Efficacy of *Bacillus thuringiensis* spray applications for control of lepidopteran pests

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It all starts here ™

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ABSTRACT

Organic insecticides play a big role in reducing the usage of chemical insecticides and their negative impact on the environment. *Bacillus thuringiensis* (Bt) spays are the only tool that organic farmers are allowed to use for the control of pests. Genetic engineering and modification of crops have been made possible with scientific advances in cell and molecular biology. These advances are used to transfer some of the Bt Cry toxins into crops for control of target species to reduce yield loss. Bt maize were commercialised for the first time in South Africa in 1998 and the economic important stem borers, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) were exposed to the Cry1Ab toxin that is found in Bt maize. *Busseola fusca* developed resistance to Cry1Ab under field conditions within eight years after it had been released. *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) is a major pest on sugarcane in South Africa and although it has not been recorded on maize in this country, is it known as a major pest of maize in other African countries. African armyworm, *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae) has a very wide distribution in Africa and is known to be an occasional pest on maize. The aims of this dissertation were to determine the efficacy of Bt spray applications for control of four lepidopteran pests and whether development of Cry1Ab resistance by *B. fusca* caused a loss in susceptibility to other Bt toxins (i.e. cross-resistance). Susceptibility bioassays with 10 day old larvae were conducted under laboratory conditions. Treatments included application of various dosage rates of Dipel® and deltamethrin as well as exposure to MON810 (maize leaves). Stemborer populations of *C. partellus, E. saccharina,* and *B. fusca* (Venda) as well as the *S. exempta* were effectively controlled by the Bt spray, Dipel®. Care should be taken not be interpret the percentage *C. partellus, E. saccharina* and *S. exempta* larvae that survived after exposure to MON810 and Bt spray treatments as development of resistance without verification of these experiments with earlier instars that are known to be more susceptible. *Spodoptera exempta* is active throughout a year in temperate zones of Africa. If *S. exempta* develop resistance to Cry toxins and Bt maize events would be released for commercial planting in these areas, *S. exempta* pose a threat added to their injuriousness. *Busseola fusca* larvae were sampled from Venda (susceptible population), Ventersdorp and the Vaalharts

Irrigation Scheme (resistant population). The Ventersdorp *B. fusca* population was controlled by MON810 and MON89034 and Bt sprays, but the percentage larvae that survived showed reduced susceptibility within the population. Dipel® treatments, MON810 and MON89034 did not provide effective control of the Vaalharts *B. fusca* population reported to be resistant to Cry1Ab, in two experiments. The high survival rates indicate a reduction in susceptibility to Cry toxins other than Cry1Ab and therefore development of cross resistance in the Vaalharts *B. fusca* population.

Keywords: *Bacillus thuringiensis*, Bt spray, *Busseola fusca*, *Chilo partellus,* cross resistance, *Eldana saccharina,* maize pests, *Spodoptera exempta*.

UITTREKSEL

Die gebruik van organiese insekdoders speel 'n groot rol om die verbruik van chemiese insekdoders en die negatiewe impak daarvan op die omgewing te verminder. Bespuiting met *Bacillus thuringiensis* (Bt) is die enigste beheeropsie of moontlikheid wat organiese boere tot hulle beskikking het. Die genetiese manipulasie van gewasse is moontlik gemaak deur die vooruitgang in sellulêre en molekulêre biologie. Dit word gebruik om gene vanaf die Bt-bakterium oor te dra na gewasse toe met die oog op die beheer van teikenspesies. Bt mielies wat Cry toksiene produseer is vir die eerste keer in 1998 in Suid-Afrika gekommersialiseer. Met die kommersialisering was die ekonomies-belangrike stamboorders, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) en *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) blootgestel aan die Cry1Ab toksien wat in Bt mielies voorkom. Binne agt jaar, het *B. fusca* weerstand in die veld teen Cry1Ab ontwikkel. *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) is 'n ernstige plaag op suikerriet in Suid-Afrika en alhoewel dit nog nie op mielies in Suid-Afrika aangeteken is nie, is dit 'n ernstige plaag op mielies in ander Afrika lande. Die kommandowurm, *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae) is baie verspreid in Afrika en is ook bekend om per geleentheid 'n plaag op mielies te wees. Die doel van die studie was om die doeltreffendheid van Bt bespuitings vir die beheer van vier Lepidoptera plae te toets, asook om vas te stel of die ontwikkeling van weerstand teen Cry1Ab deur *B. fusca* gelei het tot verminderde doeltreffendheid van ander Bt toksiene (kruisweerstand). Tien-dae oue larwes was gebruik om biologiese toetse in die laboratorium uit te voer om die vatbaarheid van die larwes te bepaal. Die behandelings het bestaan uit verskeie dosisse van Dipel®, deltametrien en blootstelling aan MON810. Die stamboorderpopulasies van *C. partellus, E. saccharina,* en *B. fusca* (Venda) was effektief beheer deur die Bt bespuiting met Dipel®. Daar moet gewaak word dat die persentasie larwes van *C. partellus, E. saccharina* en *S. exempta* wat oorleef het op MON810 en na Bt toedienings, nie as weerstandbiedend gerapporteer word, alvorens die eksperiment nie met jonger larwes, wat meer vatbaar as ouer larwes is nie, herhaal was nie. *Spodoptera exempta* is aktief deur die jaar in gemagtigde areas van Afrika. Indien *S. exempta* weerstand ontwikkel teen Cry1Ab toksiene en Bt mielies kommersialiseer verbou sou word in hierdie areas, kan *S. exempta* 'n

verdere gevaar inhou. *Busseola fusca* larwes is in Venda (vatbare populasie), Ventersdorp en die Vaalharts besproeiingskema (weerstandbiedende populasie) versamel. Die *B. fusca* populasie van Ventersdorp was beheer deur MON810, MON89034, sowel as Bt toedienings, maar die persentasie oorlewing van die larwes dui op verlaagde vatbaarheid in die populasie. Dipel$^®$ behandelings, MON810 en MON89034 het nie die *B. fusca* populasie van Vaalharts, wat as weerstandbiedend is teen Cry1Ab, effektief beheer nie. Die hoë persentasie oorlewing dui op 'n daling in die vatbaarheid vir ander Cry toksiene anders as Cry1Ab, en dus die moontlike ontwikkeling van kruisweerstand deur die *B. fusca* populasie van Vaalharts.

Sleutelwoorde: *Bacillus thuringiensis,* Bt bespuiting, *Busseola fusca*, *Chilo partellus, Eldana saccharina,* kruisweerstand, mielie plae, *Spodoptera exempta.*

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Chapter 1

Introduction and literature overview

1.1   Introduction

It is estimated that 95 % of the global population increase until the year 2050 will take place in developing countries (Cohen, 2005). Maize [(*Zea mays* L. (Poaceae)] is grown worldwide in different agricultural environments (IPB, 2013). Africa consists mainly of developing countries where maize is the staple food of many households (Thomson, 2008). The whole of Africa uses 95% of the production of maize as a food source, therefore is maize the most important cereal crop for food in sub-Saharan Africa (IPB, 2013). White grained maize is known to be the staple food of many South Africans, and yellow grained maize is grown in large quantities to serve as animal feed (Gouse *et al.,* 2005). Maize is a popular crop because it is high yielding, easy to process, readily digested, more affordable than other cereals and contains carbohydrates, proteins, iron, vitamin B and other minerals (IITA, 2009). Maize grain, leaves, stalks, tassels and ears have economic value and can be used to produce a large variety of products (IITA, 2009). Biological stresses such as mildew, rust, leaf blight, stalk and ear rots, leaf spot, maize streak virus and damage caused by various insect species result in decreased yields (IITA, 2009). Insect pests cause direct losses to yields worldwide (Mugo et al., 2011). It is estimated that Kenya loses 13.5% of its annual maize production to pests (De Groote, 2002).

1.1.1 Lepidopteran pests of maize in Africa

Many insect species are associated with maize in Africa amongst which various lepidopteran species are considered as economically important pests (Table 1.1).

Table 1.1 Lepidopteran pests of maize (*Zea mays* L.) in Africa.

Family	Species	Common name	Reference
Crambidae	*Chilo aleniellus*		Moyal & Tran, 1992
	Chilo orichalcociliellus		Seshu Reddy, 1983
	Chilo partellus	Spotted stem borer	Van Hamburg, 1979
Noctuidae	*Agrotis ipsilon*	Black cutworm	Rings *et al.,* 1975
	Agrotis longidentifera	Brown cutworm	Annecke & Moran, 1982
	Agrotis segetum	Common cutworm	Annecke & Moran, 1982
	Agrotis spinifera	Spiny cutworm	Rivnay & Yathom, 1964
	Agrotis subalba	Grey cutworm	Pretorius *et al.*, 1996
	Busseola fusca	African stem borer	Wahl, 1926
	Helicoverpa armigera	African bollworm	Jones, 1937
	Helicoverpa zea	Tomato fruitworm	Bong & Sikorowski, 1991
	Leucania loreyi	False armyworm	Ganeshan & Rajabalee, 1996
	Sesamia calamistis	African cereal stem borer	Usua, 1968
	Sesamia cretica	Pink stem borer	Gahan, 1928
	Sesamia nonagrioides botanephaga		Mohyuddin & Greathead, 1970
	Spodoptera exempta	African armyworm	Brown *et al.,* 1969
	Spodoptera exigua	Beet armyworm	Mikkola, 1970
Pyralidae	*Eldana saccharina*	Sugarcane borer	Atkinson, 1980
	Mussidia nigrivenella	Maize ear borer	Adeyemi, 1969

Stem borers such as *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae), *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) are very difficult to control due to the fact that they tunnel into the stems of plants where they are difficult to reach (Mugo *et al.,* 2011).

1.1.2　Genetically modified crops

Genetic engineering and modification of crops have been made possible with scientific advances in cell and molecular biology (Barton & Miller, 1993). With these advances it has become possible to transfer the DNA from other sources into specific crops, which can provide certain desirable traits to crops (Barton & Miller, 1993). It allows for genes that provide resistance to pests, diseases, herbicides and environmental stresses to be transferred into crops (Nap *et al.,* 2003). Insect control in agriculture has a high economic and environmental cost, and therefore it is no surprise that insect resistant transgenic plants were some of the first plant biotechnology products to reach the market (De Maagd *et al.*, 1999).

Genetically modified (GM) crops were commercialised since 1996 (James, 2012) and showed a global annual growth rate of 6%, which resulted in a record amount of GM crops been grown worldwide (James, 2012). The area planted with GM crops increased with 10.3 million hectares during the 2011/12 growing season to a total of 170.3 million hectares in 2012 (James, 2012). South Africa ranked eighth out of 28 countries in terms of area planted (2.9 million hectares) to GM crops (James, 2012). South Africa is also one of the five leading developing countries in biotech crops along with China, India, Brazil and Argentina (James, 2012).

GM crops entered the South African market in 1998 (ACB, 2012). Monsanto's insect resistant (IR) cotton, known as "Bollgard" and the insect resistant maize, MON810 were the first GM crops to be grown commercially in South Africa (ACB, 2012). MON810 maize, expresses *Bacillus thuringiensis* Cry1Ab toxins (Székács *et al.*, 2010). Monsanto also produced the first herbicide tolerant variety soybean which was cleared for commercial cultivation in 2001 (ACB, 2012). By 2004/05, 20% of maize seed sales in South Africa were genetically modified maize (ACB, 2012). The

GM maize event, MON810, was followed by an insect resistant event of Syngenta (Bt11) and Monsanto's herbicide tolerant event NK603 (ACB, 2012). Until now, GM maize, cotton and soybeans are the only genetically modified crops to be grown commercially in South Africa and 94% of all genetically modified crops planted globally consist of these three crops (ACB, 2012).

1.1.3 What is *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a bacterium that can be found in natural environments such as soil, the phyllosphere (surface of a leaf considered as a habitat), dust, plant material and insects, and proved to be an effective insect pathogen (Raymond *et al.,* 2010). With today's modern molecular methods it can be produced relatively easy in plants (Betz *et al.,* 2000). This species consists of a number of distinct subspecies, varieties and pathotypes. There were already 69 recognised serotypes and 13 sub antigenic groups, in total 82 serovars in 1999 (Lecadet *et al.*, 1999). There are more than 170 endotoxin-encoding genes identified, which indicated that the level of diversity within the species is high (Glare & O'Callaghan, 2000). *Bacillus thuringiensis* consists of diverse strains with widely different toxin profiles and it therefore has an extensive range of activity against a vast array of insects (Glare & O'Callaghan, 2000). The range of Cry toxins serve as building blocks for the development of Bt products and as part of the technical feasibility (Betz *et al.,* 2000).

Bacillus thuringiensis is a gram-positive, rod-shaped bacterium with the ability to produce crystalline inclusions during sporulation (Höfte & Whiteley, 1989). *Bacillus thuringiensis* produces parasporal crystals which in their turn consist of one or more δ-endotoxins or crystal (Cry) toxins (De Maagd *et al.,* 1999). The parasporal crystals are also the difference between Bt and other related *Bacillus* species and are what makes Bt an effective insect pathogen (De Maagd *et al.,* 1999). These endotoxins are grouped into four major classes: Cry1 which is Lepidoptera-specific, Cry2 which is Lepidoptera and Diptera-specific, Cry3 which is Coleoptera-specific and Cry4 which is Diptera-specific (Pigott & Ellar, 2007). The Cry toxins are classified according to their primary sequence similarity (Bravo & Soberón, 2008). One of the major groups of Cry toxins is known as the threedomain (3D) - Cry family and the

members of this family all share similarity in sequence and structure (Bravo & Soberón, 2008). There are, however, other Cry toxins that differ in sequence and structure from the 3D–Cry family (Bravo & Soberón, 2008). Although there are many types of Cry toxins, only a few of them are used commercially in Bt crops and Bt sprayable products (Bravo & Soberón, 2008). These Cry toxins include Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1D, Cry1E, Cry1F, Cry2Aa, Cry2Ab, Cry3A and Cry3B (Bravo & Soberón, 2008).

The Bt bacterium possesses insecticidal and occasionally wider toxicity because of the inclusion of endotoxins, haemolysis, exotoxins and enterotoxins (Glare & O'Callaghan, 2000). The formulations of Bt currently available contain ingredients other than Bt spores and crystals, which can also affect the toxicity of applications of this product in commercial spray formulations (Glare & O'Callaghan, 2000). The characterisation of Bt is not an exact science, and specific toxicity can therefore not be attributed to anything but a subspecies or product in general (Glare & O'Callaghan, 2000).

It was previously thought that microbial insecticides such as Bt would replace chemical insecticides and their negative impacts but limitations of microbes slowed down the process (Bravo *et al.,* 2011). Limitations include the narrow spectrum of activity of microbes and they are therefore only able to kill certain insect species (Bravo *et al.,* 2011). Microbes also have low environmental persistence and in the case of application, it needs to be very precise due to the pathogens being sensitive to irradiation or only specific to young larval stages of insects (Bravo *et al.,* 2011). In 2010 Bt did, however, comprise ~ 2% of the total insecticide market and was known as the most successful pathogen for the control of insects (Bravo *et al.,* 2011). Bt is therefore ultimately known as the predominant bio-pesticide of the late 20th century (Glare & O'Callaghan, 2000).

1.1.4 *Bacillus thuringiensis* transgenic maize

The forty year record of effective insect control and safe use of Bt sprays made it a suitable option to use in the development of a new pest control product, namely Bt transgenic maize (Betz *et al.,* 2000).

The first Bt maize cultivars that were released, expressed Cry1Ab for control *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Archer *et al.,* 2001; He *et al.,* 2003) and *Diatraea grandiosella* (Dyer) (Lepidoptera: Crambidae) (Williams *et al.,* 1998) in the United States of America. It was also introduced into South Africa for the control of *B. fusca* and *C. partellus* (Van Rensburg, 1999).

1.1.4.1   Advantages of Bt transgenic maize crops

Cultivation of Bt crops provide farmers with a cost effective, environmentally acceptable, low risk, cost saving pest control tool. It is an effective pest control option which results in reduced crop losses and beneficial insects are not affected by the Cry toxins (Betz *et al.,* 2000). Farmers earn a higher income due to the reduction in pesticides usage and higher yields (Gouse *et al.,* 2005). Bt is ever-present in the plant, are continuously expressed and the farmer does not have to be specific on timing of insecticide application (Navon, 2000). The timing and accuracy of the insecticide application or natural events such as rain wash-off or sunlight inactivation has therefore no effect on the efficacy of Bt crops (Betz *et al.,* 2000). Bt crops usually express sufficient quantities of Cry toxins to control insects in an effective manner before resistance development and the degree of safety of Bt crops are unmatched by any other pest control product (Betz *et al.*, 2000). Cry toxins have a narrow spectrum of activity, should be ingested to have an effect and do not have a contact action, causing the toxin to be very target specific. Exposure of humans and non-target organisms to Cry toxins are very low because it is enclosed in the plant, unlike pesticides which are applied on leaves (Betz *et al.,* 2000).

