae of *H. armigera*. Under drought stress conditions a reduction in leaf area and leaf dry weight were found in both Bt toxin expressing and non-expressing cotton plants, but no significant difference in physiological performance between Bt-expressing and non-expressing cotton plants was found. This study shows that the Bt toxin (*Cry1Ac*) level decreases in senescent plants and that drought stress did not affect the growth and development of genetically modified Bt plants when compared to non-Bt plants. Although the expression of Bt toxin (*Cry1Ac*) in Bt cotton plants decreased under drought stress no effect on the efficacy of the toxin against *H. armigera* was observed. In addition, no significant decrease of Bt toxin content was found in Bt cotton leaves after exposure to heat stress when compared to leaves from non-heat stressed plants.

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Lastly, I would like to thank my husband Manuel, my son Manuel Alexandre and my little daughter Yélène, for the encouragement and love, as well as for their patience during the long absences from them during this study.

I would not like to forget to thank my mother and brothers for their unconditional love, too.

DEDICATION

I dedicate this thesis to my father Acácio, my sister Belinha and my brothers Lucrécio and Jair, who, while no longer here in body, will always be with me in spirit within my heart.

THESIS COMPOSITION

Chapter 1 of this thesis focuses on the current knowledge about *Bacillus thuringiensis* (Bt), cotton as an important crop plant in Africa and lepidopteran pests affecting cotton plants. Further, this introductory chapter outlines the use of genetically modified plants, in particular plants carrying the Bt gene, to control cotton pests and how expression of the Bt gene may be affected by abiotic environmental stresses.

Chapter 2 describes the different experimental procedures that were applied in this study. These include the physiological characterization of cotton plants under drought and heat stress. Also, techniques to determine Bt toxin gene expression and both stability and biological activity of the expressed Bt toxin gene under drought stress in cotton are outlined.

Chapter 3 reports the results from a physiological study. In particular the first section describes the performance of non-modified cotton (non-Bt) and genetically modified (Bt) plants expressing the Bt toxin gene under drought and heat stress when compared to cotton plants not expressing the Bt toxin gene.

Chapter 4 focuses on a gene expression and protein stability study. This section is concerned with detection of Bt toxin expression by immunological techniques, such as Western blotting and the ELISA technique, in different parts of Bt cotton plants under drought and heat stress.

Chapter 5 outlines results from a study to determine the biological activity of the expressed Bt toxin gene in cotton. In this particular study the efficacy of Bt toxin expression in Bt cotton plants to control the African bollworm larvae under drought and heat stress is discussed.

Chapter 6 finally contains a general discussion in which the results obtained in this study are outlined in the light of current knowledge about Bt toxin expression and stability under drought and heat stress, as well as the biological efficacy of Bt toxin against African cotton bollworm larvae under abiotic stresses. This chapter

also outlines the new scientific achievements made in this study and the perspectives of research to be addressed in future.

The **Annexure** provides details about the composition of buffers, solutions, and chemicals used in this study.

RESEARCH AIM AND OBJECTIVES

Bacillus thuringiensis (Bt) is a gram-positive common soil bacterium that produces crystals (*Cry*) containing proteins that are toxic to certain insects, in particular the larvae of Lepidoptera and Diptera. The Bt toxin has been widely used in the past as a bioactive compound for the biological control of mainly lepidopteran pests. More recently a variety of crops, including cotton and maize, have been genetically modified to express a Bt toxin to confer resistance to lepidopteran pests. However, the effect of abiotic environmental stress, such as drought and heat, which are typical for Africa, on Bt toxin expression in a genetically modified crop has so far not been fully evaluated.

The main aim of this project was, therefore, to study the performance of cotton carrying the Bt toxin gene and the expression of the gene under abiotic environmental stress.

To achieve this aim, the project had the following three objectives:

i) To analyse physiologically the performance of genetically modified Bt cotton plants under abiotic stress and compare it with that of non-modified cotton plants.

ii) To determine Bt gene expression and stability in genetically modified cotton under abiotic stress.

iii) To determine biological activity against lepidopteran pests in genetically modified plants under abiotic stress.

CHAPTER 1: LITERATURE REVIEW

1.1 *Bacillus thuringiensis* (Bt)

1.1.1 The bacterium

Bacillus thuringiensis (Bt) is a gram-positive endospore-forming bacterium characterized by the presence of a protein crystal within the cytoplasm of the sporulating cell (Figure 1.1). The proteins within this crystal are toxic to insects, especially the larvae of Lepidoptera and Diptera. This explains the extensive use of Bt as a biological insecticide. The ecology of Bt remains unclear. However, this ubiquitous bacterium has been isolated from soil, stored grain, insect remains and plant surfaces, and it is probably best described as an opportunist pathogen (de Maagd *et al.*, 2001)

Discovered in Japan in 1901 in dead silkworm larvae, Bt was first isolated in the Thuringia region of Germany in 1911. By 1958 it was already used commercially as an insecticide in the United States and captured 95 % of the bio-pesticide market in 1989. At least 34 subspecies and 800 different strains of this bacterium are used today in a variety of sprays and dusts to control both beetle larvae and moth and butterfly caterpillars, feeding on fruits, vegetables, and other cash crops including maize, potatoes, and cotton. Together the subspecies of this bacterium colonize and kill a variety of host insects and even nematodes. But each strain does so with a high degree of specificity, which is mainly determined by the type of crystal proteins (Table 1) that the bacterium produces during sporulation (de Maagd *et al.,* 2001).

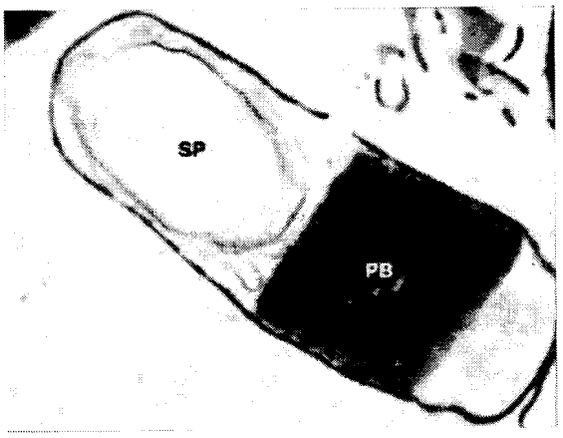


Figure 1. 1 Transmission electron micrograph of a sporulating Bt cell. δ-endotoxins are produced as regularly shaped crystals. PB denotes a protein body (crystal) and SP is a spore (de Maagd *et al.*, 2001).

1.1.2 Bt Classification

Bacillus thuringiensis (Bt) synthesizes a variety of insecticidal crystal proteins (ICPs) upon sporulation (Carozzi *et al.*, 1992). The structure of the ICPs results from the assembly of one or more types of protein subunits, the pro-toxins or δ-endotoxins. A number of different δ-endotoxins have been characterized, each having a relatively narrow insecticidal spectrum (Maizer *et al.*, 1997). In order to exert their entomopathogenic activicty, the δ-endotoxins have to be dissolved in the insect gut. Gut proteases, which specifically cleave the C-terminal of the protein (130 kDa), release smaller 60-70 kDa polypeptides, which are the active toxins (Hofte and Whiteley, 1989). These smaller polypeptides comprise a toxic motif as well as the determinants responsible for specificity, and show blocks of sequences that are remarkably well conserved among the different toxins (Sanchis *et al.*, 1989).

After the Bt toxin is ingested by the larvae of certain insect species, digestive enzymes specific to those insects dissolve the Bt *Cry* toxin and activate the toxic component. This endotoxin binds to certain receptors on the intestinal linings of the host, which causes lytic pores to form in the cell membrane of the intestine. By disrupting the ion balance of the intestine, this proliferation of pores in the insect's intestinal membrane causes the insect larvae to stop feeding and eventually to

starve to death (Levidow *et al.*, 1999). Although highly toxic to certain insects, Bt is relatively harmless to mammals, fish and birds, because their systems lack the digestive enzymes needed to convert Bt protein crystals into their active form.

Table 1. 1 Types of *Cry* toxins and the insect orders they affect.

***Cry* gene designation**	**Toxic to these orders**
CrylA(a), CrylA(b), CrylA(c)	Lepidoptera
CrylB, CrylC, CrylD	Lepidoptera
CryII	Lepidoptera, Diptera
CryIII	Coleoptera
CryIV	Diptera
CryV	Lepidoptera, Coleoptera

Four major classes of Bt crystal protein genes (*Cry* genes) have been recognized:

- Lepidoptera-specific
- Lepidoptera and Diptera-specific
- Coleoptera-specific
- Diptera-specific

Table 1.2 gives a simplified classification of the Bt genes based on the nomenclature of Hofte and Whiteley (1989).

Table 1. 2 Simplified classification of Bt toxin genes.

Toxin gene	**Molecular weight (kDa)**	**Insecticidal activity**
CrylA(a),(b),(c),B,C,D,E,F,G	130-138	Lepidoptera
CrylIA,B,C	69-71	Lepidoptera and Diptera
CrylIIA,B(b)	73-74	Coleoptera
	72-134	Diptera
CryV-CryX	35-129	Various

a) *CryI* genes

Genes of the class *CryI* encode 130-160 kDa pro-toxins, which combine to form bi-pyramidal protein crystals, which typically contain more than one gene product (Bulla *et al.*, 1975). One half of the molecule (C-terminal end) is highly conserved (70 % or more) and possibly involved in the packing of toxins within the crystalline inclusion (Hofte and Whiteley, 1989), whereas the other half (N-terminal), which is the toxic domain, is involved in ICP formation and is the more variable region (Adang *et al.*, 1987). *CryI*-type proteins typically comprise approximately 1200 aminoacids and range in size from 131.0 to 133.3 kDa (Gawron-Burke *et al.*, 1990). *CryIA* is the most common type of crystal protein found in *CryI*-producing Bt strains (Ceron *et al.*, 1994) and the *CryIA(b)* gene appears to be the most widely distributed gene amongst different Bt subspecies (Yamamoto and Powell, 1993). However, many strains produce several different types of ICPs simultaneously and the same (or very similar) crystal proteins occur in different strains (Hofte and Whiteley, 1989).

b) *CryII* genes

Genes of the class *CryII* encode 65 kDa proteins, which occur as distinct cuboidal inclusions in several Bt serovars (Yamamoto, 1983). The *CryIIA* protein is toxic to both lepidopteran and dipteran larvae, whereas the *CryIIB* toxin is toxic only to lepidopteran larvae (Widner and Whiteley, 1990). These two toxins are 87 % identical (Aronson, 1993). Another gene in this class *(CryIIC)* was cloned and sequenced by Wu *et al.* (1991).

c) *CryIII* genes

Krieg *et al.* (1983) first described a Bt variety with coleopteran activity. Since then a number of other coleopteran-active strains have been identified, encoding *CryIII*A, B, C, D and E types toxins (Ceron *et al.*, 1994). Herrnstadt *et al.* (1986) identified and isolate this toxin, which produced rhomboidal ICPs of approximately 64 kDa. For most ICP genes, transcription is concomitant with sporulation (Adams *et al.*, 1991). However, the *CryIII*A protein appears to be unique in being expressed during vegetative growth of the Bt cell (Sekar *et al.*, 1987), i.e. independently of sporulation.

d) *CryIV* and *Cyt* genes

The *CryIV* class of crystal protein genes (*CryIV*A, B,C,D) and the *Cyt* A gene are located on the same plasmid and encode proteins with molecular weights in the range of 28-140 kDa (Hurley *et al.*, 1995). These Bt crystals are composed of at least five polypeptides (Hofte and Whiteley, 1989). The proteins are all synthesised at different times during sporulation, and are added to the developing inclusion in a step-wise manner. The *CryIV* ICPs form a complex with the 27 kDa *Cyt* A protein and are only active against dipteran larvae (Koni and Ellar, 1994).

e) *cryV* genes

Tailor *et al.* (1992) discovered a new class of ICP genes (designated *CryV*), which is reportedly most closely related to *CryIB*. In host, such as lepidopteran and coleopteran insects, *CryV* genes are either cryptic or very weakly expressed.

1.1.3 Toxin specificity

Bt crystal proteins show a remarkable degree of insect specifity and the C-terminal part of the toxic fragment is considered to be responsible, via specific receptor binding (Honee *et al.*, 1991). Due to their ability specifically to bind Bt toxins, a number of proteins have being characterised as putative receptors (Maizer *et al.*, 1997). Different models have been described to explain the possible role of these receptors: the receptor itself could be a transmembrane channel that would be blocked in an open position by the toxin; the toxin and the receptor together cold form a pore; or the receptor could catalyse the insertion of the toxin in the membrane, but would have no other role in pore formation. The subsequent steps in the intoxication process include the insertion of the toxin in the apical membrane of the epithelial columnar cells, including the formation of ionic channels or non-specific pores in the target membrane. As a consequence of pore formation, a series of events leads to lesions in the plasma membrane, and finally to the destruction of the integrity of the midgut. Histological observations suggest that osmotic lyses is a common phenomenon in intoxicated insects. The insects then stop feeding and die (Maizer *et al.*, 1997).

1.2 Bt cotton

1.2.1 Cotton

The cotton genus *Gossypium* L. (Malvaceae) consists of approximately 50 species of shrubs and small trees found worldwide in both tropical and subtropical areas (Wendel and Albert, 1992). It is subdivided into four sub-genera and these further subdivided into sections and subsections. It includes four species of cultivated cotton (*G. arboreum* L., *G. barbadense* L., *G. herbaceum* L., *G. hirsutum* L. (Wendel and Albert, 1992).

Gossypium hirsutum originates from Central America and in Africa. Records indicate that cotton is a recent introduction in Africa and must have reached the continent in the 17th century (Raemaekens, 2001).

Cotton is currently the leading plant fibre crop worldwide and is grown commercially in the temperate and tropical regions of more than 50 countries. The long lint fibers are used in the production of mattresses, furniture upholstery and mops, and the shorter fibres length are a major source of cellulose for chemical industry products as well as a viscosity enhancer (thickener) in ice cream, salad dressings and toothpaste. In the chemical industry the second-cut linters are used with other compounds to produce cellulose derivatives such as cellulose acetate, nitrocellulose and a wide range of other compounds. The cottonseed forms the basis for an important oil and animal food industry.

In Mozambique cotton is a commercial cash crop, distributed all over the country, with a concentration in the north and centre, where soil fertility is good and where the rainfall is regular. The major production areas are the provinces of Nampula and Cabo Delgado in the north and Zambézia and Sofala in the centre (INAM, 2001). In Mozambique, the production and average yield are generally very low. Many constraints contribute to such low yield. These include irregular rainfall and deficient soil moisture, pests and diseases, poor agriculture practice by small-scale farmers, lack of good quality seed and the use of unimproved landraces (varieties that were domesticated by native farmers) (INAM, 2001).

