Migration patterns and survival of *Busseola fusca* larvae in maize plantings with different ratios of Bt and non-Bt seed

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I, Jaco Marais, declare that this research project which I hereby submit for the degree of *Magister Scientiae* (Environmental Science) at the North-West University, is entirely my original work and has not been submitted for a degree at any other university.

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Approval by supervisors

The supervisors of this study give permission that the data generated during the study may be used for scientific publication by the student.

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ABSTRACT

The high-dose/refuge strategy is used globally to manage insect resistance development in genetically modified crops with insecticidal properties (Bt crops). The “refuge in a bag” (RIB) strategy is also being considered for deployment against several pest species. *Busseola fusca*, the target pest of Bt maize in South Africa, evolved resistance to Cry1Ab proteins. The objective of this study was to determine whether migrating *B. fusca* larvae are effectively controlled using the RIB strategy. A field study with a single-gene event (Cry1Ab) and a “pyramid” event (Cry1A.105 + Cry2Ab2) was conducted in which the migration patterns of *B. fusca* larvae in plots with different seed mixture treatments were studied. The experiment consisted of five seed mixture ratios (5%, 10%, 15%, 20% non-Bt seed and 100 % non-Bt seed as control). Natural infestation was augmented by artificial inoculation with neonate larvae into the central non-Bt maize plant of each plot. Rate of larval survival and migration, measured in terms of increase in number of plants per plot that exhibited borer damage was recorded at weekly intervals until flowering. A laboratory study was conducted to determine larval growth and survival when simulating migration between Bt and non-Bt maize plants. A feeding experiment in which larvae were reared on different types of maize (Bt and non-Bt) was conducted and larval survival and mass recorded after a 7-day feeding period. The incidence of damaged ears, stem damage and damaged internodes per stem were recorded and relationships between these variables determined by means of correlation analyses. A review was conducted in order to identify and discuss similarities and differences between the high-dose/refuge and seed mixture strategies. This was done to determine which strategy would be the most appropriate insect resistance management (IRM) strategy against *B. fusca*. The rate of survival and migration of *B. fusca* larvae was significantly higher in the plots with maize expressing Cry1Ab and control plots, than in plots with the pyramid Bt event. Older larvae exhibited improved growth and survival in the laboratory experiment when they were transferred from non-Bt to Bt plants. Positive correlations were found between early and late season damage, although some weaker than others. Plants of the “pyramid event” suffered less late-season damage than those of the single-gene event. Since the increase in number of damaged maize plants over time is associated with migration of older and larger larvae, the observed tendencies may indicate that the assumed high-dose does not kill larvae above a certain developmental stage. The high-dose refuge strategy seems to be the better option for delaying resistance development.

Key words: *Busseola fusca*, Cry1Ab, Cry1A.105 + Cry2Ab2, larval migration patterns, “refuge in a bag” strategy, transgenic maize.