Bt crops are also compatible with other control options in integrated pest management systems (IPM) (Hillocks, 2005), and is therefore, a safe pest control option for people in surrounding areas due to reduced pesticide usage (Gouse *et al.,* 2005).

Concerns were raised about the effects of Cry toxins in Bt maize on non-target arthropods (Daly & Buntin, 2005), resulting in many studies. No significant effect of Bt maize (Cry1Ab) were reported on *Orius majusculas* (Reuter) (Hemiptera: Anthocoridae) (Zwahlen *et al.,* 2000), *Orius insidiosus* (Say) (Heteroptera:

Anthocoridae) (Pilcher *et al.,* 1997; 2005; Al-Deeb *et al.,* 2001; Bourguet *et al.,* 2002; Daly & Buntin, 2005), *Cycloneda munda* (Say) (Coleoptera: Coccinellidae) (Pilcher *et al.,* 2005), *Coleomemegilla maculata* (De Geer) (Coleoptera: Coccinellidae) (Pilcher *et al.,* 1997; 2005; Wold *et al.,* 2001; Daly & Buntin, 2005), *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Pilcher *et al.,* 1997; 2005; Lozzia *et al.,* 1998; Bourguet *et al.,* 2002), *Coccinella septempunctata* (Linnaeus) (Coleoptera: Coccinellidae) (Bourguet *et al.,* 2002; Wold *et al.,* 2001), *Metopolodium dirhodum* (Walker) (Homoptera: Aphididae), *Rhopalosiphum padi* (Linnaeus) (Homoptera: Aphididae), *Sitobion avenae* (Fabricius) (Homoptera: Aphididae), *Syrphus corollae* (Fabricius) (Diptera: Syrphidae) (Bourguet *et al.,* 2002), *Hippodamia convergens* (Guerin-Meneville) (Coleoptera: Coccinellidae), *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (Wold *et al.,* 2001), *Carphophilus* species, *Euxesta stigmatis* (Loew) (Diptera: Ulidiidae), *Chaetocnema palicaria* (Melsheimer) (Coleoptera: Chrysomelidae), *Frankliniella williamsi* (Hood) (Thysanoptera: Thripidae), *Scymnus* species and *Geocoris puntipes* (Say) (Hemiptera: Lygaeidae) (Daly & Buntin, 2005). There are also no clear reason to suspect toxicity to mammals, birds, fish and arthropods (Clark *et al.,* 2005).

1.1.4.2   Disadvantages of Bt transgenic maize crops

The disadvantages and limitations of Bt maize crops are that the Cry toxins are stomach insecticides and therefore need to be ingested by the larvae to be effective. Bt strains are instar-dependant, which means that the susceptibility of mature larvae to a lethal dose is much lower than that of young larvae (Navon, 2000). The development of resistance to Cry toxins in economically important insect species, such as *B. fusca*, is a constraint for the production of Bt maize crops (Van Rensburg, 2007).

Negative impacts of Bt maize were reported on *Danaus plexippus* (Linnaeus) (Lepidoptera: Danaidae) in the United States of America (Losey *et al.,* 1999), and *C. carnea* (Hilbeck *et al.,* 1998a,b; 1999). The concern with *D. plexippus* was resolved and the results were described as negligible due to the low exposure of *D. plexippus* larvae to Cry1Ab under field conditions (Sears *et al.,* 2001; Wolt *et al.,* 2003; Dively *et al.,* 2004; Prasifka *et al.,* 2005). If *C. carnea larvae* fed on prey that were reared

on diet containing Cry1Ab, it resulted in *C. carnea* showing a slower development rate, lower production-, and higher mortality rate (Hilbeck *et al.,* 1998a,b; 1999).

1.1.5 *Bacillus thuringiensis*: Mode of action

Insecticidal crystal inclusions are formed by a variety of insecticidal proteins called Cry or Cyt toxins (Bravo *et al.*, 2011). These toxins are produced by Bt bacteria before sporulation. Pore forming toxins are a class of bacterial toxins and the Cry and Cyt toxins forms part of this group. These toxins are secreted as water-soluble proteins, which then undergo conformational changes to be accepted in the membrane of their hosts (Bravo *et al.*, 2011).

Action by Cry proteins happens in the midgut of insects. It is, therefore, necessary to understand the basic anatomy of the insect gut and the normal physiology of the midgut where toxicity occurs (Whalon & Wingerd, 2003). Once the plant material enters the gut of the insect, the material is further broken down into smaller pieces in the foregut (Whalon & Wingerd, 2003). A sieve is formed in the lumen of the foregut by small spines that extend into the lumen (Whalon & Wingerd, 2003). These spines help to prevent any large particles from entering the midgut (Whalon & Wingerd, 2003).

Lepidopteran larvae have a very alkaline midgut, with the pH varying between 10 and 11 (Whalon & Wingerd, 2003). The high alkalinity in the midgut helps to prevent tannins from inactivating the digestive enzymes, and with dissociating tannins from leaf proteins, the digestibility of leaf tissue is enhanced. The high levels of alkalinity is maintained by the goblet cells in the midgut epithelium which secrets potassium carbonate into the lumen of the midgut (Whalon & Wingerd, 2003).

Once the Bt crystal inclusion has been ingested by the insect larvae, subsequent steps include the solubilization of the crystal proteins, the proteolytic processing of the protoxin to the active form, high affinity binding with the midgut receptors, and the irreversible insertion of the toxin into the membrane (Whalon & Wingerd, 2003).
In order for Bt to be an effective pathogen it must adjust to a few characteristics to be able to pass through the foregut of the insect. Bt would not be acceptable in its larger

vegetative state, and therefore it must present itself as a very small spore (Whalon & Wingerd, 2003). The high alkalinity in the midgut of the insect prevents the spore from germinating. The Cry δ-endotoxins play a big role in Bt-mediated toxicity (Whalon & Wingerd, 2003). The toxicity of the spore can only occur once the protoxin form of the spore is proteolytically processed. The high pH of the midgut as well as the digestive enzymes of the insect allows this transformation to occur (Whalon & Wingerd, 2003). The active Cry toxins bind to the receptors on the surface of the columnar epithelial cells in the midgut, and once it is bound, the toxin inserts into the cellular membrane (Fig. 1.1) (Whalon & Wingerd, 2003). The Cry toxins then aggregate to form pores in the membrane that lead to osmotic lysis. This damage to the midgut causes either starvation or septicaemia (Fig. 1.1) (Whalon & Wingerd, 2003).

The specificity of Cry toxins are determined by their potential to bind to the surface proteins that are located in the microvilli of larval midgut cells (Bravo *et al.*, 2011). The binding proteins for Cry1 (Lepidoptera-specific) are cadherin-like proteins, a glycoconjugate, P252, glycosylphophatidyl-inositol (GPI) - anchored aminopeptidase-N (APN), and GPI–anchored alkaline phosphatise (ALP) (Bravo *et al.*, 2011).
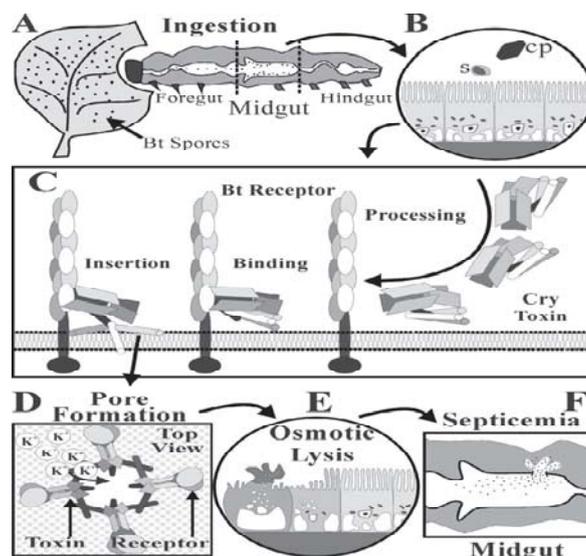

Fig.1.1 Mode of action of *Bacillus thuringiensis* Cry toxins (Whalon & Wingerd, 2003).

1.1.6 Insect resistance to *Bacillus thuringiensis*

The development of resistance to insecticides is a huge problem, because it leads to ineffective control of insect pests and disease vectors (Ferré *et al.*, 1991). Insects developed resistance to many chemical insecticides (De Maagd *et al.,* 1999) and can adapt to various toxins and control agents (Palumbi, 2001; Onstad, 2007). Insect resistance can be defined in two different ways (Moar *et al.*, 2008). The one approach is based on resistance under laboratory conditions and the other is based on field conditions (Moar *et al.*, 2008). Laboratory resistance is defined as: "a statistically significant, genetically mediated reduction in sensitivity of the target organism to the controlling agent, relative to a susceptible laboratory strain (Moar *et al.*, 2008). Laboratory resistance is therefore monitored as an increase in population LC_{50}, or as an enhanced growth or survival at a discriminating concentration compared to a susceptible colony (Moar *et al.*, 2008). Laboratory based resistance results are used in proactive resistance management programs as an early warning of reduced larval susceptibility and potential resistance problems (Moar *et al.*, 2008). Field efficacy is the ultimate proof of resistance, and field resistance can be defined as "a genetically mediated increase in the ability of a target pest to feed and complete development on one or more commercial line(s) of a Bt crop under field conditions" (Moar *et al.*, 2008). This definition allows the possibility for incomplete resistance (increased feeding but delayed or incomplete development to adult) and fitness costs (Moar *et al.*, 2008).

The first report of resistance of an insect, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) to a commercial Bt spray formulation was in 1985 (McGaughey, 1985). The rapid development of insect resistance to Bt toxins is a big concern and therefore received a lot of attention (Tabashnik, 1994).

1.1.7 Mechanisms of insect resistance to *Bacillus thuringiensis*

Ferré and Van Rie (2002) categorised the mechanisms of resistance to Bt proteins in insects into three groups. These are: (a) an alteration in binding of Cry toxins to the receptors in the midgut, namely a reduction in binding sites or a decreased binding affinity; (b) alterations in the proteolytic processing of the Cry toxins causing a

decrease in protoxin solubilisation, decreased rates of activation or increased rates of toxin degradation and (c) the rapid regeneration of the damaged midgut epithelium that prevents septicaemia (Ferré & Van Rie, 2002).

1.1.8   Insect resistance to *Bacillus thuringiensis* transgenic crops

The development of resistance to Bt toxins remains a major threat to the benefits of Bt crops (Gould, 1998; Caprio & Sumerford, 2007; Tabashnik *et al.,* 2008; 2009). For the evolution of resistance to Bt crops to occur, three conditions need to be in place. These are: variation among individuals in survival on Bt crops, inheritance of resistance traits by insects that survive on Bt crops and fitness differences consistently associated with the variation in survival on Bt crops (Endler, 1986).

Bt crops still control many target pest populations, but there have been reports of lepidopteran pests that have developed field resistance to Bt crops (Carrière *et al.*, 2010). The species are *B. fusca*, the African stem borer, in South Africa resistant to the Cry1Ab toxin in Bt maize (Van Rensburg, 2007), *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), the Fall armyworm, in Puerto Rico resistant to Cry1F toxin in Bt maize (Matten *et al.,* 2008; Storer *et al.,* 2010; 2012), *P. gossypiella*, the Pink bollworm, in western India resistant to the Cry1Ac toxin in Bt cotton (Bagla, 2010; Dhurua & Gujar, 2011) and *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae), bollworm, in Australia to Cry1Ac and Cry2Ab in Bt cotton (Downes *et al.,* 2010).

Sumerford *et al.* (2012) reported *H. punctigera, H. armigera, H. zea* and *P. gossypiella* in China not to have met the proposed standards for field evolved resistance. There was little evidence of increased adult production and sustained increase in feeding damage per larvae (Sumerford *et al.,* 2012). These standards used for assessing field evolved resistance are when changes in susceptibility to the Bt toxin are correlated with the selection pressure exerted by the Bt product, when individuals survive and complete their life cycle on the plant and if there are effects on the efficacy of Bt crops (Sumerford *et al.,* 2012).

1.1.9 *Bacillus thuringiensis* **sprayable products**

Products containing Bt were sprayed as early as 1930 for the control of insect pests, but large scale production commenced only in the late 1950's (De Maagd *et al.,* 1999). Bt sprayable products are known to be used mainly by organic farmers, gardeners and in forestry (Kunert, 2011). Cry toxins are the only active components of Bt-based microbial insecticides, which have been used as foliar sprays in agriculture and forests for several decades (Reed *et al.*, 2001), but these products have never occupied a large share of the insecticide market (De Maagd *et al.,* 1999).

Bt sprayable products are applied similar to chemical insecticides (Navon, 2000). Ground sprayers are also used for application in the case of large agricultural areas (Navon, 2000). *Bacillus thuringiensis* var. *kurstaki* was shown to be effective in controlling and eradicating insect pests, and it could therefore be chosen as a safe alternative (Glare & O'Callaghan, 2000). Three examples that show the effectiveness of *B. thuringiensis* var. *kurstaki* as an alternative to control invasive insect pests are: the eradication of *Lymantria dispar* (Linnaeus) (Lepidoptera: Erebidae) in Vancouver (1988) as well as in North Carolina (1993), and the eradication of *Orgyia thyellina* (Butler) (Lepidoptera: Lymantriidae) in Auckland, New Zealand ("Operation Evergreen" 1996) (Glare & O'Callaghan, 2000).

Chemical insecticides for the control of *Thaumatopoea pityocampa* (Denis & Schiffermüller) (Lepidoptera: Thaumetopoeidae) in pine forests in Israel, have been replaced by *B. thuringiensis* since 1987, because Bt is an environmentally friendly bio-pesticide and the use of the forest by the public for recreational purposes (Navon, 2000).

1.1.9.1 *Advantages of Bt sprayable products*

Chemical insecticides can be replaced by bio-insecticides, especially Bt sprayable products in IPM programmes (Navon, 2000). Partly because of their selectivity and short half-life, Bt Cry toxins (as well as cell bodies and spores) are generally considered to have fewer adverse impacts on the environment than many broad-spectrum and persistent chemical insecticides (see review in Schnepf *et al.*, 1998).

If compatible, Bt spray products can also be combined with other biological organisms. These include entomopathogenic microbes and nematodes, natural enemies of the pests and the use of natural insecticides to reduce pest numbers (Navon, 2000).

1.1.9.2   *Disadvantages and limitations of Bt sprayable products*

The limitations associated with the usage of Bt sprayable products include environmental factors that reduce the effectiveness of the insecticide, e.g. the inactivation by solar irradiation, through destruction of tryptophan (Navon, 2000). The product can also be washed off by rain or dew, or the microbe dosage can be diluted and the Bt protein activity can be affected by the phyllosphere and allelochemicals (Navon, 2000). Bt strains is an oral insecticide that needs to be ingested, and does not have a contact activity (Navon, 2000). Because of the cryptic behaviour of stem borers such as *B. fusca,* they escape lethal spray dosages, and spray applications are also less effective against late instar larvae (Navon, 2000). A negative economic factor can be the fact that a large amount of the product is lost due to the product dripping off the plant (Gouse *et al.*, 2005).

1.1.10 Insect resistance to *Bacillus thuringiensis* toxins under laboratory conditions

Survival of insects exposed to Bt formulations or toxins in diet or leaf dip test under laboratory conditions does not necessarily indicate that these species will survive on Bt crops (Tabashnik *et al.,* 2003). Larvae may not be able to complete their life cycle on Bt crops, even though they may have proven to be resistant to Bt formulations or toxins in diet or leaf dip bioassays under laboratory conditions (Tabashnik *et al.,* 2003). The possible reasons are: longer exposure to Bt toxins inside Bt plants, higher toxin concentrations in Bt plants than in a diet or sprays, interactions between plant chemistry and Bt toxins, production of the active form of the toxin in the Bt plant, compared to the protoxin form that is sometimes tested in laboratory bioassays. Furthermore, differences in the sets of toxins produced by Bt plants and of those tested in laboratory bioassays may also affect the outcomes of studies (Tabashnik *et al.,* 2003).