1.2.2 Cotton pests

The African cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is one of the most serious pests of cotton throughout the world (Fitt, 1989). *Helicoverpa armigera* larvae normally feed on the tender terminal leaves as neonates and subsequently on the squares and bolls (Kranthi *et al.*, 2003). However, damage to bolls is considered to be the most important type of injury. Heavy, uncontrolled infestations of cotton with *H. armigera* are capable of inflicting severe yield losses. *Helicoverpa armigera* is widely distributed in tropical and sub-tropical Africa, as well as in southern Europe, Asia, and North America. It is also extremely polyphagous, occurring on approximately 60 species of cultivated crops and 67 wild hosts in 39 families across its range of geographical distribution, making it a major pest of many important crops (Reed *et al.*, 1981; Abate and Ampofo, 1996).

However, insect control remains one of the major problems of modern agriculture, which contribute to the extensive use of chemical pesticides. It is estimated that worldwide at least 13 % of all crop production (US$ 100 billion) is lost to insects, predominantly the larvae of Lepidoptera and Coleoptera (Gatehouse and Hilder, 1994). The use of chemical insecticides (estimated at US$ 7.5 billion) was able to reduce crop loss by about 7 % (Krattiger, 1997). Alternative strategies to chemical pesticides are needed to obtain better control of insect pests and to reduce the need for intensive chemical treatments with their negative environmental impacts.

Genetic modification of crops has started to make an important contribution to the production of pest-resistant crops, because an increasing number of species become susceptible to genetic transformation. So far, two different strategies for producing such plants have been successfully used. One approach is to identify and use the insect resistance genes already present in many plants, such as inhibitors and lectins (Boulter *et al.*,1990) the other is to use genes from the insecticidal bacterium *Bacillus thuringiensis* (Perlak *et al.*, 1990).

1.3 Genetically modified plants

1.3.1 The term genetic modification

The word "Biotechnology" has been used broadly to describe all genetic modifications, particularly in the field of agriculture (Shandro, 2000). Genetic modification is an application of biotechnology involving the transfer of a gene between species in order to encourage expression of the desired trait. Plant genetic research in the last decade has focused on a large number of global agricultural challenges (Ghatnekar and Ghatnekar, 2000). Using gene technology, scientists aim to introduce, enhance or delete particular characteristics of living organisms depending on whether they are considered desirable or undesirable (CSIRO Australia, 2000).

Plants into which new pieces of DNA are introduced by procedures other than sexual crossing, are referred to as genetically modified (GM) or transgenic (Metz and Nap, 1997). The broadest definition of plant genetic engineering is changing the genetic make-up of plants to provide plants, plant products and processes for our needs. In this sense, plant genetic engineering has been around for a very long time (Boulter, 1997).

Proponents argue that advances in this new technology can produce food to feed the growing world population in the 21st century. Critics are concerned that this technology produces uncertainties about the long-term impact on the environment. A criterion by which the importance of this new technology may be evaluated is its contribution to solving or avoiding deleterious consequences of agricultural production practices. Soil erosion and agricultural residues in soil and water are two such problems, where a solution may in part be made possible with the addition of crop varieties designed to be genetically compatible with broad spectrum, high potency, environmentally safe herbicides (Goodman, 1987). Further, traditional corporations, which are among the main proponents of plant biotechnology, view transgenic crops as a way to reduce dependence on inputs such as pesticides and fertilizers (Altieri, 1999).

1.3.2 The process of plant transformation

The process of inserting foreign genetic material via genetic modification into the host plant genome and the expression of such material is called plant transformation (Hansen, 2000). For the last two decades there has been much development in plant transformation. There are two main methods of transforming a plant cell or plant tissue. Direct gene transfer is obtained by electroporation, biolistics or microinjection. The method of electroporation is based on the fact that electric pulses can open the cell membrane and allow penetration of alien DNA. Heat shock, in combination with electroporation, results in higher efficiency of the transformation. The advantage of electroporation is that it can be applied to protoplasts in angiosperms. The chemical compound polyethyleneglycol changes the pore size of the cell membrane, which enhances the probability of an alien DNA molecule penetrating the cell. This method has been used for the transfer of DNA to monocots as well as dicots, including transfer of DNA to protoplasts. The biolistic approach is using the principle of shooting particles coated with DNA into selected tissues or cells with a particle gun. The particles may consist of either tungsten or gold carrying the DNA and vary in size. This method is useful for both monocots and dicots (Simonsen and Jorgensen, 1997).

DNA transfer can also be achieved by a biological vehicle such as *Agrobacterium* spp. This technique was the earliest to be developed and has been used to produce transgenic plants for almost 20 years. Many plants are susceptible to this bacterium (Simonsen and Jorgensen, 1997). Within the last few years infection by *Agrobacterium tumefaciens* was also amended to allow DNA uptake by cereal tissue. *Agrobacterium tumefaciens* is a naturally occurring pathogenic soil bacterium that has the ability to transfer part of its tumor-inducing DNA (T-DNA) located on the *Ti* (tumor-inducing) plasmid into a plant genome, which is required for its survival (Figure 1.2). It can only infect a plant and transfer genes through wounds and causes a characteristic growth called a crown gall tumor (Figure 1.3). Researchers have, however, disarmed this tumor-inducing part of DNA and have replaced it with genes coding for useful characteristics (Gelvin, 2003). During transformation the bacterium binds to a wounded plant cell at a specific site.

Phenolic compounds exuded by the plant cell activate the virulence genes that control the excision and export of the T-DNA segment from the bacterium to the plant cell (Galun and Breiman, 1997). The traits by this technique most widely transferred so far are genes coding for resistance to herbicides, pests and pathogens (Dunwell, 1998).

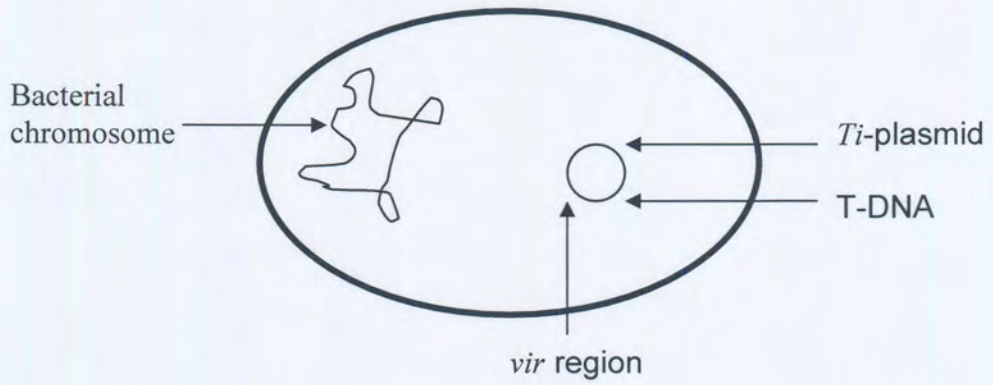


Figure 1. 2 *Agrobacterium tumefaciens* cell with *Ti*-plasmid containing T (transfer)-DNA, which is transferred into the plant genome and *vir* (virulence) region, which is required for T-DNA transfer and genome integration.

Figure 1. 3 Crown gall tumor on plant. Crown gall disease, which is caused by *Agrobacterium tumefaciens*, produces a tumor-like growth on stems of susceptible plants such as *Kalanchoe* (Zaenaen *et al.*, 1974).

1.3.3 Genetic engineering vs traditional breeding

Genetic engineering clearly differs from traditional breeding in a number of ways. Traditional breeding relies primarily on selection, using a natural process of sexual and asexual reproduction. Genetic engineering utilizes a process of insertion of genetic material. This can take place via the application of a bacterium as a gene vehicle, a gene gun or other direct gene introduction methods (Hansen, 2000). Traditional breeding has typically only recombined genetic traits within species and between different but related species. In contrast, genetic engineering can move fully functional gene traits between completely different types of organisms (Regal, 1994). Because of the wide variety of genes available for transfer as transgenes and the types of alterations that are possible by molecular techniques, gene technology is inherently different to traditional breeding methods (Rogers and Parkes, 1995).

Further, one of the disadvantages of traditional breeding is that this process is extremely time consuming. It can take more than 15 years to bring new varieties to the market (Scandizzo, 2002). Also, breeders frequently face a situation in which a resistance gene is closely linked to a gene that adversely affects the quality of a crop, i.e. where the two traits are jointly inherited. Despite these disadvantages, traditional plant breeding will, however, continue to be a vital tool for improvement of plant crops complementing the strategies of genetic engineering (Keshun, 1999).

1.3.4 Growth of GM crops

During the five-year period 1996 to 2000, the global area of GM crops increased more than 25-fold, from 1.7 million to 44.2 million hectares. This high rate of adoption by farmers reflects the growing acceptance of genetically modified plants using the technology in both industrial and developing countries. Figure 1.4 shows the estimated global area of genetically modified crops for 2000 (James, 2000), which is thought to have increased by 11 % compared to 1999.

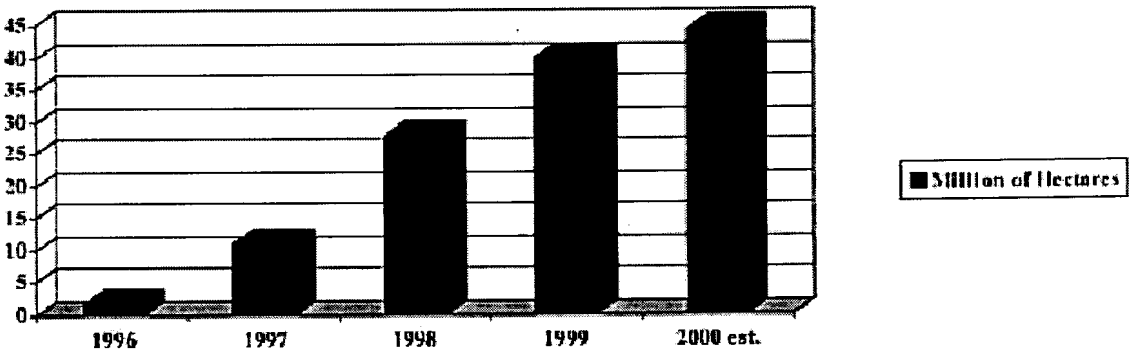


Figure 1. 4 The estimated global area of genetically modified crops for 2000 was 44.2 million of hectares. Year 2000 was the first year when the global area of GM crops almost reached 45 million of hectares (James, 2000).

In 2000, 13 countries grew GM crops (Table 1.3), but four among these had 99 % of the global GM crop area. These were the US, Canada, Argentina and China. Among these, the US had the largest GM crop area with 30.3 million hectares followed by Argentina with 10 million hectares, Canada with 3 million and China with 0.5 million hectares. In 2000, herbicide-resistant GM soybean beans occupied 58 % (25.8 million hectares) of the global area of GM crops, GM maize followed with 10,3 million hectares, GM cotton covered 5,3 million hectares and GM canola covered 2,8 million hectares. Figure 1.5 shows that out of total worldwide planting, GM soybean accounts for 36 %, GM cotton for 16 %, GM canola for 11 % and GM maize accounts for 7 % of the total respective crop planting. Herbicide-resistance as a trait for GM crops has consistently been the dominant trait during the five-year period up to the year 2000.

Table 1. 3 GM crops grown and production area in 2000.

Country	Area planted in 2000 (Millions of hectares)	GM crops grown
USA	74.8	soybean, maize, cotton, canola
Argentina	14.7	soybean, maize, cotton
Canada	7.4	soybean, maize, canola
China	1.2	cotton
South Africa	**0.5**	**maize, cotton**
Australia	0.4	cotton
Mexico	minor	cotton
Bulgaria	minor	maize
Romania	minor	soybean, potato
Spain	minor	maize
Germany	minor	maize
France	minor	maize
Uruguay	minor	soybean

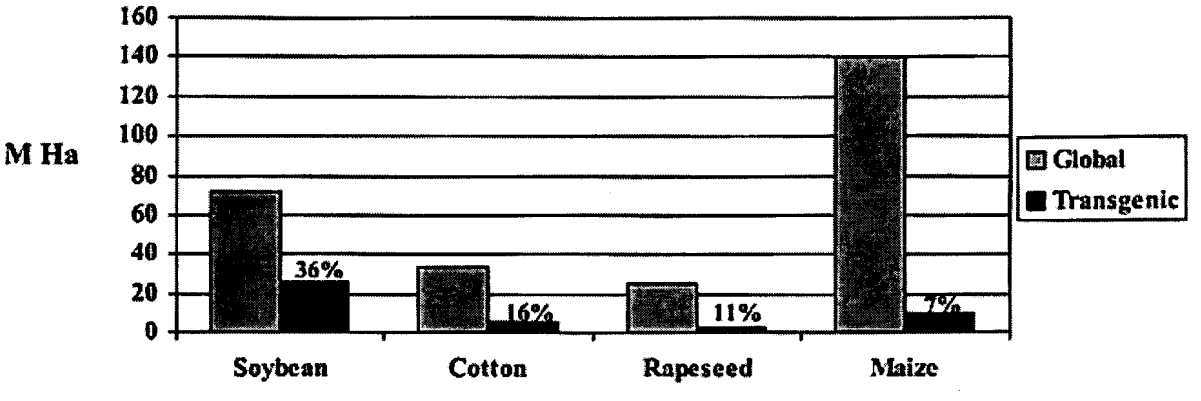


Figure 1. 5 Area of GM crops in million hectares (M Ha) in comparison to total growth of four important crops: soybean, cotton, rapeseed (canola) and maize (James, 2000).

1.3.5 Traits of GM crops

Plant biotechnology has the capacity to create a greater variety of commercial plants compared to conventional plant breeding. Genes from sexually incompatible plants, animals, bacteria or insects can now be introduced into plants. The first transgenic plant, a tobacco plant resistant to an antibiotic, was created in 1983 (Sonnewald, 2003). Others that followed include Calgene's *Flavr Savr* tomato, a variety of virus- and herbicide-resistant crop plants and insect-resistant cotton and maize expressing a Bt toxin. Currently, the major GM crops are soybean, canola, cotton and maize (Keshun, 1999).

Based on the major outcomes of the traits under modification, GM crops fall into two major categories: those with improved agronomic traits and those with altered quality traits. Herbicide-tolerant crops and Bt-based insect protected crops are the best examples of improved agronomic traits (Keshun, 1999). Wilkinson (1997) indicated that about one-third of GM crops approved or under review by regulatory agencies for commercialisation are with improved agronomic traits, including those with tolerance to a broad spectrum of herbicides. Farmers are usually increasing their acreage of herbicide-resistant crops because the use of these plants reduces the need to plough, decreases herbicide usage and can deliver a cleaner and higher grade of grain and produce (Vogt and Parish, 1999).

1.3.6 Use of Bt crops

Bt toxins are the most widely used naturally occurring agricultural pesticides. These organic insecticides are safer and more benign than the chemical pesticide they are replacing. The Bt toxin is used extensively by both organic farmers and in transgenic crops to provide environmentally safe protection against insect pests (Aroian, 2001). In addition, due to its target specificity and as it is not synthetic; the organic agricultural industry relies heavily on Bt.