UITTREKSEL

Die hoë-dosis/vlugoord insekweerstand-bestuurstrategie word wêreldwyd gebruik om weerstandsontwikkeling teen Bt gewasse te bestuur. Die “vlugoord in ’n sak” (VIS)-strategie word ook vir hierdie doel oorweeg teen verskeie insekplaagspesies, o.a. *Busseola fusca,* wat ook die teikenplaag van Bt mielies in Suid-Afrika is. Hierdie plaag het weerstand ontwikkel teen Bt mielies wat die insekdodende Cry1Ab, uitdruk. Die doel van hierdie studie was om te bepaal of die VIS strategie migrerende *B. fusca* larwes suksesvol oor tyd sal beheer. ’n Veldstudie is gedoen waarin die migrasiepatrone van larwes in persele van verskillende verhoudinge van nie-Bt: Bt mielieplante bestudeer is. Twee tipes Bt mielies, ’n enkel-geen (Cry1Ab), asook ’n baster met “gestapelde” gene (Cry1A.105 + Cry2Ab2) is gebruik. Die eksperiment het vyf saadmengselverhoudings ingesluit (5%, 10%, 15%, 20% nie-Bt saad en100 % nie-Bt saad as kontrole) wat in blokke aangeplant is. Die proef is oor twee seisoene herhaal en was onderhewig aan natuurlike infestasie, wat aangevul is met kunsmatige besmetting deur pas-uitgebroeide larwes op die sentrale nie-Bt plant in elke perseel te plaas. Larwale oorlewing en die tempo van migrasie, wat bepaal is in terme van die aantal plante wat oor tyd skade simptome vertoon, is op ’n weeklikse basis aangeteken tot en met blomtyd. ’n Laboratoriumstudie is ook gedoen om te bepaal of larwes sou oorleef en ontwikkel indien hulle van nie-Bt na Bt-plante sou migreer, soos wat onder veldtoestande oor tyd verwag word. Larwes van verskillende ouderdomme is vanaf nie-Bt na Bt-plante oorgeplaas waar hulle oorlewing en groei na ’n 7-dae periode bepaal is. Die aantal beskadigde internodes per stam asook die aantal beskadigde koppe per perseel is bepaal. Die verband tussen hierdie veranderlikes en plantskade tydens die voorblomperiode is bepaal d.m.v. korrelasies. ’n Oorsig van literatuur is gedoen om die verskille en ooreenkomste tussen die hoë-dosis/vlugoord en saadmengsel-strategie te identifiseer en te bespreek. Die tempo van oorlewing en migrasie van larwes was betekenisvol hoër in persele met enkelgeen-mielies (Cry1Ab). Ouer larwes het oorleef na die 7-dae voedingsperiode op beide die enkel- en stapelgeen-mielies. Daar was ’n positiewe verband tussen die voorkoms van skade in die voor- en na-blomperiode alhoewel sekere korrelasies swakker was as ander. Daar was ’n tendens dat skade deurgaans minder was in persele waarin stapelgeen-mielies geplant is. Die laboratoriumstudie het getoon dat ouer larwes nie doeltreffend beheer word deur die enkel-geen- of stapelgeenmielies nie. Dit blyk dat die hoë-dosis/vlugoord-strategie die beter opsie mag wees om weerstandsontwikkeling te vertraag.

Sleutelwoorde: *Busseola fusca*, Cry1Ab, Cry1A.105 + Cry2Ab2, larwale migrasiepatrone, “vlugoord in ’n sak”, transgeniese mielies.

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Chapter 1: Introduction and literature review

1.1. Introduction to genetically modified crops

Genetically modified (GM) crops have become an important part of the agricultural industry since its introduction in the late twentieth century. During 1983 scientists were able to recombine isolated genes into plant-gene sequences, heralding a new age of genetically modified crops (Stone, 2010).

China was the first to cultivate a transgenic crop on commercial scale (tobacco resistant to tobacco mosaic virus) in 1988 (Pray, 1999), while tomatoes with an altered gene to protect against rotting was introduced in the United States of America (USA) during 1994 (Harvey, 2004). In 1995 and 1996 two newly developed traits for expression by transgenic plants, namely insecticidal properties and herbicide tolerance, were introduced (Stone, 2010). The cultivation of transgenic crops was estimated at 1.7 million hectares when introduced globally in 1996 (Chapman and Burke, 2006). By 2011, 160 million hectares of transgenic crops were cultivated worldwide, meaning GM crop cultivation increased several fold since its introduction (James, 2011).

Various GM traits have been developed to produce transgenic crops fulfilling specific applications. A few of the GM traits developed include insecticidal properties, herbicide tolerance or even enhanced vitamin and mineral content (Chapman and Burke, 2006; Dill, 2005; Bouis, 2007). Maize, cotton, soybean and canola are but some of the most important transgenic crops that are cultivated (Acworth *et al*., 2008).