Table 1.2 Reported cases of resistance to Bt Cry toxins in Lepidoptera species

Species	Common name	Cry toxins	Reference
Bombyx mori	Silkworm	Cry1Ab	Atsumi *et al.,* 2012
Cadra cautella	Almond moth	*kurstaki, HD-1 (Dipel),*	McGaughey & Beeman, 1988
Diatraea saccharalis	Sugarcane borer	Cry1Ab	Huang *et al.*, 2007
Helicoverpa armigera	African bollworm	Cry1Ac	Akhurst *et al.*, 2003
Helicoverpa zea	Western corn rootworm	Cry1Ac	Tabashnik *et al.*, 2008
Heliothis virescens[a]	Tobacco budworm	Cry1Ac & Cry1Ab; Cry1Ac & Cry2A	MacIntosh *et al.* 1991; Gould *et al.,* 1992;
Homoeosoma electellum	Sunflower moth	kurstaki, HD-1	Brewer, 1991
Ostrinia nubilalis[a]	European corn borer	*kurstaki*, HD-1 (Dipel),	Huang *et al.,* 2002
Pectinophora gossypiella[a]	Pink bollworm	Cry1Aa, Cry1Ab, Cry1Ac & Cry1Fa	Tabashnik *et al.,* 2000
Plodia interpunctella[a]	Indian meal moth	*kurstaki, HD-1 (Dipel),* Bt var *entomicidus*	McGaughey & Beeman, 1988; Johnson *et al.,* 1990; McGaughey & Johnson, 1992; 1994
Plutella xylostella[a]	Diamondback moth	*kurstaki*, HD-1 (Dipel),	Ferré *et al.*,1991; Tabashnik *et al*, 1992; Tabashnik *et al*, 1994; Liu & Tabashnik, 1997
Spodoptera exigua[a]	Beet armyworm	Cry1C	Moar *et al.*,1995
Spodoptera littoralis	Cotton leafworm	Cry1C & Cry1E	Müller-Cohn *et al.*, 1996
Trichoplusia ni[a]	Cabbage looper	*kurstaki*, HD-1 (Dipel),	Janmaat & Myers, 2003

[a] - Cross resistance

Ostrinia nubilalis resistant to Dipel® (Bt var. *kurstaki*) (Huang *et al.,* 2002) and *H. virescens* resistant to Cry1Ac, Cry2A and Cry1Ab (Gould *et al.*, 2002) were not able to survive on Bt crops expressing the same Cry toxins to which they have shown resistance under laboratory conditions (Tabashnik *et al.,* 2003). *Ostrinia nubilalis* could not survive on Bt maize expressing Cry1Ab or Cry1Ac, and *H. virescens* could not survive on Bt cotton expressing Cry1Ac (Tabashnik *et al.,* 2003). Three species resistant to Bt formulations or toxins in diet or in leaf dip experiments under

laboratory conditions survived on Bt crops (Tabashnik *et al.,* 2003). These species were *H. armigera* resistant to Cry1Ac (Akhurst *et al.,* 2003) that survived on Bt cotton which expresses the Cry1Ac toxin, *P. xylostella,* resistant to Dipel (Ferré *et al.,*1991), survived on Bt broccoli which expresses the Cry1Ac and Cry1C toxins, and Bt canola which also expresses Cry1Ac. *Pectinophora gossypiella* which is resistant to Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa survived on Bt cotton which expresses Cry1Ac (Tabashnik *et al.,* 2003).

1.1.11   Cross resistance

Cross resistance to insecticides is described as when resistance to one insecticide causes resistance to another insecticide in the same insect (Tabashnik, 1994). Cross resistance to Bt can then be defined as resistance to a toxin other than to which the resistant strain was selected (Griffitts & Aroian, 2005).

Various insect species have been found to be cross resistant to Bt Cry toxins under laboratory conditions (Table 1.2). *Plodia interpunctella* proved to be resistant to Dipel[®] with 86% survival rate to Dipel[®] at 500mg/kg. This resistance also led to resistance to other Bt strains (McGaughey & Johnson, 1987). This species is cross resistant to five Bt serovars: *kurstaki, thuringiensis, galleriae, aizawai* and *tolworthi* (McGaughey & Johnson, 1987). *Plodia interpunctella* is cross resistant to Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Bt var. *entomocidus* (contains: Cry1Aa, Cry1Ab, Cry1C and Cry1D toxins), and showed limited cross resistance to Cry1Ca (McGaughey & Johnson, 1994).

Ferré *et al.* (1991) found that resistance in *P. xylostella* to Cry1Ab did not cause cross resistance to Cry1B and Cry1C. Results from a study done by (Tabashnik *et al.,* 1993) showed resistant larvae to be resistant to Dipel[®] and another Btk formulation in Hawaii, and it did lead to cross resistance against Cry1C. This species is thus, cross resistant to Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, Cry1C (Tabashnik *et al.,* 1993) and Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, Cry1J, (Tabashnik *et al.,* 1996). These results were confirmed in 2001, with the addition of limited cross resistance to Cry2Aa (Zhao *et al.,* 2001).

Since Dipel® contains more than one Cry protein, narrow cross resistance is possible. This was found for *H. virescens* where resistance to a single Bt protein lead to broad spectrum Bt spray resistance (Gould *et al.*, 1992). Resistance to Cry1Ac led to cross resistance in Cry1Ab and Cry1Aa. This was not surprising due to the similarity in structure of the toxins (Gould *et al.*, 1992). Due to the difference in the amino acid sequence of the Cry toxins (Höfte & Whiteley, 1989), it was unexpected that the cross resistance in Cry1Ac led to the cross resistance to Cry2A (Gould *et al.*, 1992). Limited cross resistance by *H. virescens* was found with Cry1Ca, Cry1Ba and Cry1Bb (Gould *et al.*, 1992), as well as with Cry1Fa (Gould *et al.,* 1995).

Trichoplusia ni was reported to be resistant to Dipel® in commercial vegetable greenhouses (Janmaat & Myers, 2003). *Pectinophora gossypiella* proved to be resistant to Cry1Ac and also showed narrow spectrum cross resistance to Cry1Aa and Cry1Ab (Tabashnik *et al.*, 2000). The results were confirmed in 2003, and limited cross resistance to Cry1Ja was found (Tabashnik *et al.,* 2003). *Ostrinia nubilalis* can develop resistance to Dipel®, but the species did not survive on Bt maize (MON810 and Bt11) (Huang *et al.*, 2002). Low levels of cross resistance were recorded between Cry1Ac and Cry2Aa with *H. zea* (Burd *et al.,* 2003). One individual out of 583 proved to be resistant to Cry1Ac and one individual out of 646 that proved to be resistant to Cry2Aa (Burd *et al.,* 2003). *Spodoptera littoralis,* resistant to Cry1C showed limited cross resistance to Cry1D and Cry1E (Müller-Cohn *et al.,* 1996). Cry1Aa and Cry1Ab proteins were also reported to have low insecticidal activity against *S. littoralis* (Müller-Cohn *et al.,* 1996).

The examples cited above indicate that development of cross resistance to different Cry toxins does occur in important pests exposed to these products. The development of cross resistance to Bt sprays, in pests exposed to crops that express Cry toxins, could have a negative impact in certain agricultural systems. For example, if cross resistance develops in pests exposed to Cry toxins in crops, organic farmers may lose the only tools (Bt-spray formulations) they are allowed to use for pest control.

Most of the above mentioned examples indicate cross resistance under laboratory conditions. No previous studies have been conducted in which evaluations were

done of possible cross resistance to Bt sprays, resulting from primary exposure to Cry1Ab producing maize. Since *B. fusca* in South Africa is highly resistant to Cry1Ab expressing maize (Van Rensburg, 2007), this allows an opportunity to study the presence of possible cross resistance in pests that are resistant to Bt maize. *Ostrinia nubilalis* that have been reported to be resistant to Dipel[®] have been evaluated for cross resistance to Bt maize producing Cry1Ab toxin but no larvae were reported to survive on the crop (Huang *et al.*, 2002).

1.2   Objectives of this study

1.2.1   General objective

The aim of this study was to evaluate the efficacy of Bt spray applications for control of three lepidopteran pests.

1.2.2   Specific objectives

The specific objectives were to determine:

- ➢ the efficacy of *Bacillus thuringiensis* spray applications for control of three lepidopteran maize pests
- ➢ if *B. fusca*, which is resistant to Bt-maize, is also resistant to Bt sprays (cross resistance)

The results of this study are presented in the form of chapters with the following titles:

- ➢ Efficacy of Bt spray applications for control of lepidopteran pests
- ➢ Evaluation of possible cross resistance in *Busseola fusca* (Lepidoptera: Noctuidae) to Cry1Ab plant-produced protein and Dipel[®].

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Chapter 2

Efficacy of Bt spray applications for control of three lepidopteran pests

2.1 Abstract

The economically important stem borers on maize in southern Africa are *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae), *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae). Transgenic maize, expressing Cry1Ab insecticidal proteins produced by the bacterium *Bacillus thuringiensis* (Bt), was introduced for control of *B. fusca* and *C. partellus* in South Africa in 1998. Stem borers can be controlled by means of insecticide sprays, however, due to their cryptic behaviour, they often escape these applications. This also account for Bt sprays. However, the efficacy of Bt sprays was evaluated for control of *C. partellus* and *E. saccharina*, because it provides an alternative for chemical insecticides with no negative impact on the environment, and is one of the few options available to organic farmers to control these pests on their crop. The African armyworm, *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae) has a very wide distribution in Africa and is known to be an occasional pest on maize. Effective biological control during the gregarious phase of *S. exempta* on pastures may reduce the environmental impact of its control. Susceptibility assays consisted of maize leaf tissue bioassays with six treatments. Ten-day old larvae were transferred to test tubes that contained leaves of the different treatments, 15 min after spray applications were done. Percentage larval survival was recorded over time. *Chilo partellus and E. saccharina* was effectively controlled by all Bt treatments as well as deltamethrin. *Spodoptera exempta* was effectively controlled by a 4 times Dipel$^{®}$ dosage rate and deltamethrin. The breakdown of Bt sprays by irradiation is a limitation, but the gregarious phase of *S. exempta* march en masse and feed vigorously which may contribute to the success of this control option.

Keywords: *Bacillus thuringiensis*, Bt spray, *Chilo partellus, Eldana saccharina,* maize pests, *Spodoptera exempta*.

2.2   Introduction

The advances in cell and molecular biology have made it possible to genetically modify crops and to express insect controlling *Bacillus thuringiensis* Cry toxins, such as Cry1Ab (Barton & Miller, 1993). Bt maize was initially developed for control of two stem borers species in North America, namely *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Archer *et al.,* 2001) and *Diatraea grandiosella* (Dyer) (Lepidoptera: Crambidae) (Williams *et al.,* 1998). Bt transgenic maize was commercialized in South Africa since 1998 to control the stem borers, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) (Gouse *et al.*, 2005). The use of biotechnology, especially GM crops, has however been a sensitive issue over the past years in other African countries (De Groote *et al.*, 2003). Millions of the people stare starvation in the face, and when this is taken into consideration, the rejection of GM crops doesn't really seem like an option (De Groote *et al.*, 2003). The Insect Resistant Maize for Africa (IRMA) project has been undertaken since 2003 by the International Maize and Wheat Improvement Centre (CIMMYT) and the Kenya Agriculture Research Institute (KARI) to lend some objective analysis to this debate (De Groote *et al.*, 2003).

Stem borers are not easy to control with insecticidal sprays, because they tunnel into the plants and often escape insecticide applications (Navon, 2000; Mugo *et al.,* 2011). The economically important stem borers on maize in southern Africa are *B. fusca*, *C. partellus*, *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Kfir, 1997; De Groote *et al.*, 2003; Mugo *et al.,* 2011).

Chilo partellus is a pest on sorghum, millet, sugarcane as well as other grasses (Singh *et al.,* 2005). Infestations by *C. partellus* can cause significant economic losses, especially in India (Singh *et al.,* 2005) and Africa (De Groote *et al.*, 2003). The species is controlled by chemical insecticides, but due to the negative impact of these insecticides on the environment, other alternatives are needed (Sharma *et al.*, 2010). Bacteria have proven to be the best alternative among the biological pesticides (Mugo *et al.,* 2011). Microbial insecticides such as Bt were assumed to replace chemical insecticides and comprise ~ 2% of the total insecticidal market in

2010, and were therefore known as the most successful insect pathogen for insect control (Bravo *et al.,* 2011). Maize expressing Cry1Ab protein was reported to be effective in controlling *C. partellus* in South Africa (Van Rensburg, 1999) and India (Hari *et al.*, 2007) under both laboratory and field conditions. Partial control of *C. partellus* on transgenic sorghum that expresses a synthetic *Bacillus thuringiensis* Cry1Ac gene was found by Girijashankar *et al.* (2005).

Eldana saccharina, the African sugarcane borer, is known to be a major pest on sugarcane and maize in Africa (Kfir *et al.,* 2002). Sorghum is attacked by *E. saccharina* in East Africa (Seshu Reddy & Omolo, 1984), as well as West Africa (Nwanze, 1984). Although it has not been recorded on maize in South Africa (Assefa *et al.*, 2008), it is known as a major pest of maize in other African countries (Girling, 1980; Kaufmann, 1983; Bosque-Pérez & Mareck, 1991). Since 1939, when *E. saccharina* was first reported in South Africa (Dick, 1945), it became a major pest on sugarcane (Assefa *et al.*, 2008) and its distribution extended past the sugarcane belt to Thohoyandou in the Limpopo province, Mkambati Nature Reserve in the Eastern Cape province as well as Boskop dam, near Potchefstroom in the North-West province, which is a major maize producing area (Assefa *et al.*, 2008). *Eldana saccharina* is a polyphagous insect and could seemingly easily move from its natural host plants to maize fields, as it did in sugarcane fields in Zimbabwe, Uganda and Kenya (Assefa *et al.,* 2008). If the species were to switch hosts, it can have serious implications for farmers (Bosque-Pérez & Mareck, 1991). During the 1985/86 growing season, maize yields in Nigeria were reduced with between 16% and 28% in the dry season (Bosque-Pérez & Mareck, 1991).

Spodoptera exempta (Walker) (Lepidoptera: Noctuidae) has a very wide distribution in Africa especially south of the Sahara and in south-western Arabia (Haggis, 1986). These countries include Kenya, Tanzania, Uganda, Ethiopia, Sudan as well as South Africa (Hamal *et al.*, 1991). It attacks maize crops during the whorl stages and can cause extreme defoliation (Capinera, 2008). All the countries mentioned above, except South Africa, do not grow Bt maize and therefore *S. exempta* does not have any exposure to the Cry toxins produced by Bt maize (De Groote *et al.*, 2003). *Spodoptera exempta* appear in sudden outbreaks, but it has never been observed that *S. exempta* can breed continuously, year after year, in the same place (Rose,

1979). *Spodoptera exempta* is an occasional pest on maize, and, due to its inconsistency to survive in an area (Rose, 1979), the population would most likely not be exposed to Bt maize for a long enough period to develop resistance. This pest is controlled with synthetic chemical insecticides. Although this method is technically effective, the environmental impact of these insecticides applied over wide areas causes concern (Grzywacz *et al.*, 2008).

Bt sprays are not registered for control on *C. partellus, E. saccharina* and *S. exempta* in South Africa. Effective biological control during the gregarious phase of *S. exempta* on pastures may reduce the environmental impact of its control and also eliminates the withholding period of grazing animals from treated areas. The aim of this susceptibility bioassay study was to determine the efficacy of Bt spray applications for control of *C. partellus, E. saccharina* and *S. exempta* in South Africa, and to compare it with the efficacy of Bt maize (MON810).

2.3   Material and Methods

Susceptibility bioassays

This laboratory study consisted of leaf tissue bioassays with six treatments. The treatments were: leaves of Bt maize (MON810) expressing the Cry 1Ab protein; leaves of non-Bt maize plants sprayed with three concentrations of Dipel®, namely 1.0, 2.0 and 4.0 times the recommended dosage rate for bollworm control on peas; water as an untreated control and deltamethrin (25g/ℓ EC), applied at the recommended dosage rate for *B. fusca* control on maize (62.5g. a.i./ha). Dipel®, containing the proteins Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab, is not registered for control of stem borers on maize. The dosage rate used was the registered dosage rate for bollworm control on peas (500g/ha) and Sprayfilm (sticker) (Protek, Heidelberg, South Africa) was added to each spray treatment at a rate of 5ml/10ℓ water.