Since the 1980s, biotechnology companies have been inserting toxin genes from the naturally occurring microbial pesticide *Bacillus thuringiensis* into other microbes or crops. Bt-maize has become very popular with American farmers for example. Maize has been modified with the Bt toxin gene to control the European maize borer, a significant pest that hides in the stalks of the plant, making it difficult to control with chemical sprays. There has been, concern that long-term exposure to such insecticidal crops could intensify selection pressure for resistant insects; if so, then this effect would shorten the useful lifespan of the product. Insect resistance might also reduce the future utility of naturally occurring microbial Bt.

In Africa, and especially South Africa, Bt cotton is the first major GM crop used at the small-scale farming level. There is further a strong intention to extend the use of Bt cotton into other parts of Africa and to use other Bt crops and crops with a genetically modified resistance to pests at a much larger scale. There might be several advantages of using a Bt crop, which include:

- improved pest management
- reduction in insecticide use
- greater yield
- improved conditions for non-target organisms.

In 2001, an estimated 12 million ha of land were planted world-wide with crops containing the Bt toxin gene. Table 1.4 lists countries that have commercialized Bt cotton and/or Bt maize. The use of Bt crop varieties has dramatically reduced the amount of chemical pesticides applied to cotton. In the USA, farmers used 450,000 kg less pesticide on Bt cotton than would have been used on conventional varieties in 1998 alone (Ismael *et al.*, 2002).

In South Africa, cotton cultivation is the main source of income in the Makhathini area, Natal province. The majority of cotton farmers are smallholders with an average farm size of 1 to 3 ha. Cotton plants account for 90 % of the cultivated land. The remaining area is used for cultivating other crops like maize and beans.

Insect pests are the main obstacles to increase of production. The bollworm complex, namely African cotton bollworm (*Helicoverpa armigera*), Red bollworm (*Diaparopsis castanaea*) and Spiny bollworm (*Earias* spp.) are the most damaging insect pests (Ismael *et al.*, 2002).

Genetically modified Bt cotton (Bollgard™) has been planted in the Makhathini area as a commercial product since 1997/1998 (Kirsten and Gouse, 2002) and 75 people started using Bt technology with only 80 ha. By 2001/2002, 2976 growers had decided to use Bt technology and 5670 ha of land is currently being used for Bt crops (Figure 1.6). This corresponds to about 5 % of South Africa's cotton growing area (Kirsten and Gouse, 2002). The rate of adoption of Bt crops by small-scale farmers is an indication of the socio-economic benefit of Bt crops in South Africa.

Table 1. 4 Countries that have commercialized Bt cotton and or Bt maize (Ismael *et al.*, 2001).

CROP	COUNTRY
Cotton	Argentina Australia China India Indonesia Mexico South Africa United States
Maize	Argentina Canada European Union South Africa United States

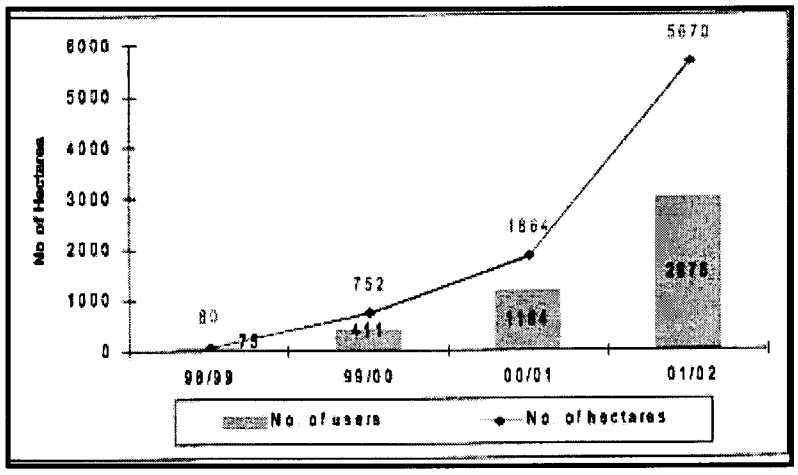


Figure 1. 6 Adoption of Bt technology by small-scale farmers (Ismael *et al.*, 2001).

1.4 Bt expression under environmental stress

1.4.1 Gene expression and stress

Conditions within any environment are not static, and plants are exposed to changes in climate to which they must adapt, or to which they must be tolerant. Any change in an environmental condition that results in a response of an organism might be considered stressful (Levitt, 1980). Koehn and Bayne (1989) defined stress as the reduction of the fitness of an organism by a change in an environmental condition that might reduce or adversely affect growth and development of an organism.

The study of stress on plants, and the way plants respond to these stresses, has become increasingly complex. With the advanced tools available to researchers today it is possible to investigate the response of plants to environmental stress at the molecular level. Stress factors may be biotic (living) or abiotic (non-living). Biotic stresses include a variety of pathogenic microorganisms, such as viruses, fungi and bacteria, as well as the effects of herbivorous insects that feed on the plant material. Abiotic stresses include water logging, drought, extreme temperature, intense light, excessive soil salinity, inadequate mineral nutrients in

the soil and also treatment with plant growth regulators and antibiotics (Smirnoff, 1998).

Drought stress, which is typical for Africa, induces several changes in metabolic processes (Table 1.5). These changes concern cell division and expansion, various factors in carbon and nitrogen metabolism and changes in activity of phytohormones. Responses to drought and salinity in barley monitored by microarray hybridization of 1463 DNA elements derived from cDNA libraries of 6 and 10 h drought-stressed plants revealed that nearly 15 % of all transcripts were either up- or down-regulated under drought stress (Ozturk *et al.*, 2002). The most drastic down-regulation was observed for photosynthesis-related functions. Plant growth and seed production is further severely affected by drought. Leaf expansion has been shown in several species to be sensitive to water stress (Hsaio, 1973; Masle and Passioura, 1987). Several studies have further demonstrated that drought inhibits cotton canopy development. Krieg and Sung (1986) determined that drought decreases the number of leaves on sympodial branches of cotton. Leaf area of glasshouse-grown cotton was also inhibited when the percentage of soil available water was less than 51 ± 15 % (Rosenthal *et al.*, 1987).

Drought stress is also known to have both positive and negative effects on protein synthesis in plants, depending on duration and intensity of the stress (Shiralipour and West, 1968; Gershenzon, 1984; Mattson and Haack, 1987; Lilburn *et al.*, 1991). However, there is a general lack of studies to evaluate Bt toxin stability/ activity under stressful environmental conditions, which are typical for Africa. Field trials of transformed Bt cotton have mostly been carried out under optimal plant growth conditions and showed good efficacy of transformed plants against lepidopteran pests.

Few studies have been carried out so far to investigate the effect of drought stress or plant senescence on the expression of Bt proteins and the relation to insect resistance under drought. When Bt and non-Bt maize plants were subjected to various soil water deficit treatments during peak European maize borer egg laying periods, a delayed leaf appearance and also reduced leaf area and plant height

were observed (Traore *et al.*, 2000). For these morphological characteristics there was no significant difference between Bt and non-Bt maize plants. However, there were significant differences between Bt and non-Bt plants for second generation maize borer damage, with Bt plants the least affected. Compared with non-Bt plants, Bt plants exhibited significantly greater total plant weight and grain yield by 9.4 %. In a further Australian study with field populations of *Helicoverpa* evidence was found of a declining level of Bt expression (*CrylAb*) in transformed cotton once plants start to age (Fitt *et al.*, 1994). Laboratory assays showed that later instars could survive on the transgenic tissues although their growth was severely retarded. Although the possible causes for the decline in efficacy of these Bt cotton plants were not fully understood, Bt expression may have been reduced by either down-regulation of the Bt gene or degradation of the Bt protein due to ageing (Daly, 1994). The growth stage of the plant seemingly has a major influence on Bt efficacy (Olsen and Daly, 2000) and the Bt concentration is also declining relative to total protein in plant during senescence (Holt, 1998). In addition, preliminary experiments have shown that the *Cry1Ac* transcript level is unstable in both immature and mature plants. Secondary products, such as phenolics and orthoquinones, that are produced or increased by plants during periods of physiological stress or physical damage, seemingly also alter the efficacy of Bt crystal proteins (Sivamani *et al.*, 1992; Navon *et al.*, 1993; Gibson *et al.*, 1995) possibly by acting on the Bt protein itself (Ludlum *et al.*, 1991).

Table 1. 5 Relative sensitivity of various plant responses to water stress.

Plant process	Relative sensitivity: very sensitive	moderately sensitive	insensitive
Growth			
Cell division	– – ——— – –		
Cell expansion	——— – –		
Carbon metabolism			
Stomatal opening	– – ——— – –		
CO_2 assimilation	– – ——— –		
Sugar accumulation	– – ——— –		
Protochlorophyll formation	———		
Nitrogen metabolism			
NO^-_3 reduction	– – ——— –		
N_2- fixation			
Proline accumulation	– ——— –		
Protein synthesis	– ——— – – –		
Protein hydrolysis	– ——— – – –		
Hormonal activity			
Indole acetic acid	– ——— –		
Gibberellin	– ——— – –		
Cytokinin	– ——— – –		
Abscisic acid	– – ——— – –		
Ethylene	– ——— – –		

Modified and adapted from Hsaio (1973), Krieg (1993)

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 Materials and Methods

2.1.1 Plants, insects and reagents

Non-genetically modified cotton seeds (*Gossypium hirsutum*, cv. Opal), as well as genetically modified commercially available cotton seeds (cv. Nuopal) expressing the Bt toxin *Cry1Ac,* were provided by CIRAD, France. The plants were grown in 5-litre plastic pots filled with a 1:1 mixture of fine sand and coconut waste.

Ready-to-hatch eggs of bollworm larvae (*Helicoverpa armigera*) were supplied by the ARC–Plant Protection Research Institute (Pretoria, South Africa). Insects were maintained in the laboratory at a temperature ranging between 25-27 °C, 16 Light: Dark hours light regime at the University of Pretoria.

Reagents used in the study were purchased either from Sigma (St. Louis, Mo.), Boehringer Mannheim (Germany), Life Technologies (UK) or BioRad (Hercules, U.S.A.). All products used were analytical or molecular biology grade (see Annexure).

2.1.2 Physiological experiments

2.1.2.1 Plant growth

Plants were grown in a glasshouse under natural light and humidity conditions and a temperature ranging between 28 and 32 °C during the growth period. During the first two weeks the plants were irrigated with tap water for acclimatization. Plants received Hoagland nutrient solutions twice weekly.

2.1.2.2 Drought stress treatment

Drought stress was induced in two-week old plants using a gravimetric method. For this purpose, half of the experimental plants were left without watering until plants showed wilting symptoms (after about 5 days) while the remaining half were maintained at 80-100 % of field capacity (the maximum amount of water that a particular soil can hold). The amount of water evaporated was monitored daily by weighing unplanted pots placed randomly between planted pots. Pots were watered with the amount equivalent to the loss of weight. This was done to bring them to the pre-determined level of moisture whenever the weight of pots fell below the lower limit established for the treatments. Drought treatment was carried out with both non-Bt and Bt cotton plants, and each plant treatment was carried out using 10 plants. Drought stress was induced by withholding watering plants until the soil moisture content reached 25-30 % of field capacity. Non-stressed control plants were watered and soil moisture content to 80-100 % of field capacity was maintained. Four weeks after treatment induction, leaf samples from both treated and untreated plants were collected from fully expanded 3rd or 4th leaf position from shoot tip, flash frozen in liquid nitrogen and either used immediately or stored at –80 °C until needed. Leaf samples were used as DNA and protein sources in the biochemical analysis.

2.1.2.3 Heat stress treatment

Plants were grown in potting soil in a greenhouse at a temperature ranging between 25 and 28 °C. Four weeks after planting, plants were transferred to a growth cabinet for heat stress treatment. To induce heat stress plants were grown for 15 days at 38/32 °C during the day and night, respectively, whereas control plants were grown in a growth cabinet at a 28/25 °C day/night cycle. The light period for both types of treatments was a 16-h photoperiod and the light intensity was set at 1000 µmol/m^2/s. Plant material from both treated and untreated plants was used as a protein source in the biochemical analysis.

2.1.2.4 Plant growth analysis

Drought stress was induced in two-week old plants using a gravimetric method as described under 2.1.2.2. For growth analysis eight treated and eight untreated Bt and non-Bt cotton plants were harvested every second week after planting. At each harvest, the number of leaves was counted and the maximum root length (MRL) determined (Hendrix *et al.*, 1991). Different parts of the plant (roots, stems, leaves, squares, flowers and green bolls) were collected separately and their fresh weight determined. The dry weight was determined after treatment of plant material for 48 hours at 80 °C in a drying oven.

The Relative Growth Rate (RGR) was determined according to Hunt (1982). In the calculation, the dry weight increase in a time interval is expressed in relation to the initial weight and the following calculation was applied with w = dry weight and T = Time:

$$RGR = (\ln W_2 - \ln W_1)/(T_2 - T_1)$$

The leaf area was measured by using a leaf area meter (Model Li-3000A, LI-COR, Inc. Lincoln, U.S.A). The Specific Leaf Area (SLA) was calculated according to Lambers and Poorter (1992), where LA = Leaf area and LW = Leaf dry weight:

$$SLA = (LA_2/LW_2) + (LA_1/ LW_1)/2$$

The Leaf Area Ratio (LAR), which expresses the ratio between the area of leaf lamina or photosynthesizing tissue and the total respiring plant tissues or total plant biomass was calculated according to Lambers and Poorter (1992), where LA = Leaf area and W = dry weight:

$$LAR= (LA_2/W_2) + (LA_1/W_1)/2$$

The Net Assimilation Rate (NAR), or unit leaf rate, which is the net gain of assimilate and mostly photosynthetic per unit of leaf area and time (Lambers and

Poorter, 1992), was calculated with LAR = Leaf Area Ratio and RGR = Relative Growth Rate as:

$$RGR = LAR \times NAR$$

The Root Weight Ratio (RWR) was calculated according to Fitter and Hay (1981) using the calculation with WR = total root dry weight and WPt = total plant dry weight

.

$$RWR = WR / WPt$$

The water content in leaves, roots and whole plants was expressed as the percentage of dry matter according to Garnier and Laurent (1994) and applying the calculation with W = dry weight and F = fresh weight.

$$\text{Water Content (\%)} = (W / F) \times 100$$

The Drought Stress Tolerance Index (DSTI) was calculated on the basis of dry weight of different plant parts for each variable according to Maiti *et al.* (1996) using the formula:

DSTI = component dry weight under drought stress / component dry weight under watered conditions.