Transgenic crops expressing insecticidal properties, introduced globally during 1996, were developed as a pest management strategy against the most important insect pests of maize and cotton (Tann *et al*., 2002; Gray, 2010). These GM crops express *Cry* proteins, encoded for by a gene transferred from *Bacillus thuringiensis* (Bt) to these crops. These proteins kill target insects when feeding on such plants (Haung *et al*., 2011). By ingesting sufficient dosages of Bt proteins, target insects die due to perforations of the mid-gut caused by the intestinal binding of *Cry* proteins (Gray, 2010).

Since its introduction, the use of Bt maize globally increased rapidly. In the USA 63 % (22.20 Mha) of the total maize produced in 2009 was Bt maize (Onstad *et al*., 2011; USDA, 2010). Adoption of Bt maize by other countries have also increased, with as much as 76 % Bt maize being cultivated during 2009 in Argentina, 74 % in Canada, 67 % in South Africa and 39 % in Brazil (Haung *et al*., 2011). The levels at which Bt proteins are expressed in tissues throughout the plant's development cycle, may affect the efficacy at which a target pest is controlled. An example is Bt cotton. Studies on Bt cotton in Australia has suggested a reduced efficacy against *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) due to sub-lethal dosages of Bt proteins, especially when larvae feed on plants after flowering stages (Olsen *et al*., 2005). These reduced levels of gene expression may be attributed to variations in plant chemistry as maturity is reached, as well as different environmental factors throughout the growing season (Olsen *et al*., 2005).

In the case of Bt maize, reduced levels of gene expression throughout the growing season could increase the risk of resistance development over time, due to pest exposure to sub-lethal dosages of Bt proteins. Different parts or tissues of a Bt plant expresses different dosages of Bt proteins. Bt maize has been reported to express higher concentrations of *Cry* proteins in the leaves compared to the grain or pollen (Andow, 2002). The feeding location of a pest insect is therefore important with regard to how much it will be exposed and therefore how it will be affected by Bt proteins. Studies have suggested that Bt events from different seed companies could also differ in gene expression throughout various plant parts (Andow, 2002).

1.2. Resistance development to Bt crops and application of IRM strategies

Since resistance development to various pesticides is a common phenomenon, there is also a concern with regard to pests developing resistance to transgenic crops expressing Bt proteins (Pimentel *et al*., 1980; Cohen *et al*., 2000). The potential occurrence of insect resistance to Bt crops has created a need for insect resistance management (IRM) strategies to delay such developments (Gould, 2000). Resistance of an insect population in the field can be defined as a decrease in genetic susceptibility to Bt proteins due to the population's exposure to it (Tabashnik, 1994b). It has been suggested that the term "field-evolved resistance" has been used rather ambiguously to describe resistance development of target pests to Bt proteins (Sumerford *et al.*, 2013).

Examples of small changes in susceptibility in laboratory bioassays to complete loss of efficacy or product failure have been included under “field-evolved resistance” (Wan *et al.*, 2012; Storer *et al*., 2010; Van Rensburg, 2007). Resistance to Bt crops can therefore take many forms and can in most cases still be managed (Sumerford *et al.*, 2013). A clear definition of the term “resistance” is an important consideration when used to describe resistance development of target pests to Bt crops. Large scale use of Bt crops increases the selection pressure asserted on pest populations, thereby favouring potential for resistance development to Bt proteins (Haung *et al*., 2011). In a scenario where a target species develops resistance to a Bt crop, the value of the particular trait is greatly reduced.

Deployment of IRM strategies in a generic fashion for all purposes and regions could pose to be problematic in terms of sustainable pest management over time. Local conditions, characteristics of agro-ecosystems and agricultural practices should be considered in order to assess the risk of resistance development as well as selecting the most appropriate IRM strategy (Head and Savinelli, 2008). IRM strategies that are tailor made for specific pests in specific geographical regions could enhance its efficacy in managing resistance development. Integrated pest management (IPM) is considered universal in principle, but site specific in its application (Kennedy, 2008). Because IRM forms a crucial part of sustainable IPM, it should be conducted in a similar manner.