The spray treatments were applied as full cover sprays to ± 15 cm pieces of maize leaf which were attached to a rope in a shaded area. The pieces were left until dry (between 5 and 10 minutes), fold in half, rolled and placed in plastic test tubes (115 x

28 mm) (Fig. 2.1). Two larvae (10 days old) were transferred to each test tube which was then closed with an aerated lid and kept in an incubator at 26 ± 1 °C and a 14L:10D photoperiod. Each treatment consisted of 10 replicates. Each replicate consisted of 20 test tubes. Survival of the larvae was assessed at four, eight and twelve days. All leaves were removed four days after commencement of the trial and replaced with an untreated piece of maize stalk of the same cultivar, which were replaced again eight days after the spray application. The experiment was terminated twelve days after application. This procedure was followed for each of the three lepidopteran species, namely *C. partellus*, *E. saccharina* and *S. exempta.*

2.3.1   *Chilo partellus*

Chilo partellus eggs were obtained from laboratory rearing colonies of the Agricultural Research Council – Grain Crops Institute (ARC-GCI). The eggs were placed in a plastic bag (Ziplock) (GLAD, South Africa) along with a wet cotton ball to prevent desiccation. These bags were kept in a rearing chamber at 26 ± 1 °C and a 14L:10D photoperiod. Neonate larvae were collected from the bags and transferred to cut pieces of the calyx of non-Bt maize plants, and kept in plastic containers. The containers were kept in the rearing chamber. Larvae from these colonies were used in the experiments when they were ten days old.

2.3.2   *Eldana saccharina*

Eldana saccharina eggs were obtained from laboratory rearing colonies from the South African Sugarcane Research Institute (SASRI), in Mount Edgecombe, KwaZulu Natal province*.* These colonies had no previous exposure to Bt maize. The eggs were placed in a plastic bag (Ziplock) (GLAD, South Africa) along with a wet cotton ball to prevent desiccation. These bags were placed in a rearing chamber at 26 ± 1 °C and a 14L:10D photoperiod. Neonate larvae were collected from the bags and transferred to cut pieces of the calyx of non-Bt maize plants and pieces of sugar cane stalks, and kept in plastic containers. The containers were kept in the rearing chamber and provided with fresh food on day four and eight. Larvae from these colonies were used in the experiment when they were ten days old.

2.3.3 *Spodoptera exempta*

Spodoptera exempta larvae were collected at Potchefstroom, (26°42'54"S; 27°06'00"E) (North-West province) in South Africa after an outbreak, placed en masse in plastic containers and reared on non-Bt maize leaves. The containers were kept in the rearing chamber at 26 ± 1 °C and a 14L:10D photoperiod and provided with fresh food. Larvae from these colonies were used when they were ten days old.

Fig. 2.1 The process followed for application of spray treatments: a) 15 cm pieces of maize leaves attached to a rope in a shaded area; b) spray treatments were applied; c) full cover sprays were applied; d) two larvae were kept on leaves in aerated test tubes.

2.3.4   Statistical analysis

The data was analyzed using STATISTICA version 11 (StatSoft, Inc., 2012). Repeated measures ANOVA were used to analyse mortality over time. Bonferroni correction was used to adjust for multi means comparisons. One way ANOVA and Tukey HSD was used to determine significant differences between treatments, twelve days after application.

2.4   Results

Susceptibility bioassays

2.4.1   *Chilo partellus*

There was a significant time x treatment interaction in terms of percentage larval survival ($F_{(10,108)}$ = 5.29; P < 0.001) (Fig. 2.2). Percentage survival decreased over time and differed significantly between the three evaluation times, namely 4, 8 and 12 days after application ($F_{(2,108)}$ = 36.77; P < 0.001). There was a significant reduction in survival of larvae after 4 days in all treatments, except for the control treatment. Mortality was similar 8 and 12 days after application of all Dipel$^®$ treatments. There were also significant differences between treatments ($F_{(5,54)}$ = 34.11; P < 0.001), with more larvae that survived in the control treatment compared to all other treatments, 12 days after application (Table 2.1). The number of surviving larvae in treatments with the recommended and double the Dipel$^®$ dosage rates, as well as the Bt maize did not differ significantly from that of deltamethrin. All these treatments effectively controlled *C. partellus* larvae (Table 2.1).

2.4.2   *Eldana saccharina*

There was a significant time x treatment interaction in terms of percentage survival ($F_{(10,108)}$ = 7.96; P < 0.001) (Fig. 2.3). Percentage survival decreased over time and differed significantly between the three evaluation times, namely 4, 8 and 12 days after application ($F_{(2,108)}$ = 122.18; P < 0.001). There was a significant reduction in survival of larvae after 4 days and no difference in larval survival 8 days and 12 days

in all the treatments, except the control (water). There were also significant differences between treatments ($F_{(5,54)}$ = 245.86; $P < 0.001$), with more larvae that survived in the control treatment compared to all other treatments, 12 days after application (Table 2.2). Survival of larvae decreased with increasing dosage rates of Dipel[®], and all of the Dipel[®] treatments were as effective in controlling *E. saccharina* larvae as Bt maize and deltamethrin. All treatments controlled *E. saccharina* larvae equally well (Table 2.2).

2.4.3   *Spodoptera exempta*

There was a significant time x treatment interaction in terms of percentage survival of larvae ($F_{(10,156)}$ = 15.17; $P < 0.001$) (Fig. 2.4). Percentage survival decreased over time and differed significantly between the three evaluation times, namely 4, 8 and 12 days after application ($F_{(2,156)}$ = 172.76; $P < 0.001$). There was a significant reduction in survival of larvae after 4 days as well as 8 days after treatment with no significant difference in mortality 8 and 12 days after application in all Dipel[®] treatments (Fig. 2.4). Significantly more larvae died 12 days after exposure to Bt maize than 4 and 8 days after exposure. There were also significant differences between treatments ($F_{(5,78)}$ = 112.93; $P < 0.001$), with more larvae that survived in the control treatment compared to all other treatments, 12 days after application (Table 2.3). Larval survival in treatments with the recommended and twice the Dipel[®] dosage rate and Bt maize differed significantly from that deltamethrin. Dipel[®] applied at 4 times the dosage rate and deltamethrin controlled *S. exempta* larvae equally well (Table 2.3).

2.5   Discussion

The tunneling behaviour of stem borers could result in larvae ingesting sub-lethal quantities of Cry toxins. For that reason, leaf tissue and not stems were used in these experiments. To compensate for this possible experimental error, the experimental procedure of using pieces of leaves that were folded, rolled and placed in aerated test tubes after being sprayed, was developed. The high mean percentage survival of *C. partellus, E. saccharina* and *S. exempta* larvae in the

untreated control group and 100% mortality in deltamethrin application indicated a good experimental design.

Larvae treated with deltamethrin died within four days after treatment in all experiments, but reaction to Bt treatments took longer. This can be ascribed to the difference in mode of action of the insecticides. Deltamethrin, a synthetic pyretroid, has a contact mode of action, indicating that the target insect should die soon after contact with the insecticide (BCPC, 2009). Bt var. *kurstaki* produces parasporal, proteinaceous, crystal-inclusion bodies during sporulation. Once the crystal toxins are solubilised in the insect, it disrupts the midgut membrane causing the insect to stop feeding and starve or die of septicaemia (Whalon & Wingerd, 2003). According to the registered technical detail of the insecticide, death should occur within 1-4 days (BCPC, 2009).

When Bt maize was introduced into South Africa, *C. partellus,* one of the economically important stem borers species, was also exposed to Bt maize (Van Rensburg, 1999). First instar *C. partellus* larvae were effectively controlled by Bt maize, expressing Cry1Ab, causing 100% mortality in South Africa (Van Rensburg, 1999) and India (Hari *et al.*, 2007). Singh *et al.* (2005) reported Cry1Ab to prevent first instar larvae from progressing to the second instar and the few that reached the second instar, were small, causing less damage to plants and they did not survive more than three days in the field. However, 15-20% survival of 15-day old *C. partellus* larvae were found in India (Hari *et al.*, 2007). This can be explained by Bt strains being less effective against later instars (Navon, 2000). A mean survival rate of 13 % was found for *C. partellus* in the current study on Bt maize (Cry1Ab) after 10-day old larvae fed for 12 days. A high level of susceptibility of *C. partellus* larvae to Cry1Ab expressed in maize plants was demonstrated in this study as well as that of Hari *et al.* (2007), although the larvae were 10 days old at the time of first exposure to the protein. There is no report of resistance to any Cry protein by *C. partellus*, even though it has been exposed to Bt Cry toxins in South Africa, and a single Cry protein is therefore still sufficient to control this pest species. It was therefore also expected and confirmed that multiple Cry toxins also control *C. partellus* effectively. These results confirm the findings of Sharma *et al.* (2010) in India that the combination of Cry toxins effectively controlled *C. partellus*. The

synergism of Bt *Cry* toxins for control of *C. partellus* was studied by Sharma *et al.* (2010) who evaluated survival of neonate *C. partellus* larvae exposed to Cry1Aa, Cry1Ab and Cry1Ac toxins alone and in combination. In the single Cry toxin tests, Cry1Ab proved to be the most effective. It is three times more toxic than Cry1Ac, and Cry1Ac five times more toxic than Cry1Aa (Sharma *et al.,* 2010). The combination of Cry1Ab and Cry1Ac proved to be the most toxic combination, being two times more toxic than Cry1Ab (Sharma *et al.,* 2010).

High mortality rates of *E. saccharina* larvae indicated no resistance and low levels of tolerance to Cry toxins. It could be explained by the fact that it has not previously been exposed to Bt maize, resulting in larvae still being susceptible to all Cry proteins they were exposed to, namely Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab in Dipel® and Cry1Ab in Bt maize.

The effective control of *E. saccharina* by all Bt treatments (Cry1Ab in maize plants and Bt var. *kurstaki* in Dipel®) indicates that larvae, although being already 10 days old by the time of first exposure, are susceptible to these Bt proteins. These results are similar to those of Tounou *et al.* (2005) who found 0% survival of *E. saccharina* on Cry1Ab.

Bioassays proved that Cry1Ab, found in Bt maize is more toxic than Cry1C, Cry2A and Cry1Ac to *E. saccharina,* with 0% larvae that survived at the lowest dosage (Tounou *et al.,* 2005). *Eldana saccharina* moths are more attracted to maize plants for oviposition than sugarcane, where maize and sugarcane were planted in close proximity under greenhouse conditions (Keeping *et al.,* 2007). Bt maize could therefore be able to be used as a dead end trap crop if implemented under field conditions with the same efficacy (Keeping *et al.,* 2007).

Spodoptera exempta was effectively controlled by Bt maize as well as Dipel® in this study. Increased mortality in the control group might be explained by the larvae that were kept in isolation instead of gregarious during the experiment. Survival rate of larvae decreased with increasing dosage rates of Dipel®. This was expected because the lethal dose of Bt is instar dependant and the susceptibility of late instar larvae is low (Navon 2000). Late discovery of larvae, when already in later instars,

can therefore be problematic for control with Bt sprays. Broza *et al.* (1991) reported effective control of *S. exempta* with Bt var. *entomocidus,* Bt var.*aizawai* and Bt K26-21. Cry1Ab as well as a combination of Cry1Aa, CryAb, Cry1Ac and Cry2A provided effective control of *S. exempta* (Broza *et al.,* 1991). Similar results were reported by Hamal *et al.* (1991) for *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) with Bt var. *entomocidus,* Bt var. *aizawai* and Bt K26-21 isolate being effective in control. Second and third instar larvae were controlled effectively under field conditions but the mortality rate of fourth to sixth instar larvae was between 50 and 80% (Broza *et al.,* 1991).

Laboratory studies proved that Cry1C and Cry1E are highly toxic to neonate *S. exempta* (Bai e*t al.,* 1993). The results also proved that Cry toxins are instar specific with the LC_{50} value being ten times higher with third instar larvae (Bai *et al.,* 1993). The four Cry toxins, Cry1Aa, Cry1Ab, Cry1B and Cry1D inhibited the growth rate of *S. exempta* larvae, while Cry1Ac showed no activity (Bai *et al.,* 1993). It was also reported that other *Spodoptera* spp. are not controlled effectively by Cry1A toxins (Fitt *et al.,* 2004). Dipel[®] contains Cry1Aa, Cr1Ab, Cry1Ac, Cry2Aa and Cry2Ab, and proved to be effective against *S. exempta* under laboratory conditions in this study, similar to results reported by Broza *et al.* (1991), with Cry1Aa, CryAb, Cry1Ac and Cry2A. *Spodoptera exempta* larvae are most active at relatively high temperatures, 24 – 32 °C (ARC, 2013), which usually prevails with intensive solar irradiation. Bt sprays become, however, inactive due to solar irradiation (Navon, 2000).

Although larvae from all three species survived when exposed to MON810 and other Bt treatments, it cannot be interpreted as development of possible resistance, because it was not neonate larvae that have been exposed to these Cry toxins. *Spodoptera exempta* is continuously active in temperate zones during a year. Resistance development is, therefore a threat should Bt maize be planted commercially in the temperate zone of Africa

It can be concluded that no development of resistance to either maize expressing Cry1Ab proteins, or to the Bt spray, Dipel[®], (Bt var. *kurstaki*) by *C. partellus, E. saccharina* and *S. exempta* were observed. This finding confirms the *status quo* with regard to the three insect species and their resistance to Cry toxins in South Africa.

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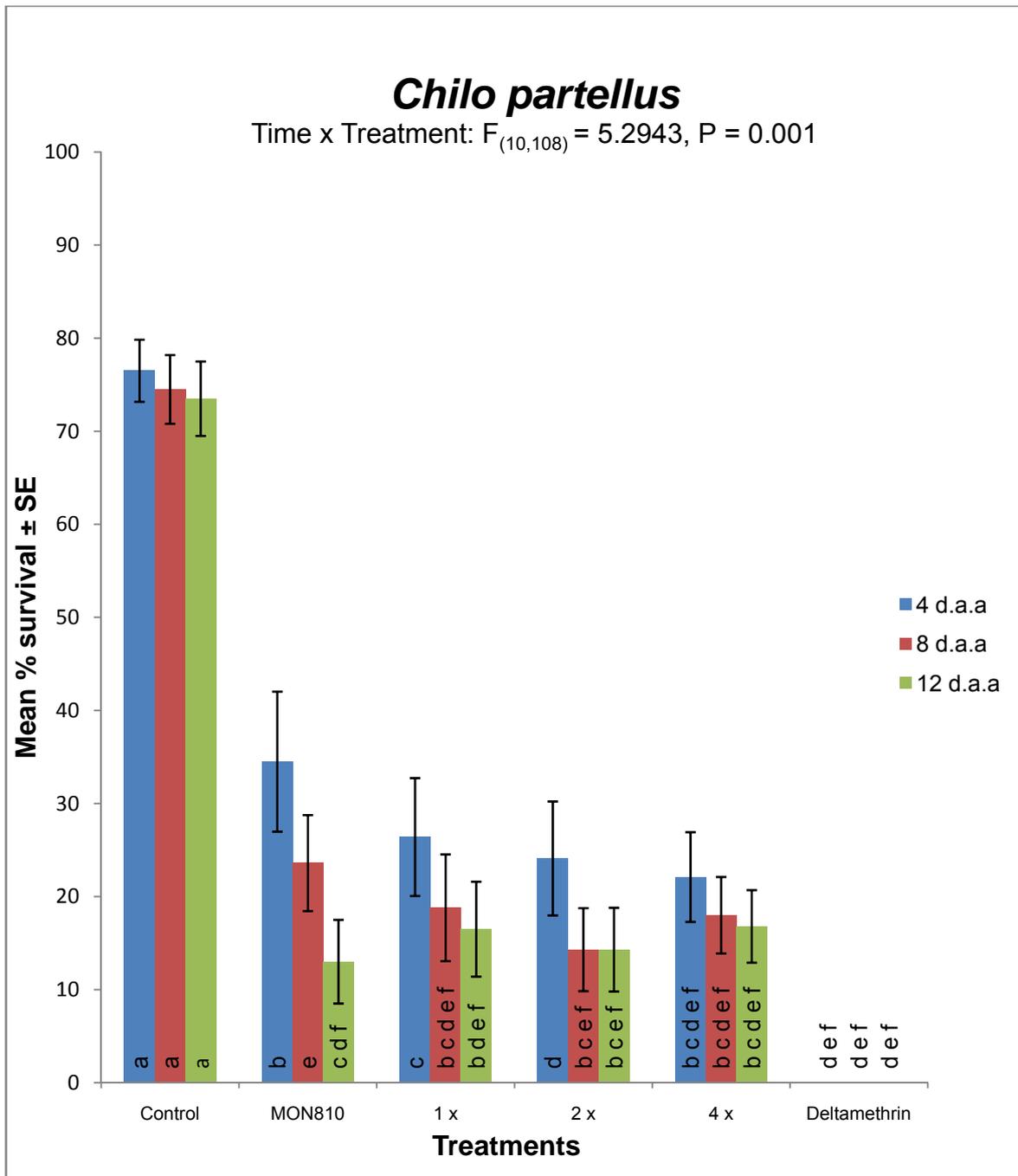
Fig. 2.2 Mean percentage survival of *Chilo partellus* larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 1x, 2x and 4x. (d.a.a. = days after application).