2.1.3 Biochemical experiments

2.1.3.1 Protein analysis

For protein analysis, cotton leaf material was homogenized in liquid nitrogen in extraction buffer which contained either 50 mM Tris-HCl, pH 8.9 (buffer A) or 50 mM sodium phosphate, pH 6, containing 10 mM β-mercaptoethanol (buffer B). Buffer B, which has an acidic pH, was specifically used to prevent Bt toxin degradation.

The protein concentration of extracts was determined according to the method of Bradford (1976). The colour reagent (200 µL) was diluted with 800 µL water, a crude plant extract (2 µL) was added to this reaction mixture and the mixture was left at room temperature for 20 min. The absorbance of the reaction mixture was determined with a spectrophotometer (Pharmacia, Sweden) at a wavelength of 595 nm. The protein concentration was determined using a standard curve with known quantities of bovine serum albumin used as a protein.

2.1.3.2 SDS-PAGE gel electrophoresis

SDS-PAGE gel electrophoresis was carried out according to the method described by Sambrook *et al.* (1989). Total soluble protein of leaf extracts was separated on 12 % SDS-PAGE gel. Samples were first incubated at 95 °C for 5 min in loading buffer containing 62.5 mM Tris-HCl, 2 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol and 0.001 % (w/v) bromophenol blue (pH 6.8) and then loaded onto the SDS-PAGE gel. Electrophoresis was performed using the Bio-Rad Mini-protean II system at constant current and varying voltage. Samples were run at 100 V through the stacking gel and at 200 V through the resolving gel until the blue front of the loading dye reached the bottom of the gel.

2.1.3.3 Western blotting

After extraction of soluble protein from leaves, samples were run on SDS-PAGE for protein separation. Western blotting was carried out as described in the technical booklet for the ECL Plus Western Blotting System (Amersham, UK). After electrophoresis, the PAGE gel was equilibrated for 20 min in transfer buffer containing 25 mM Tris and 192mM glycine in 15 % methanol, pH 8.2. A polyvinylidene difluoride (PVDF) membrane (PVDF-Plus, Micron Separations Inc., U.S.A) was cut to size and pre-wet in 100 % methanol for 5 sec, after which the membrane was washed in distilled water for 5 min, and equilibrated in transfer buffer for 15 min. Transfer of proteins was done in a BioRad Mini protein II transfer apparatus filled with transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20 %

methanol), at 4 °C and 60 V for 1 h. After transfer, the membrane was washed once for 5 min in Tris-buffered saline (TBS), pH 7.6. Thereafter the membrane was incubated in a 5 % fat free milk powder/TBS buffer solution containing 0.1 % (v/v) Tween 20 for 1 h to block non-specific binding sites. The membrane was again washed three times for 10 min each in the same milk powder/TBS/Tween solution, and once for 10 min in TBS containing 0.1 % (v/v) Tween 20 only (TTBS). The membrane was then incubated in primary antibody in TTBS containing 5 % (w/v) fat free milk powder at room temperature, on an orbital shaker, overnight. Primary antibodies directed against Bt *Cry1Ac*, raised in rabbit, were obtained from CIRAD, France. To remove all unbound antibodies, the membrane was washed four times for 10min each in TTBS. Detection was done using the ECL Plus Western Blotting Detection System as outlined by the supplier (Amersham, UK). This system detects immobilized specific antigens conjugated to horseradish peroxidase (HRP)-labeled antibodies. The membrane was incubated in secondary antibody (HRP)-labeled goat anti-rat antibody) diluted in TTBS containing 5 % (w/v) fat free milk powder for 2 hours, at a dilution of 1:5000. Afterwards, the membrane was washed four times for 10 min each in TTBS. Detection reagent (2 mL) containing a Lumigen PS-3 acridan substrate was added onto the membrane, and incubated for 5 min at room temperature. The substrate reacts with the HRP, and generates acridinium ester intermediates. These intermediates react with peroxide (present in the detection reagent) under slightly alkaline conditions and produce a sustained, high intensity chemiluminescence. Excess detection reagent was drained off by touching the edge of the membrane against tissue paper. The membrane was wrapped in clean Saran Wrap, and placed in an X-ray film cassette. A sheet of autoradiography film (Hyperfilm ECL, Amersham Pharmacia Biotech, UK) was placed on top of the membrane in a dark room under safe red light conditions. After exposure, the film was developed, rinsed in tap water, and fixed.

2.1.3.4 *Cry1Ac* detection

Cotton leaves (1g fresh weight) were homogenised with a mortar and pestle in 1 ml extraction buffer. For homogenisation of bolls and flowers, wide slices across the centre and through the entire fruiting form were cut with a razor blade and the 1g fresh weight of flower or boll piece was determined before homogenisation.

The amounts of *Cry1Ac* toxin in leaves, flowers and bolls were determined using an enzyme-linked immunosorbent assay (ELISA) as described by the supplier Strategic Diagnostic, Inc (U.S.A). For the assay, plant samples with 200 µg of protein were used. For the immuno-reaction ELISA plates were incubated at room temperature (20-30 °C) for 60 min with a *Cry1Ac* toxin monoclonal antibody as outlined by the supplier. At the end of the incubation period, the plate was quickly inverted and the content of wells discarded. After washing of plate wells a colour solution was added, a colour reaction occurred over a 10 min time period and colour reaction was finally measured in an ELISA plate reader at 620 nm. Since the supplier does not provide any *Cry1Ac* standard for the assay, the measured optical density using an ELISA plate reader only represented relative values. All samples were analysed in duplicates.

2.1.3.5 DNA analysis

2.1.3.5.1 Genomic DNA isolation

Genomic DNA isolation was done according to the method described by Gawel and Jarret (1991). Fresh leaves (1.5 g) were frozen in liquid nitrogen, homogenised with a mortar and pestle and transferred into a pre-heated extraction buffer (10 ml) containing 100 mM Tris-HCl (pH 8); 1.4 mM NaCl; 20 mM EDTA; 0.1 % mercaptoethanol and 2 % CTAB. The mixture was incubated at 65 °C for 30 min followed by addition of a chloroform:isoamyl alcohol (24:1) mixture (8 ml), and the homogenate incubated for 15 min at room temperature. After centrifugation for 5 min at 10 000 x g at room temperature, the clear supernatant was transferred to a new micro centrifuge tube and an equal volume of ice-cold iso-propanol was added to precipitate the DNA. After mixing, the DNA was centrifuged for 10 min at 4 °C and the pellet then washed with 70 % ethanol. The DNA pellet was dried for

5-10 minutes and then re-suspended in 50 µl sdH_2O (sterile distilled water). Any contaminating RNA was removed by addition of 2.5 µl of a 10 µg/ml stock solution of RNase and incubated at 37 °C for 30 min. The DNA was recovered by the addition of 1/10 volume of 3M sodium acetate (pH 6.8) and two volumes of 96 % ethanol to the DNA-containing solution as outlined by Sambrook *et al.* (1989), and finally DNA was dissolved in 100 µl sdH_2O and the DNA run on an agarose gel.

2.1.3.5.2 Gel electrophoresis of DNA

DNA molecules were separated on a 1 % agarose gel according to the method of Sambrook *et al.* (1989). Samples were loaded in a loading buffer containing 50 % (v/v) glycerol, 1xTAE buffer and 1 % (w/v) bromophenol blue. TAE buffer (1x) was also used to fill up electrophoresis cells. Agarose gels were run at 100 V and 80 mA to separate the DNA.

2.1.3.5.3 Polymerase chain reaction (PCR)

The PCR reaction mixture was made up as stated in Annexure 1, and contained reaction buffer, deoxyribonucleotides, primers, and polymerase. Primer annealing occurred as outlined in the text (Chapter 6). Primers were designed from the *Cry1Ac* sequence (Tengel *et al.*, 2001) and commercially synthesised (Roche, Switzerland). The forward primer was Cry1Ac-LI-KK: 5'–TCA CCG AGC ACA ACA AGA AG-3' and the reverse primer was Cry1Ac-RI-KK: 5'–CAT CGA CAG GCT TGA ACT CC-3'. The melting temperature for primer Cry1Ac-LI-KK was 57.3 °C, and for primer Cry1Ac-RI-KK it was 59.4 °C.Temperature cycles were as specified in Table 2.1.

Table 2. 1 PCR cycle temperatures and number of cycles.

Temperature	Duration	Number of Cycles	Purpose
94 °C	4 min	1 (First cycle)	Complete denaturation of dsDNA to yield single stranded templates
95 °C	30 sec	30	Denaturation of dsDNA
60 °C	30 sec		Annealing of primers to ssDNA
72 °C	30 sec		Polymerase extends primer by incorporating dNTPs into growing strand
72 °C	1 min	1 (Last cycle)	Final elongation of dsDNA products

2.1.4 Insect experiments

2.1.4.1 Plant material

The efficacy of genetically modified cotton (*Cry1Ac*) under drought stress against first-instar larvae of *H. armigera* was determined using leaves, flowers and immature green bolls of cotton plants. For the control non-modified cotton plants were used.

Plants were two weeks old when drought stress was imposed based on a gravimetric method as described in 2.1.2.2. Two types of experiments were carried out. In the first experiment insects were fed on leaves derived from six-week-old plants before exposure to drought stress to determine the biological efficacy of Bt toxin against first-instar larvae of *H. armigera* at the vegetative stage. In the second experiment insects were fed on different plant material Trial 1: mature leaves, flowers and immature green bolls, Trial 2: young leaves after exposure to drought stress when 50 % of plants flowered (10 weeks old) to determine the biological efficacy of Bt toxin against first-instar larvae of *H. armigera* at the reproductive stage. Plants used for the two experiments were selected from two different sets of plants.

2.1.4.2 Insect feeding

Cotton leaves were collected from the third node (numbered from the top of the plant). For the first experiment, leaves from six-week-old cotton plants (one leaf from each plant) were placed individually into plastic Petri dishes (17.5 cm in diameter and 1.5 cm high). For the second experiment with leaves, flowers and immature green bolls, plant material was freshly excised from individual plants and placed individually into glass vials (8.2 cm height and 2.2 cm in diameter). Vials were closed with a nylon-mesh lid. Three first-instar *H. armigera* larvae were placed on each leaf, flower or boll and placed in each Petri dish or vial, resulting in 30 larvae per treatment. Petri dishes or vials were kept in a growth chamber at a temperature ranging between 28 and 25 °C and a 16:8 hours light/dark cycle. Leaves in Petri dishes or vials were removed and replaced with a fresh leaf daily. Five days after introduction the surviving larvae were counted and their individual fresh weight determined (Daly and Fitt, 1998).

2.1.5 Data analyses

Data analyses were carried out using STATISTICA 6.1 (StatSoft. Inc.©, Tulsa, Oklahoma, U.S.A, 2004).

For physiological study, differences in plant growth parameters under stressed and non-stressed conditions were analysed using one-way analysis of variance (ANOVA) or Student's *t*-test. The level of significance was set at 5 % (Zar, 1996).

For biochemical study, differences in Bt *Cry1Ac* content under stressed and non-stressed conditions were analysed using one-way analysis of variance (ANOVA). The level of significance was set at 5 % (Zar, 1996).

Mortality of *H. armigera* larvae feeding on different plant parts was analysed using the χ^2 – test (Bailey, 1994). The Bonferroni adjustment was used when performing multiple statistical significance tests on the same data (Sokal and Rohlf, 1995). ANOVA was used to determine the effect of drought stress and presence of Bt on

weight of *H. armigera* larvae and Bt content in different plant parts. Due to skewness and heterogeneity of data, which could not be improved by transformation, the level of significance was set at 1 % for the analyses.

The percentage corrected mortality of insect larvae feeding on genetically modified cotton plants was calculated using Abbott's formula (Abbot, 1925) with T % = percentage of dead larvae on genetically modified cotton plants and C % = percentage of dead larvae on non-genetically modified cotton plants:

$$\text{Corrected \% mortality} = 100 \times (T\,\% - C\,\%) / (100\,\% - C\,\%)$$

CHAPTER 3: PHYSIOLOGICAL PLANT PERFORMANCE

3.1 Objectives

This part of the study focused on the physiological characterization of genetically modified (Bt) and non-modified cotton plants. The objectives of this part was to analyse physiologically the performance of genetically modified Bt cotton plants under abiotic stress and compare it with that of non-modified cotton plants.

3.2 Results

3.2.1 Plant growth analysis

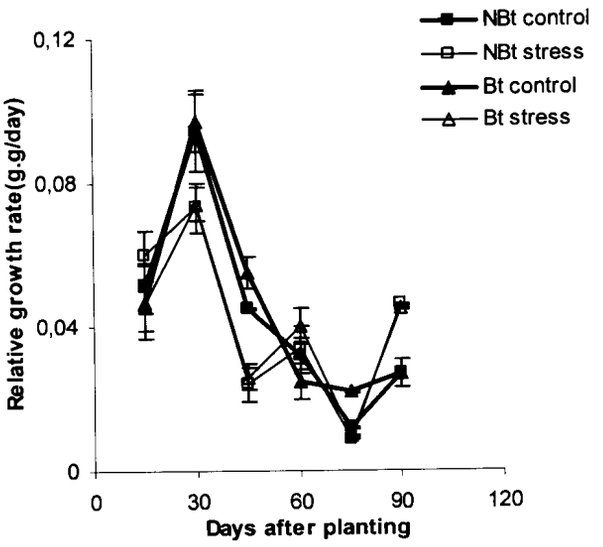
3.2.1.1 Relative growth rate and net assimilation rate

When plants were exposed for four weeks to drought stress a reduction in plant height and leaf number was found (Figure 3.1). Non-genetically modified and genetically modified cotton plants showed two stages of growth at the vegetative stage (0-60 days after planting) and at the reproductive stage (60-105 days after planting). The vegetative stage covers the time from germination to flowering whereas the reproductive stage covers the time from flowering to the formation of bolls.

Figure 3.2A shows the relative growth rate (RGR) of plants. During the vegetative stage the RGR on a total dry weight basis was reduced under drought stress by 18 % (non-modified) and 27 % (genetically-modified) plants when compared to well-watered plants. However, these decreases in RGR were statistically not significant ($(p \geq 0.05)$ between the different types of plants (genetically modified and non-modified) or treatments. When the net assimilation rate (NAR) was measured, a similar decrease in NAR was measured as found for RGR, with no significant difference between treatments and plants ($p \geq 0.05$) (Figure 3.2B).

A B

Figure 3. 1 Drought-stressed (A) and non-stressed (B) genetically modified cotton plants after four weeks of exposure to drought stress.



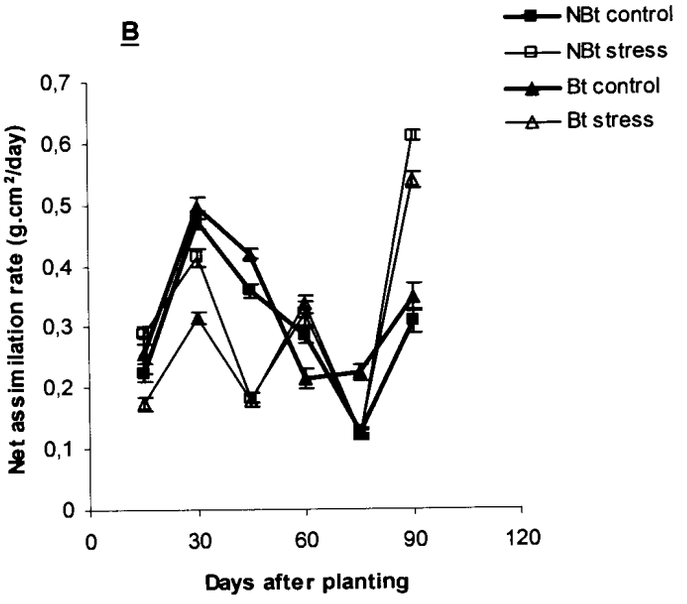


Figure 3. 2 (A) Effect of drought stress on relative growth rate (RGR) and (B) net assimilation rate (NAR) of genetically modified (Bt) and non-genetically modified (NBt) cotton plants. Each data point represents the mean of measurements from eight individual plants (± SD).

3.2.1.2 Leaf dry weight, leaf area and root growth

Drought stress significantly reduced the leaf dry weight by 52 % and 45 % for genetically modified and non-genetically modified cotton, respectively, 60 days after planting and exposing plants to drought stress 45 days after planting when compared to non-stressed plants ($p \leq 0.05$) (Figure 3.3A). However, there was no significant difference between the genetically modified and non-modified plants ($p \geq 0.05$). The leaf area was also significantly reduced by 41 % and 53 % for genetically modified and non-modified cotton, respectively, 60 days after planting and exposing plants to drought stress 45 days after planting when compared to non-stressed plants ($p \leq 0.05$) (Figure 3.3B). However, there was again no significant difference between the genetically modified and non-modified plants ($p \geq 0.05$). Both the specific leaf area (SLA), which is a measure of the transpiring leaf area (Lambers and Poorter, 1992), and the leaf area ratio (LAR), which is a measure of the photosynthetic capacity of the leaf area (Lambers and Poorter, 1992), were not significantly different between treatments or between the two types of plants used in the experiments ($p \geq 0.05$) (Figures 3.3C and D).

At the vegetative stage the root dry weight was significantly reduced under drought stress by 17 % and 51 % on non-genetically and genetically modified cotton, respectively ($p < 0.05$) (Figure 3.4A). During the reproductive stage of cotton plants (60 to 105 days after planting), plants under drought stress showed a significant reduction ($p \leq 0.05$) in the root dry weight of both genetically modified and non-modified plants when compared to non-stressed plants (Figure 3.4A). The root dry weight ratio (RWR) was significantly increased ($p \leq 0.05$) in non-genetically modified and genetically modified cotton plants under drought stress during the vegetative stage period (60 days after planting) (Figure 3.4B), indicating a change in biomass partitioning between roots and leaves increasing the proportion of dry matter to the roots (Fitter and Hay, 1981). However, there was no significant difference between the genetically modified and non-modified plants for either root dry weight or RWR ($p \geq 0.05$).

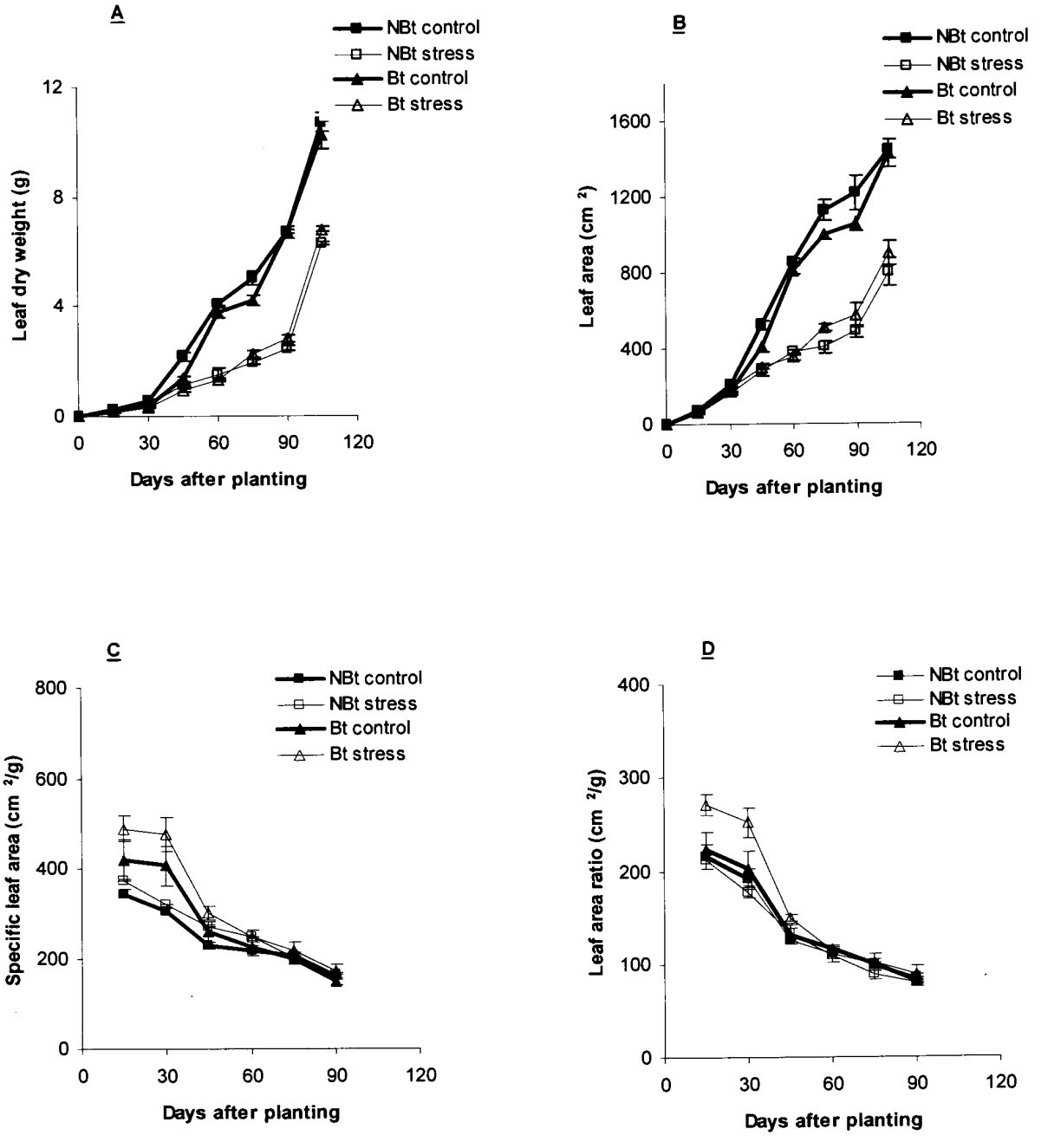


Figure 3. 3 (A) Effect of drought stress on leaf dry weight, (B) leaf area, (C) specific leaf area (SLA) and (D) leaf area ratio (LAR) of non-genetically modified (NBt) and genetically modified (Bt) cotton plants. Each data point represents the mean of measurements from eight individual plants (± SD).

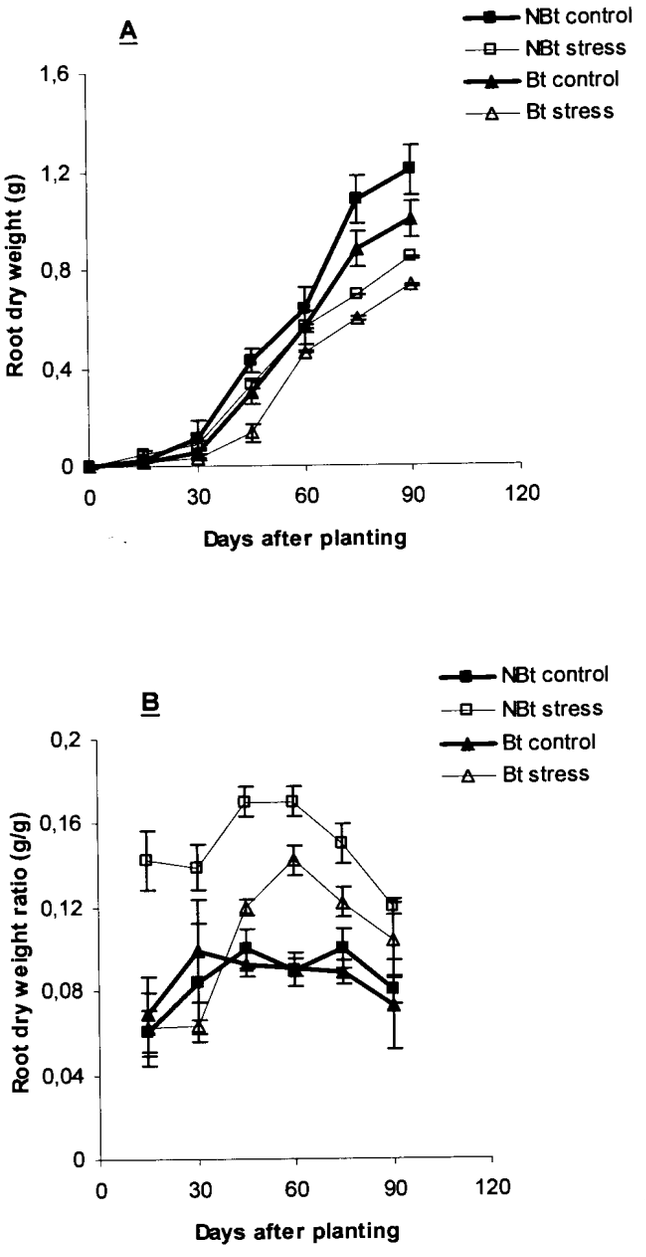


Figure 3. 4 (A) Effect of drought stress on root dry weight and (B) root dry weight ratio (RWR) of non-modified (NBt) and genetically modified (Bt) cotton plants. Each data point represents the mean of measurements of eight individual plants (± SD).

3.2.1.3 Drought stress tolerance index, total dry matter accumulation and final dry matter distribution

When the drought stress tolerance index (DSTI) was measured, which is an indicator of the level of drought tolerance of a plant (Maiti *et al.*, 1996), four different parameters were used for the determination of tolerance (leaf dry weight, leaf area, root dry weight and total plant dry weight), and curve patterns were similar for both genetically modified and non-modified plants (Figure 3.5). This indicates that the two types of plant have a similar degree of tolerance to drought stress.

Figure 3.6 shows that exposure of plants to drought stress significantly reduced dry matter accumulation ($p \leq 0.05$) 60 days after planting when plants were exposed to drought stress for 45 days. However, no significant difference ($p \geq 0.05$) was found between non-modified and genetically modified cotton plants. Also in the final distribution of dry matter in different parts of the plant 105 days after planting no difference was found between non-modified and genetically modified cotton plants (Figure 3.7).

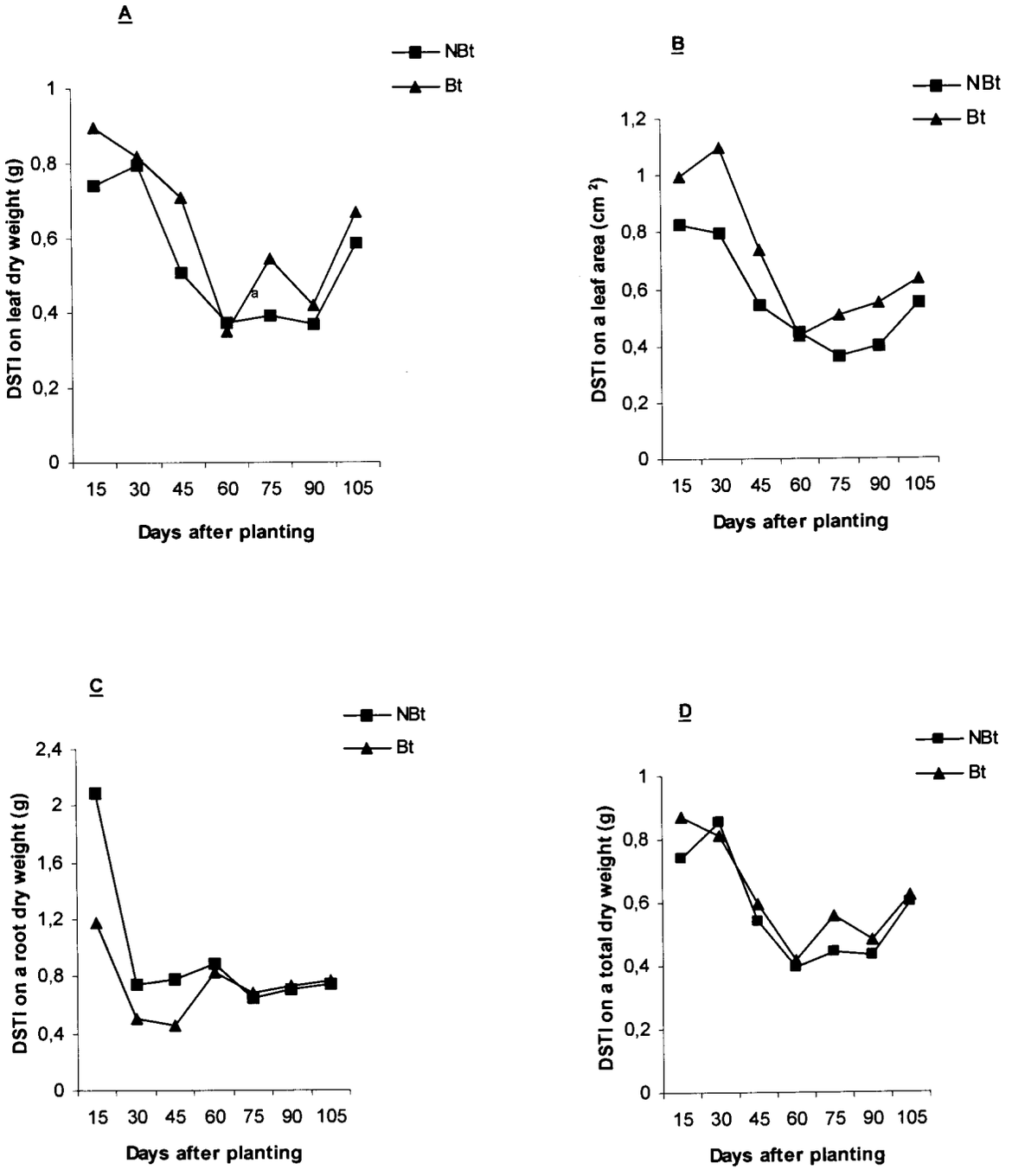


Figure 3. 5 (A) Drought stress tolerance index (DSTI) based on leaf dry weight, (B) leaf area, (C) root dry weight and (D) total dry weight of non-genetically modified (NBt) and genetically modified (Bt) cotton under drought stress conditions.