Table 2.1 Mean percentage survival of *C. partellus* larvae from Potchefstroom, 12 days after application.

Treatment	Mean % survival (±SE)
Control (water)	73,5 ± 4,0a*
1 x Dipel®	16,5 ± 5,1bc
2 x Dipel®	14,3 ± 4,5bc
4 x Dipel®	16,8 ± 3,9b
MON 810	13,0 ± 4,5bc
Deltamethrin	0,0 ± 0,0c

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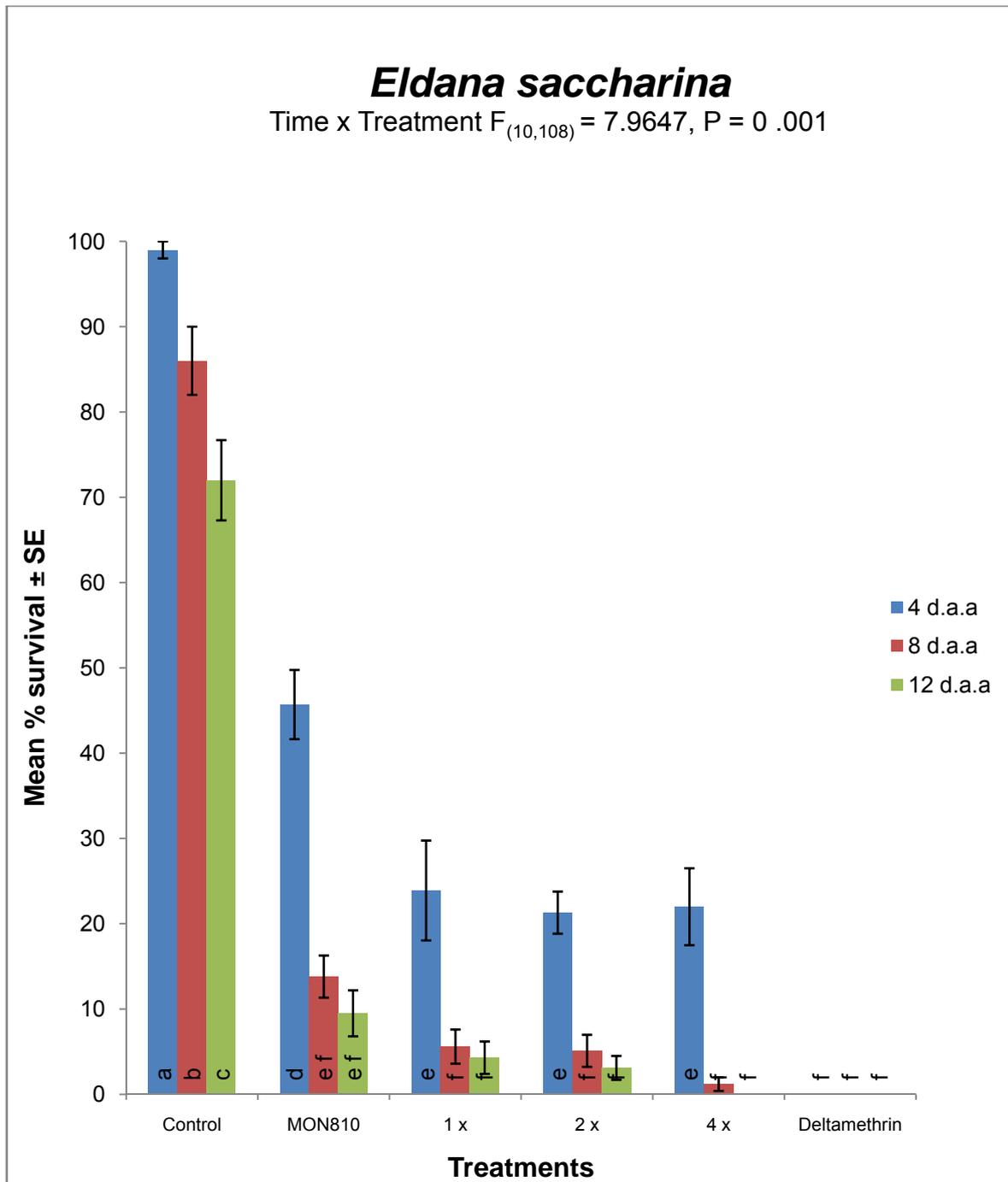 do not differ significantly at P = 0.05 (Tukey's HSD).

Fig. 2.3 Mean percentage survival of *Eldana saccharina* larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 1x, 2x and 4x. (d.a.a. = days after application).

Table 2.2 Mean percentage survival of *E. saccharina* larvae, 12 days after application.

Treatment	Mean % survival (± SE)
Control (water)	72,0 ± 4,7a*
1 x Dipel®	4,3 ± 1,9b
2 x Dipel®	3,1 ± 1,4b
4 x Dipel®	0,0 ± 0,0b
MON 810	9,5 ± 2,7b
Deltamethrin	0,0 ± 0,0b

*Means within a column followed by the same letter

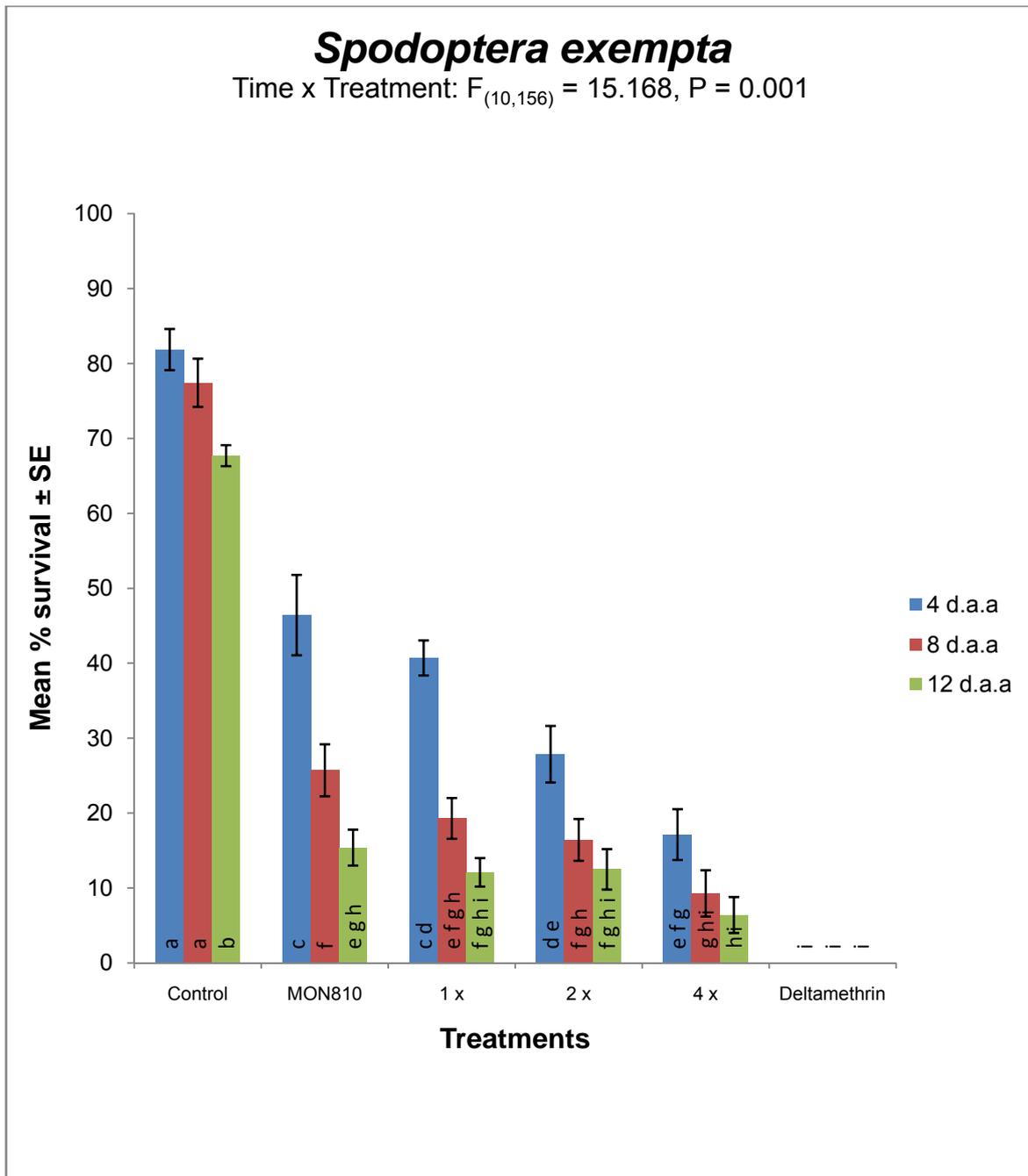do not differ significantly at P = 0.05 (Tukey's HSD).

Fig. 2.4 Mean percentage survival of *Spodoptera exempta* larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 1x, 2x and 4x. (d.a.a. = days after application).

Table 2.3 Mean percentage survival of *S. exempta* larvae, 12 days after application.

Treatment	Mean % survival (±SE)
Control (water)	67,7 ± 1,4a*
1 x Dipel®	12,1 ± 1,9cd
2 x Dipel®	12,5 ± 2,7cd
4 x Dipel®	6,4 ± 2,4bd
MON 810	15,4 ± 2,4c
Deltamethrin	0,0 ± 0,0b

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

Chapter 3

Evaluation of possible cross resistance in *Busseola fusca* (Lepidoptera: Noctuidae) to Cry1Ab plant-produced protein and Dipel[®]

3.1   Abstract

Bt transgenic maize were commercialized in South Africa since 1998 to control the economically important stem borers, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae). The first report of field resistance by *B. fusca* to *Bt* maize which expresses the Cry1Ab protein was during the 2006 growing season in the Christiana region in South Africa. The aim of this study was to determine whether development of Cry1Ab resistance by *B. fusca* led to loss of susceptibility to other Bt toxins (i.e. cross-resistance). Susceptibility assays consisted of leaf tissue bioassays with six treatments. Treatments were 1, 5 and 10 times the recommended dosage rate of Dipel[®] for bollworm control on peas, and deltamethrin, all applied separately to non Bt maize leaves, exposure to Cry1Ab producing maize as well as to pyramided maize producing both Cry1A.105 and Cry2Ab2 proteins. Ten-day old *B. fusca* larvae were transferred to test tubes that contained leaves of the different treatments. Percentage larval survival was recorded over a 12 day period. High mortality rates of *B. fusca* larvae from the Venda (susceptible) population, could be explained by the low levels of exposure to Bt maize in this area, resulting in larvae still being susceptible to all Cry proteins they were exposed to. The *B. fusca* population from Venterdorp was less effectively controlled, but the Dipel[®] treatments, and the two Bt maize treatmenst did not provide effective control of the Vaalharts *B. fusca* population reported to be resistant to Cry1Ab in both these experiments. These results indicate a reduction in susceptibility to Cry toxins other than Cry1Ab and therefore the possible development of cross resistance in the Vaalharts *B. fusca* population.

Keywords: *Bacillus thuringiensis*, *Busseola fusca*, cross resistance, resistance.

3.2 Introduction

Organic insecticides based on formulations of the soil bacterium *Bacillus thuringiensis* (Bt) became an important part of pest management (Tabashnik, 1994). Bt is the most successful insect pathogen used for insect control and comprises ~ 2% of the total insecticidal market (Bravo *et al.,* 2011). It is therefore seen as one of the most important sources of insect control agents in modern agriculture (Raymond *et al.,* 2013), but it is mostly active against the larval stages of insects (Raymond *et al.,* 2010).

A series of Cry proteins are effective against a wide range of insects including Lepidoptera, Coleoptera, Diptera, Hymenoptera and nematodes (De Maagd *et al.,* 2001). Early assumptions were that the four main classes of toxins (Cry1, Cry2, Cry3, Cry4) were order specific (Höfte & Whiteley, 1989). However, the spectrum and assumed toxicity of Cry proteins are very diverse and include species across different orders (Hilbeck & Schmidt, 2006). A single Cry protein, such as Cry1Fa, can for example have an effect on a wide variety of organisms, and distantly related Cry proteins like Cry1Aa and Cry2Aa, can have similar activity spectra (Griffitts & Aroian, 2005). Some strains such as Bt var. *kurstaki* (Btk) HD1 express more than one Cry protein with Cry1Aa, Cr1Ab, Cry1Ac and Cry2Aa (Bravo *et al.,* 2011). Other Bt strains include Btk HD73, Bt var. *aizawai* HD137, Bt var. *san diego* and Bt var. *tenebrionis* (Soberón *et al.,* 2009). Btk products are specifically intended for the control of lepidopterans, which are important crop pests (Soberón *et al.,* 2009). Bt Cry toxins play an important role in diminishing the use of chemical insecticides, and have also shown to be effective as an insect control tool, especially with the development of transgenic plants that express Cry proteins (James, 2012).

The first Bt maize events which were released, expressed only a single Cry protein, namely Cry1Ab (Baumgarte & Tebbe, 2005). A stacked gene maize product (MON89034) followed, which expresses two Cry proteins (Cry1A.105 and Cry2Ab2), and therefore provides a more effective insect resistant management tool (Van den Berg *et al.,* 2013).

A primary concern for the long time usage of Bt toxins is the development of resistance by insects (Tabashnik, 1994; Gould, 1998; Gahan *et al.,* 2001). The development of insect resistance poses a threat for the efficacy of especially Bt crops (Bravo *et al.,* 2011). The mode of action of Cry1A toxins are based on two hypotheses namely the pore-forming model (Bravo *et al.*, 2004) and the signalling model (Zhang *et al.*, 2006). Insects have developed mechanisms of resistance against the mode of action of Cry1A toxins that bind to the extracellular domain of cadherin proteins that cross over the midgut membrane (Vadlamudi *et al.*, 1995). The most common mechanisms of resistance that insects have developed is where the Bt toxin that binds to the midgut receptors is disrupted, while at least three lepidopteran pests of cotton, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) (Gahan *et al.*, 2001), *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Morin *et al.*, 2003) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Xu, *et al.*, 2005), have developed resistance through mutations of the midgut cadherin proteins that bind the Cry1A protein. Understanding how the insects react to the attack of Cry proteins will make it possible to develop more efficient Bt spray products (Bravo *et al.,* 2011).

Environmental factors, such as solar irradiation, play a vital role in reducing the effectiveness of Bt spray products like Dipel[®] (Navon, 2000). The dosage of the spray application can also be reduced by rain, or washed off (Navon, 2000). Bt spray is intended for neonate and young larvae and the effectiveness is reduced against older larvae (Bai *et al*, 1993, Navon, 2000; Bravo *et al.,* 2011). Bt spray as with other Bt Cry toxins need to be ingested by the larvae and does not have a contact action (BCPC, 2009). Bt spray is not intended for stem borers, due to the fact that they tunnel into the plant and cannot be reached by the Bt spray (Navon, 2000).

Cross resistance is described as when resistance to one insecticide leads to resistance to other insecticides by the same insect (Tabashnik, 1994). Cross resistance to Bt can be defined as resistance to a toxin other than to which the resistant strain was selected (Griffitts & Aroian, 2005).