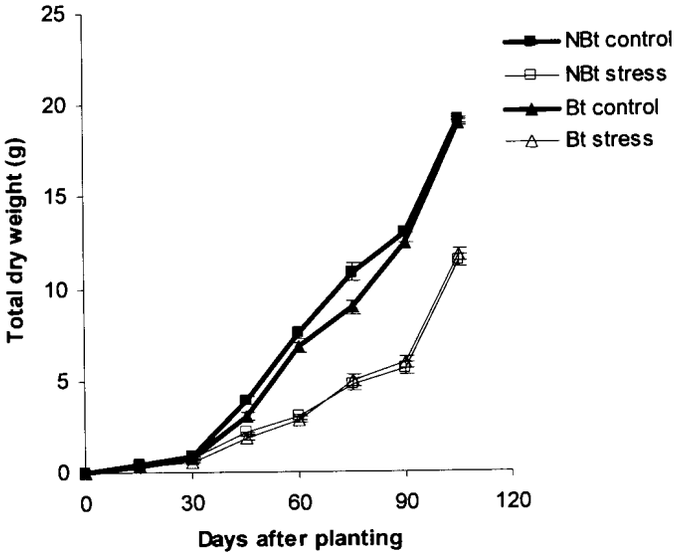


Figure 3. 6 Effects of drought stress on total dry weight in non-genetically modified (NBt) and genetically modified (Bt) cotton plants. Each data point represents a mean of eight plants (± SD).

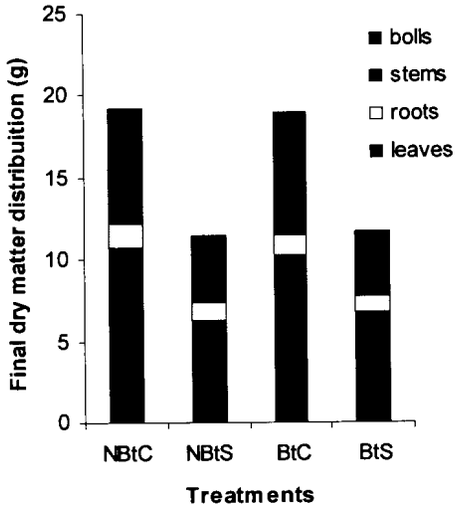


Figure 3. 7 Effects of drought stress on absolute final dry matter distribution (105 days after treatment) of non-genetically modified (NBt) and genetically modified cotton (Bt). NBtC and BtC represent well-watered controls and NBtS and BtS represent plants after drought-stress treatment.

CHAPTER 4: BIOCHEMICAL CHARACTERIZATION AND Bt EXPRESSION

4.1 Objectives

This chapter of the study focused on the biochemical characterization of genetically modified (Bt) and non-modified cotton plants. The objectives of this chapter was to determine Bt gene expression and stability in genetically modified cotton under abiotic stress.

4.2 Results

4.2.1 Total protein production

Total soluble protein was significantly reduced ($F_{1,54}$ = 13.1, $p < 0.0001$) in older plants (Figure 4.1A and B; ML) when compared to younger plants (Figure 4.1A and B; YL). However, no significant difference ($F_{1,54}$ = 9.3, p = 0.071) in total protein content was found in any plant part measured between genetically modified and non-modified cotton plants in either well-watered or drought stress-treated plants.

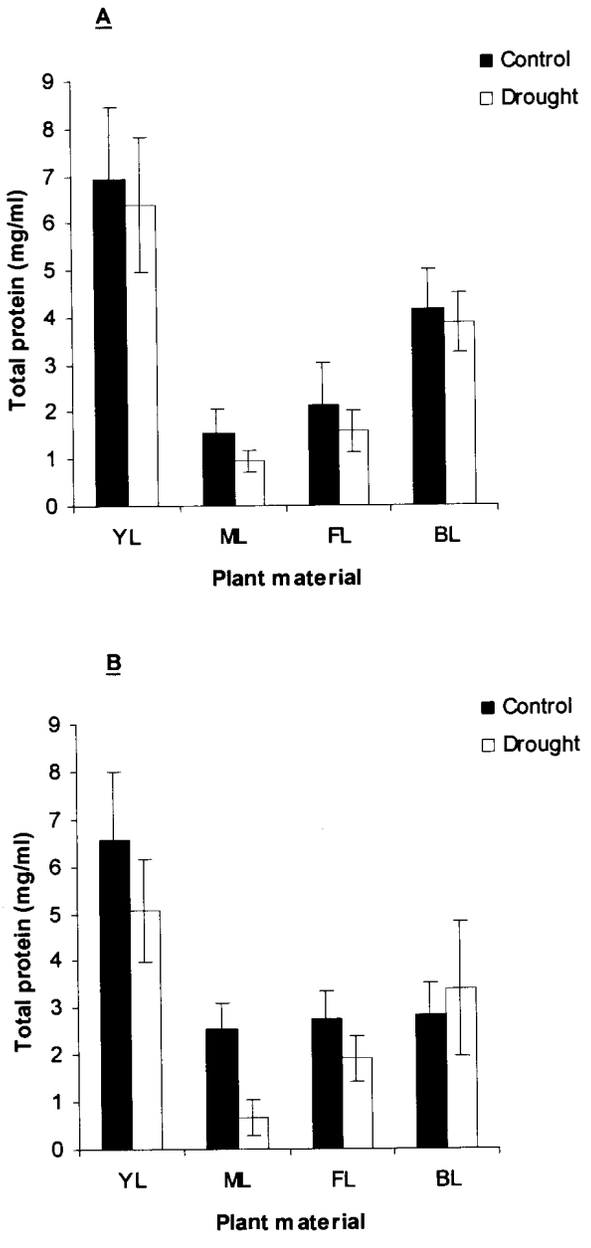


Figure 4. 1 The effects of drought stress on total soluble protein in genetically modified (Bt) cotton plants (A) and in non-modified plants (B) for different plant material with leaves from plants exposed to stress for 30 days (YL), leaves from plants exposed to stress for 60 days (ML), flowers (FL) and bolls (BL) from plants exposed to stress to 60 days. "Control" represents well-watered plants and "Drought" represents plants after drought-stress exposure. Data represent the mean of 10 individual plants (± SD) for the control group and for the drought group.

4.2.2 Expression and stability of Bt toxin

The expression and stability of Bt toxin (*Cry1Ac*) was immuno-detected by Western blotting. When an antiserum against the Bt toxin (*Cry1Ac*) was used to detect the toxin in genetically modified cotton plants before exposure to drought stress, a single band with the predicted size of about 65 kDa was detected (Figure 4.2). No immuno-reaction was detected with the *Cry1Ac* antiserum in non-modified cotton plants (Figure 4.2).

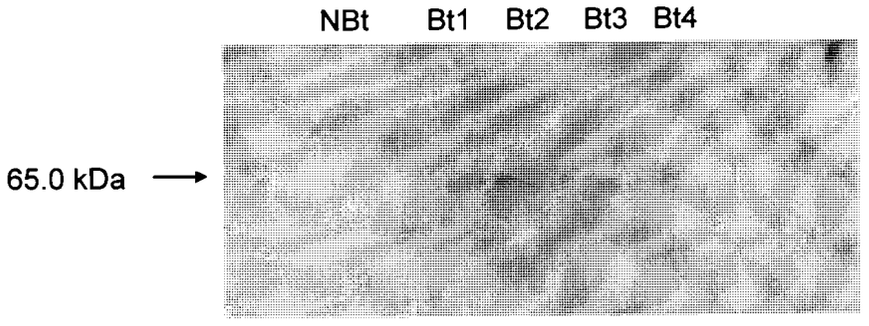


Figure 4. 2 Detection of Bt toxin (*Cry1Ac)* expression by Western blot analysis in leaves of non-genetically modified (NBt) and four different genetically modified (Bt) cotton plants before drought stress treatment. Each sample loaded onto the SDS-PAGE contained 100 µg protein.

After exposure to drought stress expression of the Bt toxin could not be clearly detected despite using a protein amount of 100 µg protein for SDS-PAGE, which also caused the immuno-detection of several other unspecific protein bands (Figure 4.3).

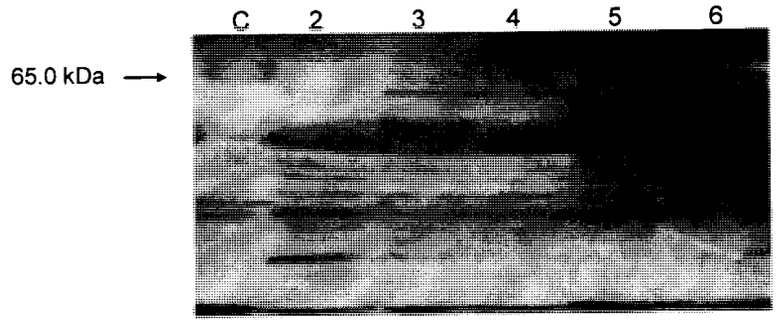


Figure 4. 3 Detection of Bt toxin (*Cry1Ac*) expression by Western blot analysis in plant extract from non-genetically modified (NBt) and genetically modified (Bt) plants under drought stress and non-drought conditions, with *Cry1Ac* antiserum. C represents positive control, lane 2 represents negative control extract derived from a non-modified plant; lanes 3 and 4 extracts derived from genetically modified cotton plants under drought stress and lanes 5 and 6 extracts from genetically modified well-watered cotton plants. Each sample loaded onto the SDS-PAGE contained 300 µg protein.

4.2.3 Detection of Bt toxin expression by ELISA under stress

Figure 4.4 shows a typical ELISA plate for the detection of Bt toxin (*Cry1Ac*) with the SDI *Cry1Ac* monoclonal antibody system.

Bt toxin (*Cry1Ac*) expression could be clearly identified by ELISA testing in all parts of genetically modified Bt cotton plants with the highest expression in younger leaves when compared to more mature leaves, flowers and bolls (Figures 4.5 and 4.6). This indicates that Bt is expressed differently in individual parts of the plant and that leaf ageing decreases Bt expression. However, expression of Bt toxin differed greatly between individual plants. Individual parts of the plants exposed to drought stress always showed a lower Bt toxin content than parts of the group of well-watered plants. However, this content was not significantly different ($F_{1,54}$ = 13.1, p = 0.054) between the two different groups of plants. Also, no significant decrease ($F_{1,54}$ = 9.7, p = 0.068) of Bt toxin content was found in Bt

cotton leaves after exposure to a temperature of 38/32 ºC (day/night time temperature) when compared to leaves from a group of plants exposed to 27/25 ºC (day/night time temperature).

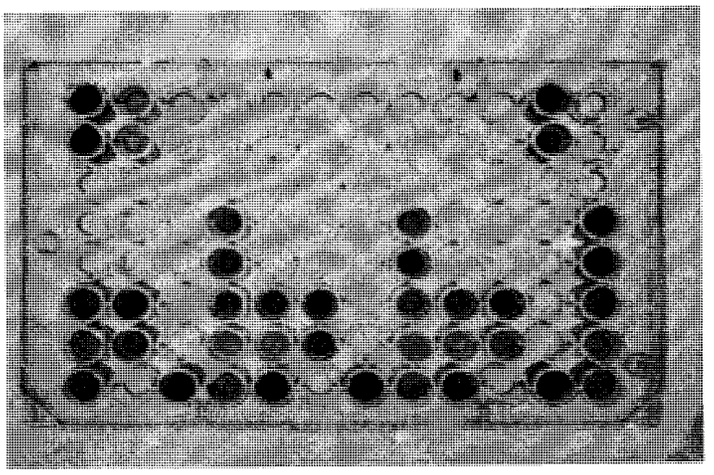


Figure 4. 4 Typical ELISA plate for the detection of Bt toxin (*Cry1Ac*) with the SDI *Cry1Ac* monoclonal antibody system. Development of a blue colour in the wells indicates presence of the Bt toxin. Intensity of blue colour was measured in an ELISA reader at 620 nm.

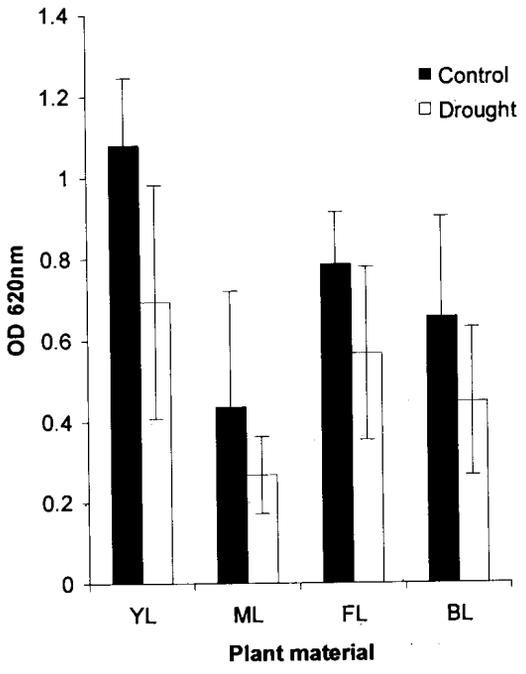


Figure 4. 5 The effect of drought stress on *Cry1Ac* expression in different parts of Bt cotton plants under well-watered (control) and drought stress conditions (drought). For detection of Bt expression leaves from plants exposed to stress for 30 days (YL) and leaves (ML), flowers (FL) and bolls (BL) from plants exposed to stress for 60 days were used. For measurement of Bt content in different plant material an equal amount of protein (200 µg protein) was used for each sample. Data represent the mean of 10 individual plants (± SD) for the "control" group and for the "drought" group.

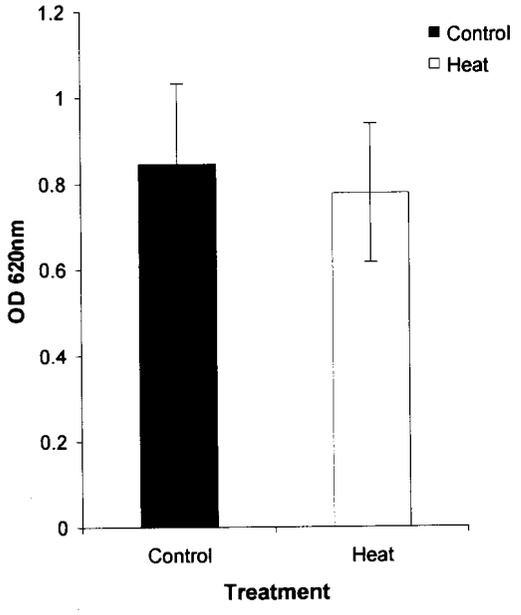


Figure 4. 6 The effect of heat stress on Bt toxin expression in leaves under well-watered (control) and heat stress conditions (heat). For detection of Bt expression leaves from plants exposed to stress for 30 days were used. For measurement of Bt content in different plant material an equal amount of protein (200 µg protein) was used for each sample. Data represent the mean of 10 individual plants (± SD) for the "control" group and for the "heat" group.

4.2.4 Detection of Bt toxin *Cry1Ac* by PCR

For *Cry1Ac* detection by PCR, primers were designed from the *Cry1Ac* sequence reported by Tengel *et al.* (2001) using the web Primer program (http://genome-www2.stanford.edu'cgi-bin/SGD/web-primer) for primer design. The forward primer used for *Cry1Ac* detection was *Cry1Ac*-Li-Cry1Ac-F 5'-GAAACCGGTTACACTCCCATCGAC-3'KK: and the reverse primer *Cry1Ac* -RI-Cry1Ac-R 5'-CTCTGCATAGATTTGGTAGAGATTG-3'KK. A 250bp DNA fragment containing the *Cry1Ac* coding sequence was successfully isolated and separated on a 1 % agarose gel (Figure 4.7).

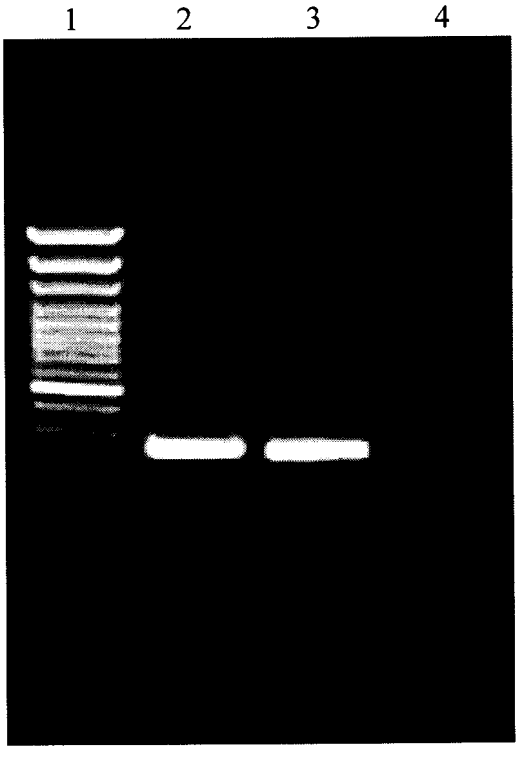


Figure 4. 7 Detection of *Cry1Ac* toxin presence in leaves. Lane 1 represents molecular weight marker (1000bp, Roche, Switzerland). Lanes 2 and 3 represent the *Cry1Ac* fragment (250bp) from genetically modified cotton plants and Lane 4 represents non modified cotton, separated on a 1 % agarose gel, and stained with ethidium bromide.

CHAPTER 5: BIOLOGICAL CHARACTERIZATION

5.1 Objectives

This chapter of the study focused on the results of biological characterization of genetically modified (Bt) and non-modified cotton plants. The objectives of this chapter was to determine biological activity against lepidopteran pests in genetically modified plants under abiotic stress.

5.2 Results

5.2.1 Insect feeding

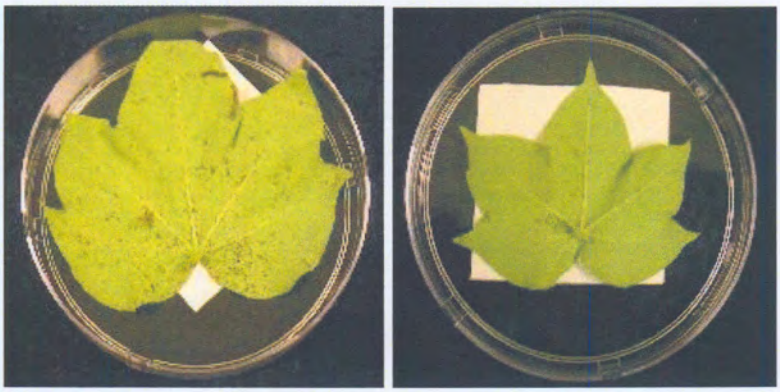
Newly hatched first-instar larvae of *H. armigera* were allowed to feed for five days on leaves of either genetically modified or non-modified plants under different environmental conditions. Figures 5.1A and B show the setup of the laboratory experiments with Petri dishes in which plant leaves from drought-treated and well-watered plants were used for biological activity testing.

Figures 5.2A-C show the data for weight of first-instar larvae of *H. armigera* after feeding on mature leaves, flowers and bolls of Bt and non-Bt plants under well-watered and drought conditions. Statistical differences between plant parts were very high. Therefore, plant parts were analysed separately.

The weight of *H. armigera* first-instar larvae feeding on mature leaves was approximately 27 % lower on Bt cotton plants compared to non-Bt cotton plants ($F_{1,57}$ = 8.6, p = 0.005) (Figure 5.2A). The weight of first-instar larvae feeding on mature leaves of drought-stressed plants was approximately 26 % lower compared to larvae feeding on mature leaves of well-watered non-Bt and genetically modified Bt cotton plants ($F_{1,57}$ = 8.6, p = 0.005).

The weight of *H. armigera* larvae feeding on flowers of Bt and non-Bt cotton plants was similar ($F_{1,70}$ = 3.8, p = 0.06) (Figure 5.2B). Drought stress did not reduced the

weight of larvae feeding on flowers on non-Bt and Bt cotton plants ($F_{1,70} = 0.45$, $p = 0.5$).



A B

Figure 5. 1 Typical insect feeding experiment with leaves of a genetically modified cotton plant grown either under drought stress (A) or under well-watered conditions (B). Three first-instar *Helicoverpa armigera* larvae were transferred to each leaf and allowed to feed for five days.

The weight of *H. armigera* larvae feeding on bolls was approximately 26 % lower on Bt cotton when compared to non-Bt cotton plants ($F_{1, 69} = 21$, $p < 0.0001$) (Figure 5.2C). However, drought stress did not affect the weight of larvae feeding on bolls of Bt and non-Bt plants ($F_{1, 69} = 21$, $p = 0.6$).

In a separate trial, the weight of first-instar larvae feeding on young leaves of Bt cotton was 96 % lower compared to larvae feeding on non-Bt plants ($F_{1,112} = 323.37$, $p < 0.0001$). The weight of larvae feeding on young leaves of drought-stressed plants was 32 % lower compared to larvae feeding on well-watered plants (($F_{1,112} = 16.77$, $p < 0.0001$) (Figure 5.2D).

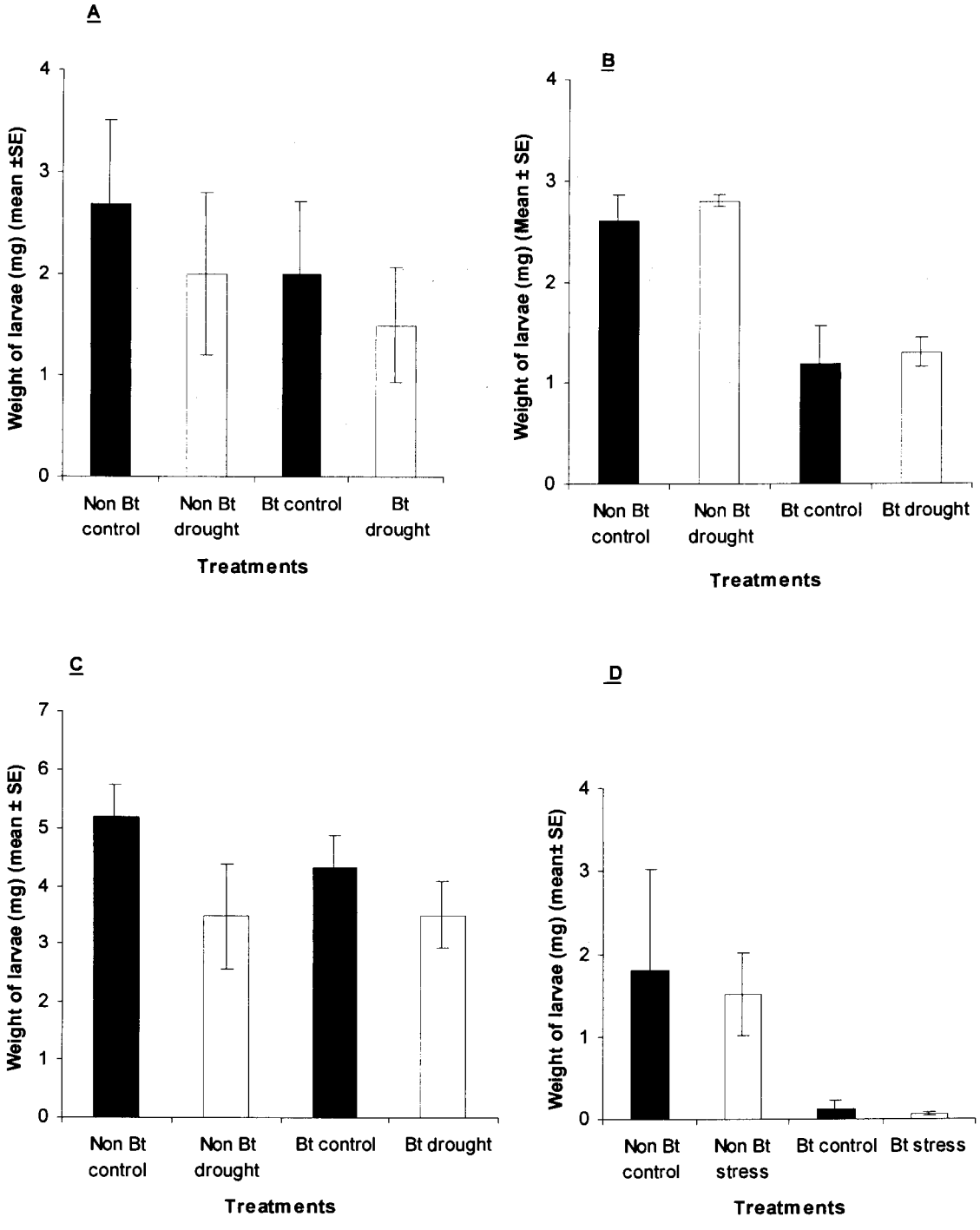


Figure 5. 2 Weight of *Helicoverpa armigera* larvae calculated as fresh weight gain after feeding on Trial 1 (A) mature leaves, (B) flowers and (C) bolls of non-Bt and Bt cotton plants and Trial 2 (D) young leaves.

The percentage corrected mortality of *H. armigera* larvae after feeding on young leaves was higher (87 %) compared to mature leaves (48 %), flowers (35 %) and bolls (45 %) (Figure 5.3). Drought stress slightly increased mortality of larvae feeding on younger leaves, flowers and bolls by 12 %, 13 % and 5 %, respectively, compared to the control. In contrast, the percentage corrected mortality of *H. armigera* larvae feeding on mature leaves was reduced (18 %) under drought stress conditions.

The percentage mortality of *H. armigera* larvae feeding on *Bt* plants was significantly higher when compared to larvae feeding on non-*Bt* plants (χ^2 = 35.7; *df* = 1; $p < 0.001$). Likewise, the mortality of larvae feeding on drought-stressed plants (χ^2 = 18.2; *df* = 3; $p < 0.05$) and well-watered plants (χ^2 = 15.2; *df* = 3; $p < 0.05$) was significantly higher on *Bt* compared to non-*Bt* cotton plants. However, drought stress did not increase mortality of larvae feeding on either Bt plants (χ^2 = 0.2; *df* = 3; $p > 0.05$) or non-Bt plants (χ^2 = 0.71; *df* = 3; $p > 0.05$). The percentage mortality of larvae feeding on flowers, mature leaves and bolls from *Bt* plants was higher compared to non-*Bt* plants (χ^2 = 9.0; *df* = 3; p= 0.001), (χ^2 = *12.0; df = 3; p= 0.001)* and (χ^2 = 15.4; *df* = 3; p= 0.001), respectively.

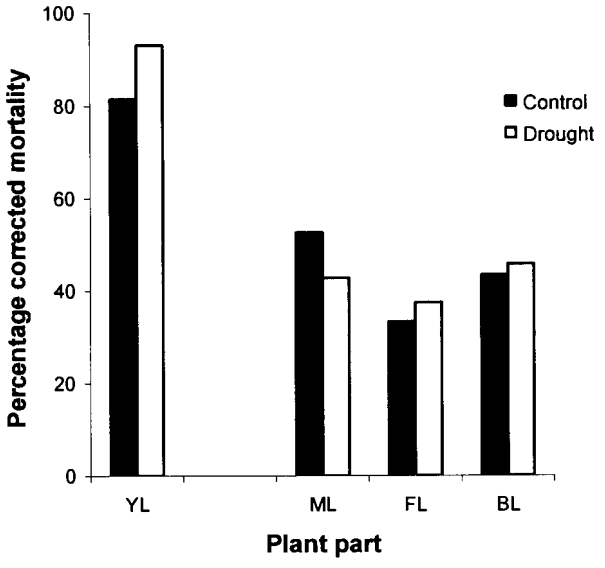


Figure 5. 3 Percentage corrected mortality of *H. armigera* larvae after feeding on young leaves (YL), mature leaves (ML), flowers (FL) and bolls (BL) of Bt cotton plants.

CHAPTER 6: DISCUSSION

6.1 Physiological plant performance

In this study, reduction in leaf area was found in both Bt toxin expressing and non-expressing cotton plants. In addition, under drought stress both types of plants had reduced leaf dry weight and leaf area and the delay of leaf appearance it is well documented. Environmental stresses, such as drought, high salinity and low temperature, have adverse effects on plant performance. In particular the expansion of leaves is very sensitive to lack of moisture and responds more rapidly with a decrease to changes in leaf water status than photosynthesis and transpiration (Michelena and Boyer, 1982; Muchow, 1985; Hoogenboom *et al.*, 1987). Reduction of leaf expansion is further associated with an increase in leaf thickness, resulting in a higher ratio of photosynthesis to transpiring area. However, whatever physiological parameter was measured in this study, no difference in physiological performance was found between Bt-expressing and non-expressing cotton plants. This confirms recent findings by Traore *et al.* (2000) that Bt-expressing and non-expressing plants are not significantly different in their growth performance under drought stress.

6.2 Biochemical characterization and Bt expression

In general, plants respond and adapt to stress through various biochemical and physiological processes, thereby acquiring stress tolerance (Shinozaki *et al.*, 2003). This can be based on the production of proteins or of secondary compounds. In this study, no significant difference in total protein content was found in different plant parts of either Bt-expressing or non-expressing cotton plants under both stress and non-stress conditions. In contrast, stress affected the expression of the Bt toxin. Bt toxin presence, which was identified by application of the PCR technique, was determined in this study by either the ELISA technique or by Western blotting. Using Western blotting, Bt expression was only identified in young non-stressed plants. However, this technique is less sensitive than ELISA and might not be suitable for detection of Bt expression. ELISA was therefore

applied as the standard method throughout the study. Under drought stress Bt toxin levels in transgenic cotton plants decreased. However, such a decrease was not found under heat stress. Using ELISA, Bt (*Cry1Ac*) expression was also detected in both non-stressed and stressed transgenic plants. The detection of *Cry1Ac* expression in these plants by ELISA testing also showed that all parts of genetically modified Bt cotton plants clearly express the toxin.