Various insects such as *H. virescens* (Gould *et al.,* 1992), *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Huang *et al.,* 2002), *P. gossypiella* (Tabashnik *et al.,*

2000), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (McGaughey & Johnson, 1987), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Tabashnik *et al.,* 1992; Tabashnik *et al,* 1993; Liu & Tabashnik, 1997)*, Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Müller-Cohn *et al.,* 1996), *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Burd *et al.,* 2003), *Trichoplusia ni* (Hübner)(Lepidoptera: Noctuidae) (Janmaat & Myers, 2003) had been evaluated for resistance against Bt toxins under laboratory conditions and found to be resistant to more than one Cry toxin. *Plutella xylostella* has proven resistance against Dipel[®] (Ballester *et al.*, 1994), Javalin[®] (Bt var. *kurstaki*) (Tang *et al.*, 1996) and Florbac[®] (Bt var. *aizawai*) (Wright *et al.,* 1997) under field conditions and *T. ni* proved to be resistant to Dipel[®] (Janmaat & Myers, 2003) in greenhouse studies.

Dipel[®] is the most used *B. thuringiensis* var. *kurstaki* (HD-1 strain) product and is applied for control of more than hundred species of Lepidoptera globally (Navon, 1993). Dipel[®] contains the following Cry proteins: Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab and it is available in various formulations, namely dry flowable, emulsifiable suspension and sand granules (Valent Biosciences, 2013).

Plodia interpunctella proved to be resistant to Dipel[®] with 86% survival rate to Dipel[®] at 500mg/kg, and it also led to resistance to other Bt strains (McGaughey & Johnson, 1987). This species is resistant to five Bt serovars: *kurstaki, thuringiensis, galleriae, aizawai* and *tolworthi* (McGaughey & Johnson, 1987). *Plodia interpunctella* is resistant to Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Bt var. *entomocidus* (contains: Cry1Aa, Cry1Ab, Cry1C and Cry1D toxins and showed limited cross resistance to Cry1Ca (McGaughey & Johnson, 1994).

Resistance in *P. xylostella* to Cry1Ab, did not cause cross resistance to Cry1B and Cry1C (Ferré *et al.,* 1991). Resistant larvae proved to be resistant to Dipel[®] and another Btk formulation in Hawaii, and it did lead to cross resistance against Cry1C (Tabashnik *et al.,* 1993). This species is thus, cross resistant to Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, Cry1C (Tabashnik *et al.,* 1993) and Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, Cry1J proteins (Tabashnik *et al.,* 1996). These results were confirmed in 2001, with the addition of limited cross resistance to Cry2Aa (Zhao *et al.*, 2001). Since Dipel[®] contains more than one Cry protein, narrow cross resistance is

possible. This was found for *H. virescens* where resistance to a single Bt protein lead to broad spectrum Bt resistance (Gould *et al*., 1992). Resistance to Cry1Ac led to cross resistance in Cry1Ab and Cry1Aa, but was not surprising due to the similarity in structure of the toxins (Gould *et al*., 1992). Due to the difference in the amino acid sequence of the Cry toxins (Höfte & Whiteley, 1989), it was unexpected that the resistance in Cry1Ac led to the cross resistance in Cry2A (Gould *et al*., 1992). Limited cross resistance was found with Cry1Ca, Cry1Ba and Cry1Bb (Gould *et al*., 1992), as well as with Cry1Fa (Gould *et al.,* 1995). *Pectinophora gossypiella* proved to be resistant to Cry1Ac and also showed narrow spectrum cross resistance to Cry1Aa and Cry1Ab (Tabashnik *et al*., 2000). *Ostrinia nubilalis* can develop resistance to Dipel®, but the species did also not survive on Bt maize expressing Cry1Ab proteins (Huang *et al*., 2002). Low levels of cross resistance were recorded between Cry1Ac and Cry2Aa with *H. zea* (Burd *et al.,* 2003). One individual out of 583 proved to be resistant to Cry1Ac and one individual out of 646 that proved to be resistant to Cry2Aa (Burd *et al.,* 2003). *Spodoptera littoralis,* resistant to Cry1C showed limited cross resistance to Cry1D and Cry1E (Müller-Cohn *et al.,* 1996). Cry1Aa and Cry1Ab proteins also proved to have low insecticidal activity against *S. littoralis* (Müller-Cohn *et al.,* 1996).

Busseola fusca (Fuller) (Lepidoptera: Noctuidae)*,* is a maize stalk borer indigenous to Africa (Annecke & Moran, 1982). Certain theories state that this insect probably originated in central Africa and moved southwards with maize (Annecke & Moran, 1982). This pest attacks maize as well as other graminaceous plants with moderately thick stems (Annecke & Moran, 1982).

Busseola fusca and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) are economically important pests of maize and are the target pests of Bt maize (Cry1Ab) in South Africa (Kruger *et al.* 2011). The first report of resistance by *B. fusca* to *Bt* maize which expresses the Cry1Ab protein in the field was during the 2006 growing season in the Christiana region in South Africa (Van Rensburg, 2007). Field evolved resistance being a decrease in the susceptibility of a population due to the continued exposure to the toxin in the field (National Research Council, 1986; Tabashnik, 1994). The resistance to Bt maize by *B. fusca* developed within eight years after the first release of Bt maize in South Africa in 1998 (James, 2012). Field resistance by *B.*

fusca to Bt maize, also in the refuge area, was confirmed in the 2008/2009 growing season in the Vaalharts area, 50km from the original site in Christiana (Kruger *et al.* 2011). The aim of this chapter was to determine whether development of Cry1Ab resistance by *B. fusca* led to loss of susceptibility to other Bt toxins (i.e. cross-resistance).

3.3 Material and Methods

Collection sites for experimental larvae

Busseola fusca larvae were collected from Venda (22° 57′ S, 30° 29′ E) (Limpopo province), Ventersdorp (26°19′ S, 26°49′ E) (North-West province) and the Vaalharts Irrigation Scheme (28°01′ S, 24°43′ E) (Northern Cape province) in South Africa.

Venda population

Busseola fusca eggs were obtained from laboratory rearing colonies from the Agricultural Research Council – Grain Crops Institute (ARC-GCI) in Potchefstroom.

Ventersdorp population

Busseola fusca pupae were obtained from laboratory rearing colonies from the Agricultural Research Council – Grain Crops Institute (ARC-GCI) in Potchefstroom.

Vaalharts population

Busseola fusca populations were collected during January 2013 at farms in the Vaalharts area. Infested maize plants from refuge areas of Bt maize crops were sampled from individual rows, approximately 20 m apart. The plants were dissected and the larvae removed. Pooled larvae per row served as F_0-individuals for a replication. These larvae were reared through to adults on non-Bt maize. Moths used from the Vaalharts population were therefore from the field-collected population (F_0-generation).
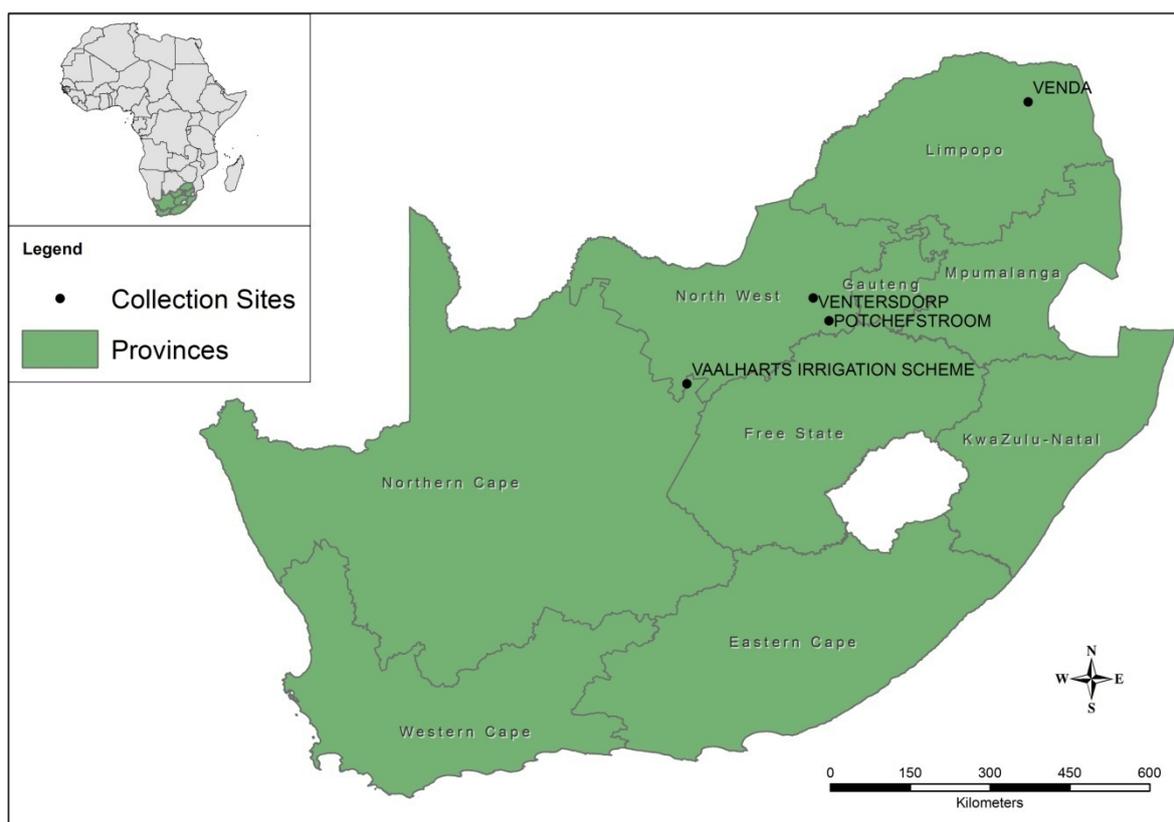
Fig.3.1 Map of collection sites in South Africa

Oviposition by moths under laboratory conditions

Moths of each population were confined as one male-female pair per oviposition chamber. The oviposition chamber and method used for laboratory oviposition by *B. fusca* moths were as described by Kruger *et al.* (2012). Oviposition chambers were 30 cm high and 15 cm in diameter and covered with a fine gauze mesh to prevent escape of moths. A 20 cm long piece of maize stem with bases of leaves intact was placed in an upright position in the container. Plastic containers were filled with approximately 5 cm of crusher stone (7 mm diameter) as substrate to keep the maize stems upright. Stems were inserted 3-4 cm into the substrate. Water was added up to a level three-quarters of the height of the substrate to provide humidity to moths and to keep stems fresh. Oviposition chambers were kept at ambient temperature in the laboratory. Egg batches were removed from each stem at 2-day intervals by cutting off a small piece of the leaf with the egg batch attached to it. The eggs were placed in a plastic bag (Ziplock) (GLAD, South Africa) along with a wet cotton ball to prevent desiccation. These bags were placed in a rearing chamber at

26 ± 1 °C and a 14L:10D photoperiod. Neonate larvae were collected from the bags and transferred to the calyx of non-Bt maize in plastic containers. The containers were kept in the rearing chamber and provided with fresh food on day four and eight after putting them into containers. Larvae from these colonies were used when they were ten days old.

3.3.1 Susceptibility bioassays

3.3.1.1 Preliminary bioassay

The susceptibility assay consisted of leaf tissue bioassays with six treatments. The treatments were leaves of BT maize (expressing Cry1Ab protein); leaves of non-Bt maize plants sprayed with five concentrations Dipel®, namely half the recommended dosage rate, 1.0, 1.5, 2.0 and 5.0 times the recommended dosage rate for bollworm control on peas; water as an untreated (negative) control and deltamethrin (25g/ℓ EC), applied at the recommended dosage rate for *B. fusca* control on maize of 62.5g. a.i./ha (positive control). Dipel® is not registered for control of stem borers on maize. The dosage rate used was the registered dosage rate for bollworm control on peas (500g/ha) and Sprayfilm (sticker) (Protek, Heidelberg, South Africa) was added to each spray treatment at a rate of 5ml/10ℓ water. For ease of reading, the Dipel® concentrations will be referred to as 1.0, 1.5, 2.0 and 5.0 times "the recommended dosage rate" from this point onwards. The spray treatments were applied as full cover sprays to 15 cm-pieces of maize leaves which were attached to a rope in a shaded area. The pieces were left until dry (between 5 and 10 minutes), fold in half, rolled and placed in plastic test tubes (115 x 28 mm). Two larvae (10 days old) from the Ventersdorp population were transferred to each test tube that was closed with an aerated lid and kept in an incubator at 26 ±1 °C and a 14L:10D photoperiod. Each treatment consisted of 10 replicates. Each replicate consisted of 20 test tubes. All leaves were removed at 4 days after commencement of the trial and replaced with an untreated piece of maize stalk of the same cultivar they were exposed to during the first four days and replaced again at 8 days after the spray application. Survival of the larvae was assessed at four, eight and twelve days after treatment. The experiment was terminated twelve days after application of treatments.

3.3.1.2 Bioassay with high dosage rates

3.3.1.2.1 Venda population

The same procedure was followed as in the preliminary experiment (see 3.3.1.1) except for the Dipel® dosage rates that were adjusted to the recommended, 5 and 10 times the dosage rate registered for bollworm control on peas. (Referred to as 1, 5 and 10 times "the recommended dosage rate" onwards). The test tubes were kept in a rearing chamber at 26 ±1 °C and a 14L:10D photoperiod and survival of the larvae was recorded at four, eight and twelve days after treatment.

3.3.1.2.2 Ventersdorp population

The same procedure was followed as in 3.3.1.2.1

3.3.1.2.3 Vaalharts population

The same procedure was followed as in 3.3.1.2.1

3.3.2 Statistical analysis

The data was analyzed using STATISTICA version 11 (StatSoft, Inc., 2012). Repeated measures ANOVA were used to analyse mortality over time. Bonferroni correction was used to adjust for multi means comparisons. Mortality on day 12 was analysed by means of one way analysis of variance and the significant differences between the means were determined using Tukey's post hoc test.

3.4 Results

3.4.1 Susceptibility bioassays

3.4.1.1 Preliminary bioassay

There was a significant time x treatment interaction ($F_{(12,112)}$ = 2.90; P < 0.002) in terms of percentage survival (Fig. 3.2). Percentage survival decreased over time and differed significantly between the three evaluation times ($F_{(2,112)}$ = 69.38; P < 0.001). There were also significant differences between treatments ($F_{(6,56)}$ = 93.28; P < 0.001), with more larvae that survived in the control treatment compared to 1, 1.5, 2 and 5 times Dipel® dosage rates applied, 12 days after application (Table 3.1). Although these treatments differed from the control treatment, survival rate was still high, which indicated ineffective control of *B. fusca* larvae. Percentage survival after application of 0.5 times the Dipel® dosage rate did not differ significantly from the negative control treatment. All treatments differed significantly from deltamethrin (positive control), with deltamethrin being the only treatment that effectively controlled *B. fusca* larvae (Table 3.1).

3.4.1.2 Bioassay with high dosage rates

3.4.1.2.1 Venda population

There was a significant time x treatment interaction in terms of percentage survival ($F_{(12,84)}$ = 32.95; P < 0.001) (Fig. 3.3), that decreased over time and differed significantly between the three evaluation times, namely 4, 8 and 12 days after application ($F_{(2,84)}$ = 238.54; P < 0.001). There was also a significant reduction in survival of larvae on both the maize varieties after 4 days (Fig 3.3). Treatments differed significantly ($F_{(6,42)}$ = 82.36; P < 0.001), with more larvae that survived in the control treatment compared to all other treatments, 12 days after application (Table 3.2). The number of larvae that survived after application of Dipel® at 1 and 5 times the dosage rate was significantly higher than that on the two Bt maize varieties and deltamethrin. Dipel® applied at 10 times the dosage rate, the two Bt maize varieties

and deltamethrin controlled *B. fusca* larvae similarly with no significant differences between them (Table 3.2).

3.4.1.2.2 Ventersdorp population

There was a significant time x treatment interaction in terms of percentage survival ($F_{(12,82)}$ = 9.47; P < 0.001) (Fig. 3.4). Survival decreased over time and differed significantly between the three evaluation times, namely 4, 8 and 12 days after application ($F_{(2,82)}$ = 176.65; P < 0.001). There was a significant reduction in survival of larvae on the two Bt maize varieties at 4 days and onwards. There were also significant differences between treatments ($F_{(6,41)}$ = 13.627; P < 0.0000), with more larvae that survived in the control treatment compared to all other treatments, 12 days after application (Table 3.3). Control with the recommended Dipel® dosage rate differed significantly from that of deltamethrin. Dipel® applied at 5 and 10 times the dosage rate, the two Bt maize varieties and deltamethrin controlled *B. fusca* larvae similarly with no significant differences between them (Table 3.3).

3.4.1.2.3 Vaalharts population (Without MON89034)

There was a significant time x treatment interaction in terms of percentage survival ($F_{(10,96)}$ = 3.45; P < 0.001) (Fig. 3.5). It decreased over time and differed significantly between the three evaluation times, namely 4, 8 and 12 days after application ($F_{(2,96)}$ = 52.91; P < 0.001). There were also significant differences between treatments ($F_{(5,48)}$ = 143.30; P < 0.001), with more larvae that survived in the control treatment compared to 1, 5 and 10 times the Dipel® dosage rate, 12 days after application (Table 3.4). Although these treatments differed from the control treatment, the survival rate was high, indicating ineffective control of *B. fusca* larvae. All treatments differed significantly from deltamethrin, with deltamethrin being the only treatment that controlled *B. fusca* larvae effectively (Table 3.4).

3.4.1.2.4 Vaalharts population (With MON89034)

There was a significant time x treatment interaction ($F_{(12,70)}$ = 3.98; P < 0.001) in terms of percentage survival (Fig. 3.6), that decreased over time and differed

significantly between the three evaluation times, namely 4, 8 and 12 days after application ($F_{(2,70)}$ = 48.45; P < 0.001). There were also significant differences between treatments ($F_{(6,35)}$ = 28.43; P < 0.001), with more larvae that survived in the control treatment compared to 5 and 10 times the Dipel[®] dosage rate as well as on the Bt maize variety MON89034, expressing two Cry proteins , 12 days after application (Table 3.5). Although these treatments differed from the control treatment, the survival rate was high, indicating poor control of *B. fusca* larvae. All treatments differed significantly from deltamethrin, with deltamethrin being the only treatment that controlled *B. fusca* larvae effectively (Table 3.5).

3.5   Discussion

Lepidoptera species can develop resistance to Bt Cry toxins under laboratory conditions (McGaughey, 1985). The commercialization and rapid adoption of Bt transgenic crops increased the concern that Lepidoptera target pest species will quickly develop resistance against these Cry toxins (Gould, 1998).

Bt transgenic maize, expressing Cry1Ab, were commercialized in South Africa since 1998 (ACB, 2012), with the purpose to control economically important stem borers (Gouse *et al.*, 2005), including *B. fusca* (Van Rensburg, 1999). The first report of field resistance by *B. fusca* to Bt maize was reported from Christiana, North-West province in South Africa in 2007 (Van Rensburg, 2007), followed by a second report one year later (Kruger *et al.*, 2011). This report was based on observations by farmers in the Vaalharts Irrigation Scheme, Northern Cape province in South Africa, about 50km from Christiana (Kruger *et al.*, 2011). There is probably more than one reason for resistance development, but one of the main factors that contributed to the development of resistance in the Vaalharts Irrigation Scheme was the poor level of compliance of refuge standards (Kruger *et al.*, 2011). This happened between 1998 and 2006 in the area where resistance was first recorded in South Africa (Kruger *et al.*, 2009).

The knowledge that the Vaalharts *B. fusca* population is resistant to Cry1Ab present in MON810 (Kruger *et al.*, 2011), created the opportunity to test for possible cross resistance by *B. fusca* to Bt Cry toxins. Dipel[®] DF, registered for control of *H.*

armigera, but not for *B. fusca*, contains the active ingredient Bt var. *kurstaki*, (Tabashnik, 1994).

The high mean percentage survival of *B. fusca* larvae in the untreated control group (negative control) and 100% mortality in the positive control group (deltamethrin application) indicated a good experimental design. The half-dosage rate was omitted from experiments after no significant difference in efficacy between the untreated control group and the group that received half the dosage rate were found in the preliminary experiment. The rate was too low for evaluation of efficacy for control of *B. fusca* larvae. Similar efficacy of the 1.5 and 2.0 times dosage rate compared to 1.0 time dosage rate and a high survival rate of ±68% with twice the dosage rate also indicated a rate too low for *B. fusca* control. The experimental dosage rates were adjusted to 1, 5 and 10 times the recommended dosage rate for bollworm control on peas, for all experiments following the preliminary experiment.

Not more than 50% of larvae from any of the *B. fusca* populations treated with the different Dipel treatments (except for 10 times the dosage rate applied to larvae from Venda), died within four days after treatment. This could be explained by the size of the larvae been evaluated (10 days old). The susceptibility of late instar larvae to Bt is very low (Navon, 2000). Larvae transferred to MON810 and MON89034 were also exposed to the Cry toxins for the duration of the study compared to the sprayed treatments that received unsprayed stems after four days. The Cry toxin rates expressed by these Bt plants and Bt sprays are also not known, a difficulty also acknowledged by (Tabashnik *et al.,* 2003).

High mortality rates of *B. fusca* larvae from the Venda population, 12 days after application, could be explained by the low levels of exposure to Bt maize in this area, resulting in larvae still being susceptible to all Cry proteins they were exposed to, namely Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab in Dipel[®]; Cry1Ab in Bt maize (MON810) as well as Cry1A.105 and Cry2Ab2 in MON89034. Larval survival rate decreased with increasing dosage rates of Dipel[®], to a level where 10 times the recommended rate was as effective in controlling *B. fusca* larvae as Cry1Ab and Cry1A.105 and Cry2Ab2 expressing maize and deltamethrin. Effective control of the

Venda population by the two maize varieties confirmed its susceptibility to Cry toxins. In a study conducted in Kenya, *B. fusca* and *C. partellus* also died soon after ingestion of plant tissue expressing Cry1Ab proteins (Mugo *et al.*, 2011) confirming susceptibility of these two stem borer species in Kenya to the Cry1Ab toxin.

The Ventersdorp population was controlled similarly with 5 and 10 times the Dipel® dosage rate, the two maize varieties and deltamethrin. A higher percentage of larvae did, however, survive compared to the Venda population. The Ventersdorp population has been exposed over time to Bt maize over large areas planted by commercial farmers (Pers. obs). The survival rate of larvae was lower compared to the Vaalharts population that has proven resistant to Cry1Ab Bt maize.

The Vaalharts *B. fusca* population resistant to Bt maize, (Cry1Ab), could still be effectively controlled with deltamethrin. The percentage survival on maize plants expressing Cry1Ab was similar in the two experiments with *B. fusca* larvae from Vaalharts after 12 days (81 and 80% respectively). These survival rates were also similar to those in the untreated control treatment indicating the high levels of resistance present in the population. Dipel® did not provide effective control of the Vaalharts *B. fusca* population in any of the experiments. Survival rates were high, even with 10 times the recommended dosage rate for bollworm control on peas. This is in contrast to survival of *B. fusca* larvae from the susceptible (Venda) population after treatment with Dipel®. The evolution of resistance is a heritable decrease in a population's susceptibility to a toxin (Tabashnik, 1994). Therefore the reduced susceptibility of the Vaalharts population to Dipel® indicates the possible development of cross resistance to the Cry proteins Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab contained in Dipel, as shown under laboratory conditions.

This finding indicates that the polyphagous lepidopteran species, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), a target species on Bt cotton (Cry1Ac) and a non-target species on Bt maize in South Africa, should also be evaluated for possible cross resistance. It is an important pest on vegetables and if found to be cross resistant, organic farmers may lose their only available control option.

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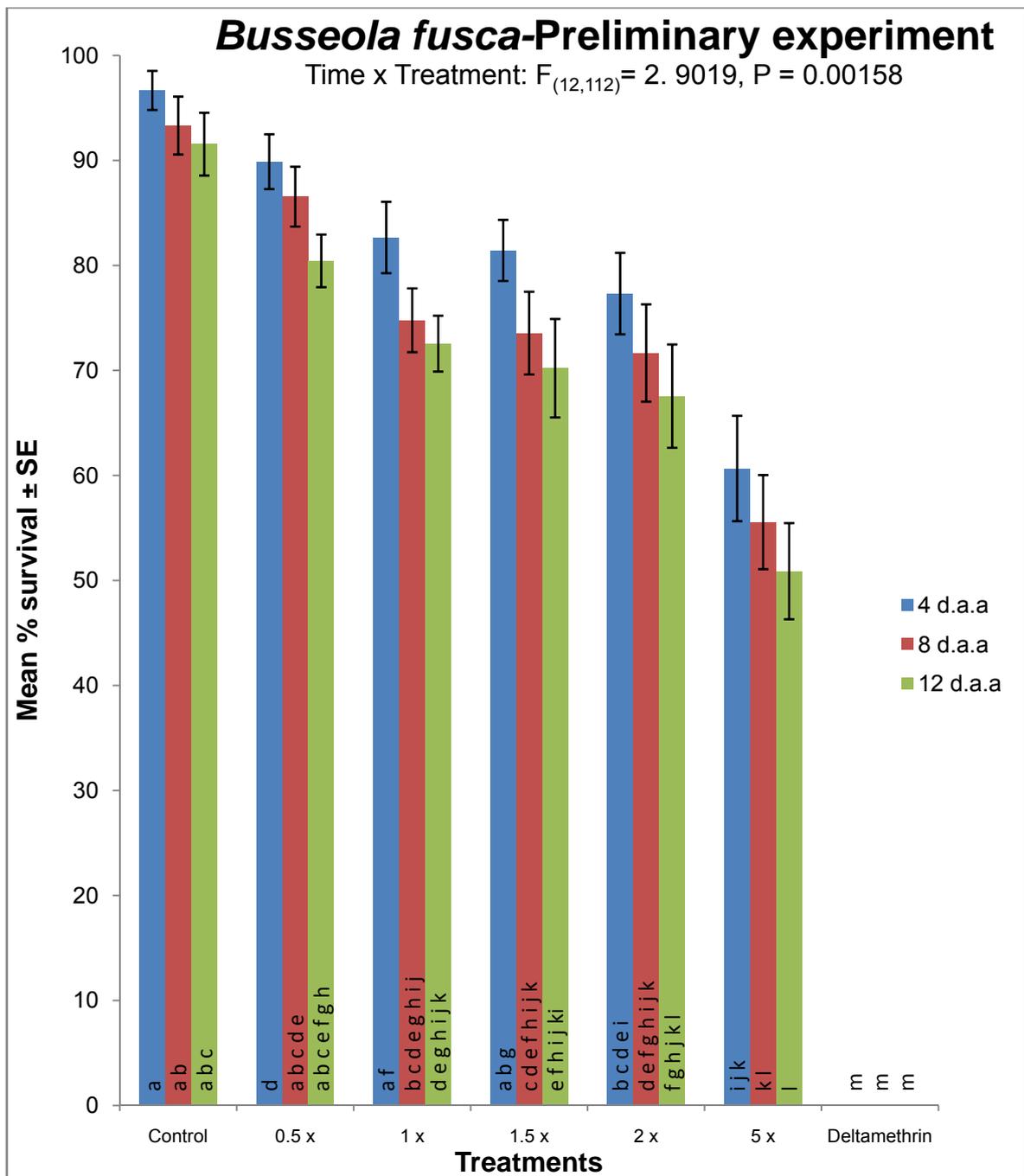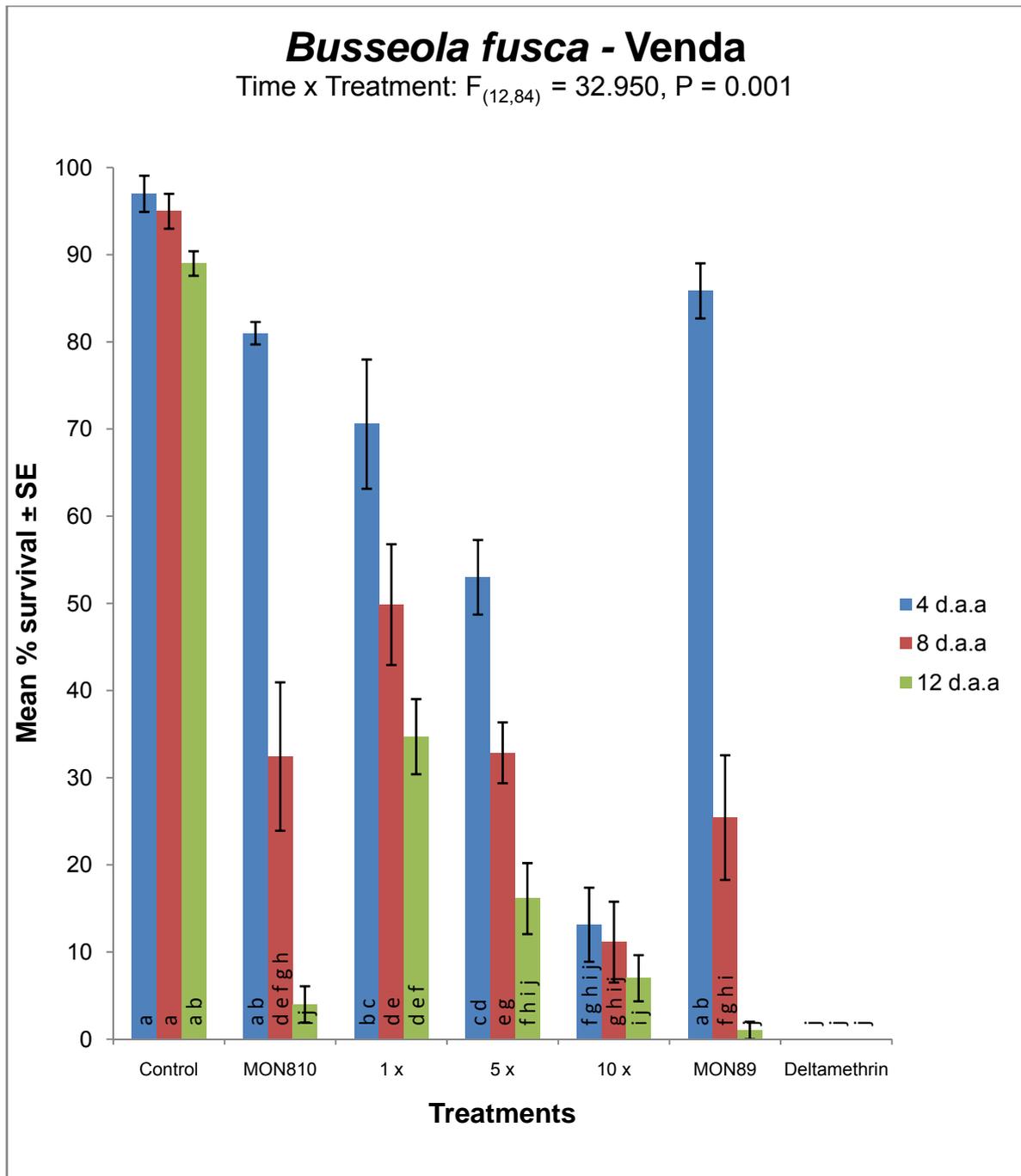
Fig. 3.2 Mean percentage survival of *Busseola fusca* larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 0.5x, 1x, 1.5x, 2x and 5x. (d.a.a. = days after application).

Table 3.1 Mean percentage survival of *B. fusca* larvae in the preliminary experiment, 12 days after application.

Treatment	Mean % survival (± SE)
Control (water)	91, 56 ± 2,99a*
0,5 x Dipel®	80,44 ± 2, 51ab
1 x Dipel®	72,56 ± 2,66b
1,5 x Dipel®	70,22 ± 4,69b
2 x Dipel®	67,56 ± 4,92b
5 x Dipel®	50,89 ± 4,58c
Deltamethrin	0 ± 0d

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

Fig. 3.3 Mean percentage survival of *Busseola fusca* (Venda) larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 1x, 5x and 10x. (d.a.a. = days after application).

Table 3.2 Mean percentage survival of *B. fusca* larvae from Venda, 12 days after application.