Drought stress has both positive and negative effects on protein synthesis in plants, depending on duration and intensity of the stress (Shiralipour and West, 1968; Gershenzon, 1984; Mattson and Haack, 1987; Lilburn *et al.*, 1991). Differences in soil moisture content have been identified to contribute to a decrease in total soluble protein and Bt toxin (*Cry1Ab*) concentrations (Sachs *et al.*, 1998). However, Bt expressing plants had greater grain and biomass yields than their non-Bt counterparts. However, decrease of Bt expression under drought stress was not significant, but a clear trend to lower Bt expression was found after exposure of transgenic cotton plants to drought stress. In this study unfortunately, experiments to determine if Bt toxin degradation occurring as a consequence of drought stress were not successful, despite several attempts to identify possible degradation products by Western blotting and also loading high amounts of protein on SDS gels. The statistical analysis of plants was affected by a high variability in expression levels between the ten individual plants used. Among the plants tested, some were even found with non-detectable Bt toxin levels, this fact may contribute to high variability. Such plants were, however, not used in the experiments. In addition, in an attempt to measure Bt expression in cotton material collected from non-irrigated cotton of small-scale farms, no detectable Bt expression was found in any of the transgenic material collected using the ELISA technique. Due to expression variability, it is therefore vital in any experimental study to test all plants before inclusion in an experimental design, as done for this study, to reduce any high variability of expression levels in the experiments. Such variability has also been reported by other research groups (Greenplate, 1999). Toxin levels may not only vary within a single plant due to environmental conditions, but might also vary throughout the growing season. Also, terminal leaves express more Bt toxin when compared to flowering structures (Greenplate, 1999; Greenplate *et al.*, 2000; Adamczyk *et al.*, 2001). Unfortunately, variable Bt toxin expression might

contribute to the development of insect resistance to the Bt toxin; in addition, temporary toxin expression might further cause loss of efficacy of the Bt toxin for insect control (Mellon and Rissler, 1998).

Factors that might influence Bt expression in a transgenic plant are still not completely understood. This includes site-of-gene insertion, cultivar or parental background, and various environmental conditions. They contribute to variable Bt toxin expression, so that variability was even found among replicates within a location (Sachs *et al.*, 1998). In general, environmental factors, such as temperature and moisture influence plant gene expression and any factor that affects gene expression and protein synthesis or degradation is also likely to influence Bt toxin expression. Results of several research groups (Holt, 1998; Fitt, 1998; Sachs *et al.*, 1998; Greenplate, 1999) have shown that the Bt (*Cry1Ac*) toxin level decreases in various plant parts throughout the growing season. Mahon *et al.* (2002) further found that cold treatment of transgenic cotton plants resulted in reduction of the efficacy of the Bt toxin. Variable expression levels among various plant parts might also support many consultant's and growers observations that more bollworms are found feeding on and damaging Bt cotton fruit (i.e. squares buds, flowers and bolls) than green square buds, square and boll bracts (Adamczyk *et al.*, 2001). Field trials with *H. zea* further showed that larvae on flowers apparently feed on pollen with low toxin content (Greenplate, 1997).

In the present study it was also found that Bt expression significantly decreased during plant senescence. This result is in agreement with a previous finding that levels of Bt expression decline once plants start to age. Bt concentrations decline significantly after squaring, possibly due to declining production of mRNA (Fitt *et al.*, 1994). It is well documented that the promoter activity driving gene expression is controlled by DNA methylation. This process might occur during plant maturation and it is also controlled by the tissue type in which a transgene is expressed (Kloti *et al.*, 2002; Elmayan and Vaucheret, 1996). Promoter methylation can be evident in the exon/intron region of a promoter and is thereby thus associated with transcriptional gene silencing, but can also occur in the transcribed region and so is associated with post-transcriptional gene silencing (Meng *et al.*, 2003).

6.3 Biological characterization

The percentage mortality of *H. armigera* larvae feeding on cotton plants expressing the Bt toxin was significantly higher in the present study when compared to larvae feeding on plants not expressing the Bt toxin. The study therefore confirms that plants expressing the Bt toxin are highly toxic to *H. armigera* (Pietrantonio and Heinz, 1998). Further, mortality was always greater in transgenic Bt-expressing plants when compared to non-Bt plants, whether plants were exposed to drought stress or not.

A significant increase in mortality of *H. armigera* larvae was found with larvae feeding on young leaves. This result was probably associated with an increased Bt toxin level (*Cry1Ac*) in leaves at the vegetative stage or the lower level of secondary compounds in younger leaves (Olsen *et al.*, 1998). Drought stress did not significantly reduce larval mortality, although a clear trend towards lower Bt expression levels was found in plants. In contrast, insect mortality clearly decreased when feeding on senescent plant material.

Maintaining insect mortality under drought stress might be explained in two ways. First, the level of Bt toxin produced is highly sufficient to kill larvae, despite the fact that plants are exposed to drought stress. Secondly, it is well documented that under drought stress the metabolism of plants is changing and secondary metabolites are produced as a response to stress. Production of such metabolites might significantly contribute to maintaining the mortality rate under drought stress and thus be additive to the Bt toxin effect. Plants respond to drought by changing the concentration and levels of secondary compounds. This includes production of phenolics, orthoquinones, and tannins (Sivamani *et al.*, 1992; Navon *et al.*, 1993; Gibson *et al.*, 1995; Ludlum *et al.*, 1991). Tannins are reported to be potent in their antibiotic effect and adversely affect the growth and development of lepidopteran larvae (Kranthi *et al.*, 2003). In cotton, stress metabolites like lasinilene and cadalene as well as phenolic polymers in the lignin fraction are produced under biotic stress and drought is also able to promote the production of poisonous metabolites in legumes (Dubery and Slater, 1997; Anderson *et al.*, 2001). The possibility of metabolite involvement as an additive effect under stress has also

been suggested by Adamczyk *et al.* (2001). Overall, additional production of anti-insecticidal metabolites might result in Bt plants that can be used under drought conditions without compromising on efficacy against bollworm.

The percentage mortality of *H. armigera* larvae feeding on mature leaves was higher in the present study when compared to flowers and bolls of Bt expressing cotton plants. High larval mortality was, however, found in larvae feeding on mature leaves expressing only a very low level of the Bt toxin. Additionally, mature leaves under drought significantly reduced the mortality of *H. armigera* larvae feeding on such leaves, indicating a direct relation between Bt-levels and larval mortality. However, factors including low nutritional value or increased levels of secondary compounds (e.g., condensed tannins) in leaves of mature plants might have also contributed to a higher mortality (Olsen *et al.*, 1998).

Overall, this study confirms previous research results but also provided new insights. The study clearly shows that the Bt toxin (*Cry1Ac*) level decreases in senescent plants and that drought stress does not affect the growth and development of genetically modified Bt plants when compared to non-Bt plants (Fitt, 1998; Sachs *et al.*, 1998; Greenplate *et al.*, 2000; Adamczyk *et al.* 2001). This study provided as a new result that under drought stress the expression of Bt toxin (*Cry1Ac*) in Bt cotton plants decreases but does not affect the efficacy of the toxin against *H. armigera*.

6.4 Future perspectives

The application of Bt technology is proving to be among the most effective techniques in the management of destructive stem borers that are currently causing great yield losses across Africa. Through the use of genetically modified cotton plants expressing the Bt toxin, farmers in Africa might be able in the future to maximise crop production at reduced use of chemical pesticides, which are often expensive and also toxic. However, stable expression of a transgene is among the essential requirements for the successful implementation of the

transgenic technology and for it to be part of a strategy to provide effective protection of plants against stressful environments.

The present study formed part of a continuous body of research in molecular plant physiology to investigate the influence of abiotic stresses typical for Africa on Bt expression in transgenic plants. The results obtained clearly showed that drought or heat stress contributes, in comparison to natural senescence, only little to the decline in Bt efficacy against *H. armigera.* However, one should always be cautious to translate data generated from a laboratory study directly into a field situation with a variety of stresses and different stages of plant maturation (Bianchi *et al.*, 2000). Certainly, additional investigation of other environmental stresses and stress combinations is required to ascertain that transgenic plant performance is not compromised by these stresses and that optimal activity of the Bt toxin in transgenic plants is ensured.

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ANNEXURE

Buffers for protein work

50 mM Tris-HCl (pH 8.9) (Buffer A)

606 mg Tris was added to 180 mL distilled water. The pH was adjusted to pH 8.9 with 0.1 M HCl and the final volume made up to 200 mL.

50 mM sodium acetate (NaAc)/ 10 mM β-mercaptoethanol (pH 5.4) (Buffer B)

820 mg NaAc was added to 180 mL distilled water. The pH was adjusted to pH 5.4, and the final volume made up to 200 mL. β-mercaptoethanol was diluted to a 50 mM stock solution on the day of use (35 µL β-mercaptoethanol added to 10 mL dH_2O), and 100 µL of this stock solution was added to 400 µL of the 50 mM NaAc extraction buffer to obtain a final concentration of 10 mM β-mercaptoethanol.

Transfer buffer for Western blotting (pH 8.2)

25 mM Tris, 192 mM glycine, 15 % methanol. 3.03 g Tris and 14.4 g glycine were dissolved in 150 mL methanol. The solution was brought to 1 L with dH_2O. The solution was stored at 4°C.

10x Tris buffered saline (TBS) (pH 7.6)

24.23 g Tris and 292 g NaCl were dissolved in 700 mL dH_2O, pH was adjusted to pH 7.6 with HCl, and the final volume was brought to 1 L with dH_2O. For further use the buffer was diluted 10-times and stored at 4 °C.

Gel electrophoresis systems

30 % Acrylamide mix (stock solution):

29 g acrylamide and 1 g bisacrylamide were dissolved in warm distilled water and brought to 100 mL with dH_2O to give a final ratio of 29:1 (w/w) acrylamide:bisacrylamide. The solution was stored at 4 °C.

1 M Tris-HCl, pH 6.8 (stock solution):

12 g Tris was dissolved in 80 mL dH_2O, titrated to pH 6.8 with 1 M HCl and brought to a final volume of 100 mL with dH_2O.

1.5 M Tris-HCl, pH 8.8 (stock solution):

18.17 g Tris was dissolved in 80 mL dH_2O, titrated to pH 8.8 with 1 M HCl and brought to a final volume of 100 mL with dH_2O.

Table A.1 Polyacrylamide gel (non-denaturing) 6 %

Stock solution	**6 % Resolving gel**
30 % acrylamide mix	2 mL
1.5 M Tris (pH8.8)	2.5 mL
10 % Ammonium persulphate	0.1 mL
TEMED	8 µL
Water	5.4 mL

Tris glycine electrophoresis buffer for non-denaturing PAGE gel:

Electrophoresis buffer stock (5x) (0.125 M Tris, 1.25 M glycine, pH 8.3): 15.1 g Tris and 94 g glycine were dissolved in 900 mL dH_2O. The pH was adjusted to pH 8.3, and the final volume made up to 1 L. The solution was stored at 4 °C and diluted five times before use.

Loading buffer for non-denaturing PAGE gel:

Buffer [50 mM Tris-HCl pH 6.8, 10 % (v/v) glycerol, 100 mM dithiothreitol (DTT), 0.01 % (w/v) bromophenol blue]: 1.7 mL 1.5 M Tris (pH 6.8) and 5 mL glycerol were mixed. Bromophenol blue (2.5 mg) was added and the solution was brought to a final volume of 50 mL with dH_2O. DTT was added to the solution on the day of use from a 1 M stock solution, to bring the final concentration of DTT to 100 mM.

Table A.2 SDS-PAGE gel (5-10 %) (Sambrook *et al.*, 1989)

Stock solution	**Stacking gel**	**10 % Resolving gel**
30 % acrylamide mix	0.33 mL	1.7 mL
1M Tris (pH 6.8)	0.25 mL	-
1.5M Tris (pH 8.8)	-	1.3 mL
10 % SDS	0.02 mL	0.05 mL
10 % Ammonium persulphate	0.02 mL	0.05
TEMED	2 µL	0.002 mL
Water	1.4 mL	1.9 mL

Table A.3 SDS-PAGE gel (5-12 %)(Sambrook *et al.*, 1989)

Stock solution	**Stacking gel**	**12 % Resolving gel**
30 % acrylamide mix	0.33 mL	2.0 mL
1M Tris (pH 6.8)	0.25 mL	-
1.5M Tris (pH 8.8)	-	1.3 mL
10 % SDS	0.02 mL	0.05 mL
10 % Ammonium persulphate	0.02 mL	0.05 mL
TEMED	2 µL	2 µL
Water	1.4 mL	1.6 mL

Electrophoresis buffer for SDS PAGE gel:

Electrophoresis buffer stock (5x) [0.125 M Tris, 1.25 M glycine, pH 8.3, 0.1 % (w/v) SDS]: 15.1 g Tris and 94 g glycine were dissolved in 900 mL dH_2O. The pH was adjusted to pH 8.3 and 50 mL of a 10 % (w/v) stock solution of SDS was added. The final volume was made up to 1 L with dH_2O. The solution was stored at 4 °C and diluted five times before use.

Loading buffer for SDS PAGE gel:

1-time Buffer [50 mM Tris-HCl pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 100 mM DTT, 0.01 % (w/v) bromophenol blue]: 1.7 mL 1.5M Tris (pH 6.8) and 5 mL glycerol were mixed. From a 10 % (w/v) SDS stock solution (10 mL) was added. Bromophenol blue (2.5 mg) was added and the solution was brought to a final volume of 50 mL with dH_2O. DTT was added to the solution on the day of use from a 1 M stock solution, to bring the final concentration of DTT to 100 mM.

Table A.4 Staining solution for PAGE gels:

Chemical	Concentration	Weight/ volume
Coomassie Brilliant Blue R-250	0.1 %	0.1 g
Water	65 %	65 mL
Propan-2-ol	25 %	25 mL
Glacial Acetic Acid	10 %	10 mL
Total Volume		100 mL

1 % Agarose gel for separation of DNA

1 g Agarose was added to 100 mL TAE buffer and heated in a microwave until boiling. After solution had cooled down to 60 °C, 2 μL of a 10 mg/mL stock solution of ethidium bromide was added. The ethidium bromide intercalates between the DNA basepairs and fluoresces under UV light so that the DNA can be visualised.

A 50-times stock solution was prepared by mixing the ingredients and adjusting the pH to pH 8.5. This stock solution was stored away from light, and diluted 50 times for use.