Treatment	Mean % survival (± SE)
Control (water)	89,00 ± 1,41a
1 x Dipel®	34,71 ± 4,31b
5 x Dipel®	16,14 ± 4,07c
10 x Dipel®	7,00 ± 2,65cd
MON810	4,00 ± 2,08d
Mon 89	1,00 ± 1,00d
Deltamethrin	0 ± 0d

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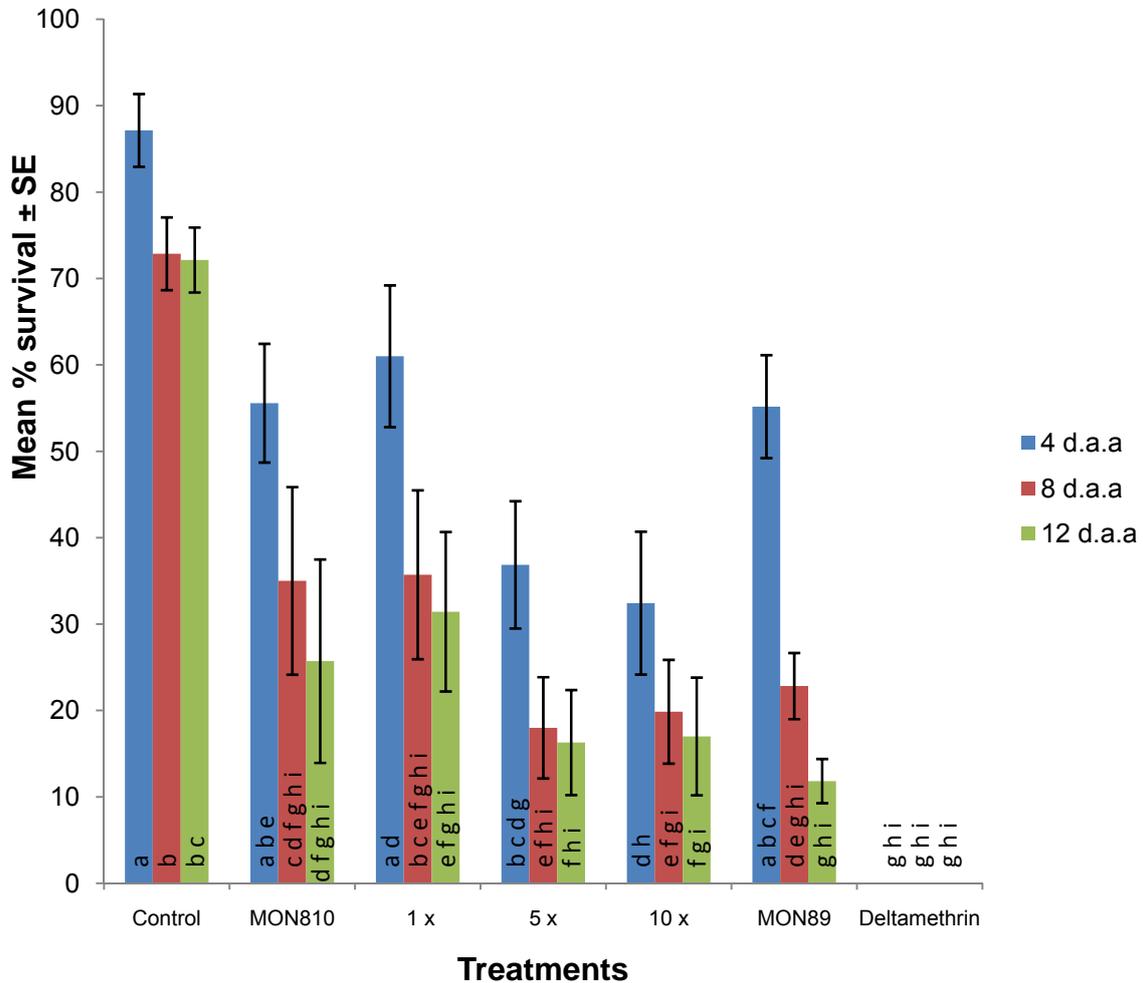 do not differ significantly at P = 0.05 (Tukey's HSD).

Fig. 3.4 Mean percentage survival of *Busseola fusca* (Ventersdorp) larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 1x, 5x and 10x. (d.a.a. = days after application).

Table 3.3 Mean percentage survival of *B. fusca* larvae from Ventersdorp, 12 days after application.

Treatment	Mean % survival (± SE)
Control (water)	72,14 ± 3,76a
1 x Dipel®	31,43 ± 9,23b
5 x Dipel®	16,29 ± 6,08bc
10 x Dipel®	17 ± 6,81bc
MON810	25,71 ± 11,77bc
Mon 89	11,83 ± 2,56bc
Deltamethrin	0 ± 0c

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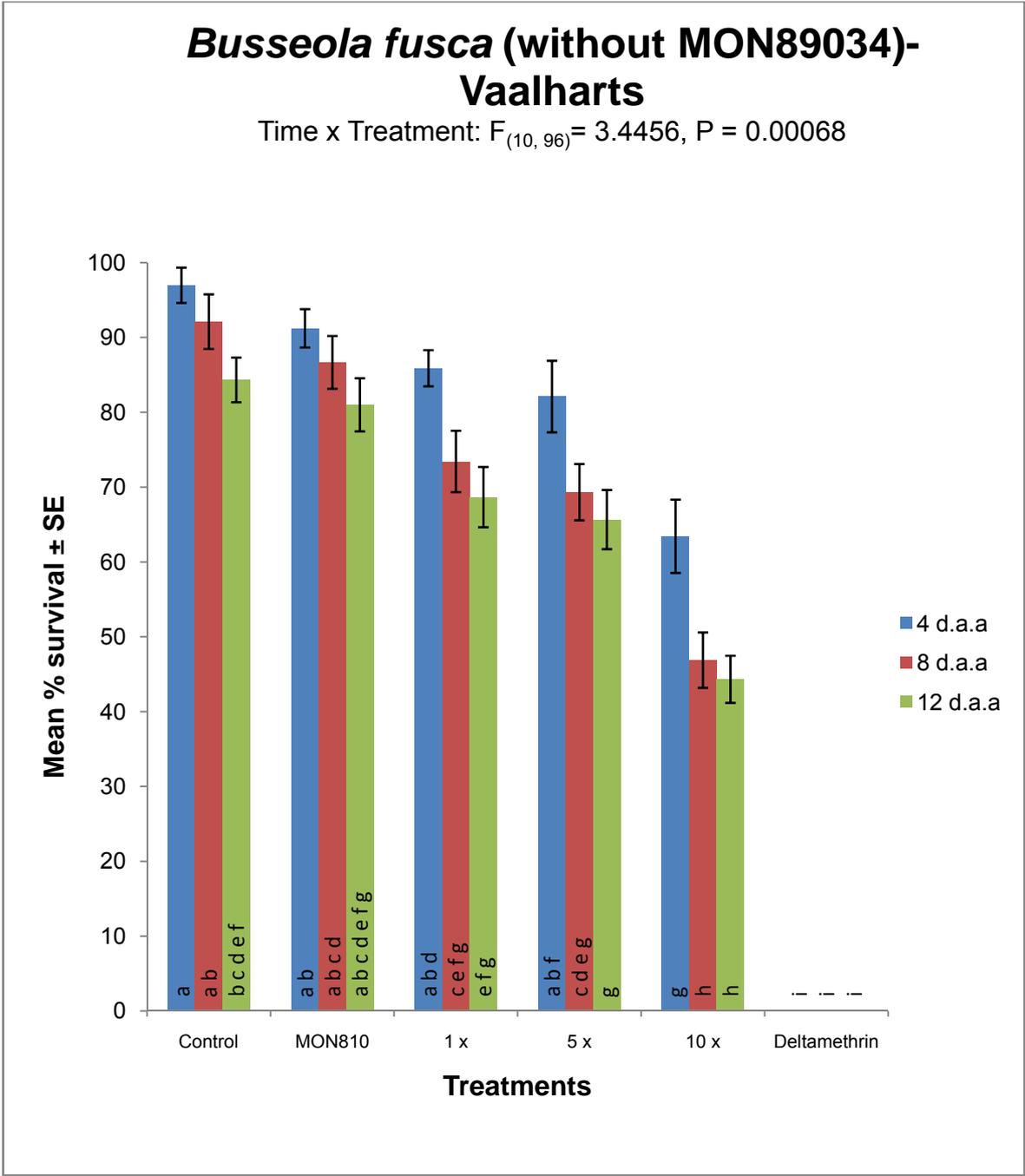do not differ significantly at P = 0.05 (Tukey's HSD).

Fig. 3.5 Mean percentage survival of *Busseola fusca* (Vaalharts Irrigation Scheme) larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 1x, 5x and 10x. (d.a.a. = days after application).

Table 3.4 Mean percentage survival of *B. fusca* larvae from Vaalharts Irrigation Scheme, 12 days after application.

Treatment	Mean % survival (± SE)
Control (water)	84,33 ± 2,98c
1 x Dipel®	68, 67 ± 4,03ab
5 x Dipel®	65,67 ± 3,95a
10 x Dipel®	65,67 ± 3,16a
MON810	81,00 ± 3,55bc
Deltamethrin	0 ± 0d

*Means within a column followed by the same letter

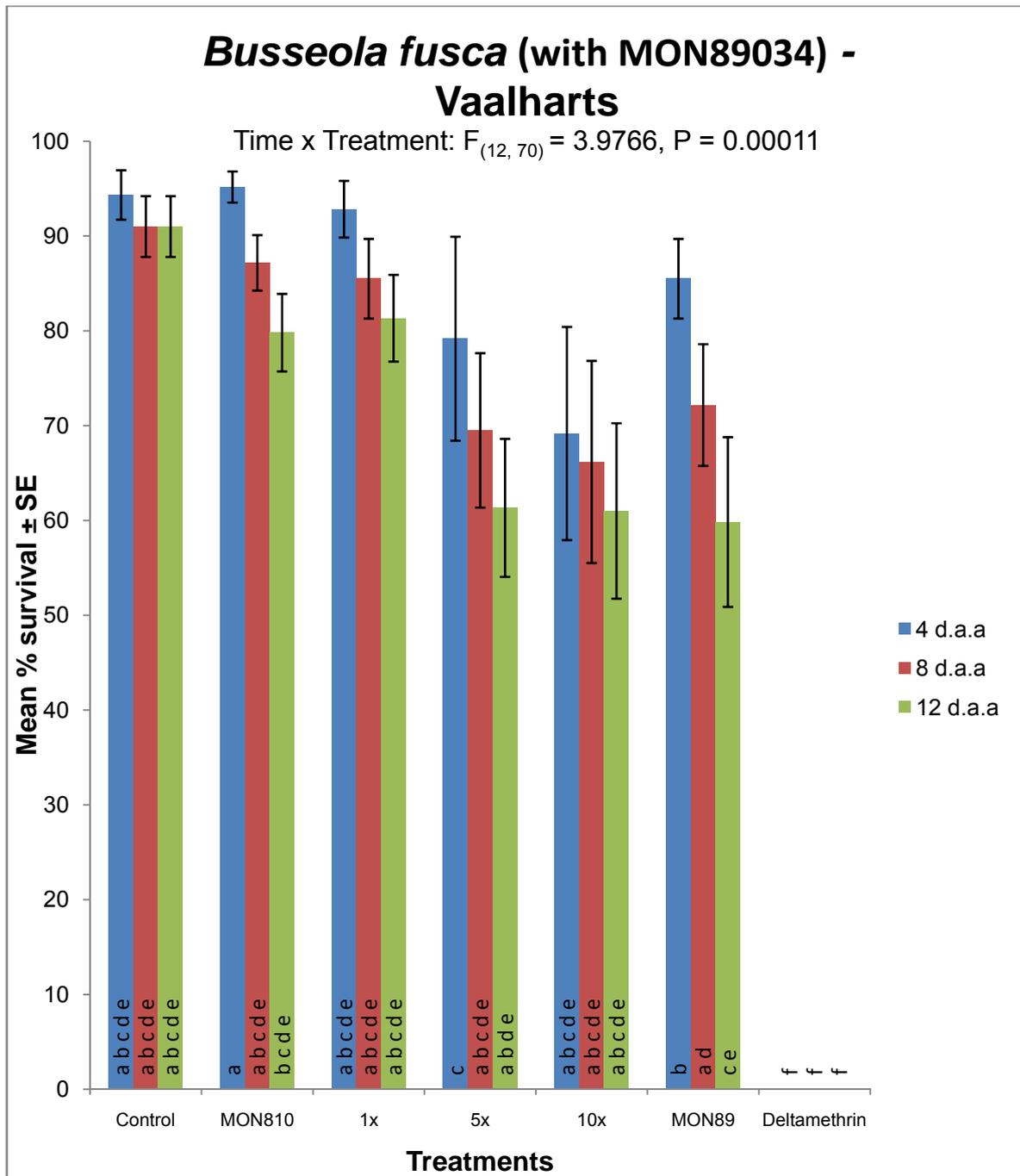do not differ significantly at P = 0.05 (Tukey's HSD).

Fig. 3.6 Mean percentage survival of *Busseola fusca* (Vaalharts Irrigation Scheme) larvae after 4, 8 and 12 days of feeding on maize exposed to different treatments. The dosage rates of Dipel® are indicated as 1x, 5x and 10x. (d.a.a. = days after application).

Table 3.5 Mean percentage survival of *B. fusca* larvae from Vaalharts Irrigation Scheme, 12 days after application.

Treatment	Mean % survival (± SE)
Control (water)	91,00 ± 3,21b
1 x Dipel[®]	81,33 ± 4,57ab
5 x Dipel®	61,33 ± 7,28a
10 x Dipel®	61,00 ± 9,25a
MON810	79,83 ± 4,09ab
MON89034	59,83 ± 8,95a
Deltamethrin	0 ± 0c

*Means within a column followed by the same letter

do not differ significantly at P = 0.05 (Tukey's HSD)

Chapter 4

Conclusions

Chemical control on maize [(*Zea mays* L. (Poaceae)] was the norm for control of the stem borer species, *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) before the advent of Bt maize in South Africa. Bt maize has been grown to control these lepidopterous stem borers in South Africa since 1998. The first report of field resistance of *Busseola fusca* (Lepidoptera: Noctuidae) to Bt maize (Cry1Ab) was made during the 2006 cropping season in South Africa (Van Rensburg, 2007), eight years after the first release of Bt maize (MON810) in South Africa (James, 2012).

The aims of this dissertation were to determine the efficacy of Bt spray applications for control of four lepidopteran pests, including three stemborer species, *C. partellus*, *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) and *B. fusca*. The experimental design was adapted to compensate for the sub-lethal dose that might be ingested due to the tunnelling behaviour by exposing experimental larvae to the spray treatment for the first 4 days by folding and rolling maize leaves after application. It was also investigated whether development of Cry1Ab resistance by *B. fusca* caused a loss in susceptibility to other Bt toxins (i.e. cross-resistance). If cross resistance develops in pests exposed to Cry toxins in crops, organic farmers may lose the only tools (Bt-spray formulations) they are allowed to use for pest control.

Susceptibility bioassays with 10-day old larvae were conducted under laboratory conditions. Treatments included application of various dosage rates of Dipel$^{®}$ and deltamethrin as well as exposure to MON810. A single Cry protein is still sufficient to control *C. partellus* in South Africa in contrast to *B. fusca* that are not effectively controlled in the Northern Cape area by Cry1Ab. It was therefore also expected and confirmed that multiple Cry toxins also control *C. partellus* effectively (Chapter 2).

High mortality rates of *E. saccharina* larvae indicated no resistance and low levels of tolerance to Cry toxins. It could be explained by the low levels of exposure to Bt maize, resulting in larvae still being susceptible to all Cry proteins they were exposed

to, namely Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab in Dipel® and Cry1Ab in Bt maize (Chapter 2).

Spodoptera exempta was effectively controlled by Bt maize as well as Dipel® in this study. This lepidopteran species is active throughout a year in temperate zones of Africa. If *S. exempta* develop resistance to Cry toxins and Bt maize events would be released for commercial planting in these areas, *S. exempta* pose a threat added to their injuriousness (Chapter 2). Care should be taken not be interpret the percentage *C. partellus, E. saccharina* and *S. exempta* larvae that survived after exposure to Bt maize and Bt spray treatments as development of resistance without verification of these experiments with earlier instars that are known to be more susceptible.

Busseola fusca larvae were sampled from Venda (susceptible population), Ventersdorp and the Vaalharts Irrigation Scheme (resistant population). The *B. fusca* population from Venda, was effectively controlled by maize expressing Cry1Ab (MON810), Cry1A.105 and Cry2Ab2 (MON89034) and Bt spray (especially high dosage rate). The Ventersdorp *B. fusca* population was controlled by both maize varieties and Bt sprays, but the percentage larvae that survived showed reduced susceptibility within the population (Chapter 3). Dipel® treatments, and the two Bt maize varieties did not provide effective control of the Vaalharts *B. fusca* population reported to be resistant to Cry1Ab, in two experiments. The high survival rates indicate a reduction in susceptibility to Cry toxins other than Cry1Ab and therefore possible development of cross resistance in the Vaalharts *B. fusca* population (Chapter 3). This finding indicates that the polyphagous lepidopteran species, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), a target species on Bt cotton (Cry1Ac and Cry2Ab2) and a non-target species on Bt maize in South Africa, should also be evaluated for cross resistance. It is an important pest on vegetables and if found to be cross resistant, organic farmers may lose their only available control option.

4.1 References

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