Unforeseen pest management costs could be avoided if IRM strategies are effectively deployed (Andow, 2002). Deployment of IRM strategies to prolong resistance development not only benefits a producer from an economic point of view, but also that of the seed industry. The Bt product’s shelf life could be extended with less development costs incurred for newer Bt event development (Andow, 2002). Prevention of cross-resistance between Bt crops and Bt sprays is another justification for development of appropriate IRM strategies. Bt sprays may still be used in organic farming practices, thereby making the prevention of cross-resistance of great importance since Bt sprays are a major pest management tool in this type of agriculture (Andow, 2002).

Resistance to insecticidal crops expressing Bt proteins has been relatively limited. A few cases of field resistance has been documented including: (1) *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) resistant to Bt maize expressing Cry1Ab in South Africa, (2) *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) in Puerto Rico resistant to Cry1F expressed in Bt maize, (3) *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) resistant in some areas of the USA to Cry1Ac and Cry2Ab expressed in Bt cotton and (4) *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) resistant to Bt maize expressing Cry3Bb1 in the USA (Van Rensburg, 2007; Tabashnik *et al.*, 2009; Gassmann *et al*., 2012; Tabashnik *et al.*, 2013).

Resistance to Bt proteins may develop due to many reasons. These reasons could include exposure to sub-lethal dosages of Bt proteins, non-compliance to refuge requirements, dominant nature of resistance genes or a non-random mating pattern between pest adults (Murphy *et al*., 2010; Kruger *et al*., 2009; Campagne *et al.*, 2013; Bates *et al.*, 2005).

Advances in GM crop technology facilitated “stacking” or “pyramiding” of multiple genes. The inclusion of multiple Bt genes (pyramiding), or combination of insecticidal traits with herbicide tolerance traits within a single event (gene stacking) are examples of this technology (Onstad *et al*., 2011; Bates *et al*., 2005). An important benefit of pyramided Bt genes within a single event is enhanced delay of resistance development by target species (Bates *et al.,* 2005). By providing improved management of a pest complex as well as having the ability to use smaller refuges (5 % to 10 % according to models), pyramided gene technology may also enhance the efficacy of various insect resistance management (IRM) strategies (Bates *et al.,* 2005). Deploying pyramided gene hybrids reduces the number of different seed types a producer must use to control different pest complexes, while from an economic perspective, money can be saved due to lower insurance and input costs (Onstad *et al.,* 2011). Purchase costs of stacked gene products are, however, generally more expensive than single gene events. Also, proper consideration of intended use should be practiced in order to prevent unnecessary deployment of stacked gene / pyramided products.

1.2.1 High-dose/refuge strategy

Economic and easy to deploy IRM methods are needed to delay resistance development of target species to Bt crops. Some IRM strategies apply a non-Bt refuge concept. One such example is the high-dose/refuge strategy that consists of two components.

First, according to the United States Environmental Protection Agency (EPA) (cited by Goldstein *et al.,* 2010)*,* Bt plants deployed in a high-dose/refuge strategy are designed to produce insecticidal proteins at a dosage 25 times the concentration needed to control target insects. Concentrations of insecticidal proteins expressed by Bt plants must therefore be more than what is needed to control susceptible insects. Secondly, a separate refuge consisting of non-Bt plants must be planted alongside the Bt crop (Gould, 2000). A refuge size of 20 % (chemical control allowed) or 5 % (chemical control prohibited) can be selected (Haung *et al*., 2011).

Susceptible insects are benefitted in the non-Bt refuge, thereby providing potential mates to resistant individuals surviving in the Bt crop areas (Gould, 2000). The frequency of resistance alleles are therefore reduced, decreasing the numbers of resistant homozygous genes being transferred to next generations (Kruger *et al*., 2009).

The diluted heterozygous resistance genes can then be controlled by the crop with high-dose Bt protein expression. The inability of a Bt event to deliver such a high-dose expression to a specific target pest, could be countered with the deployment of larger separate refuges (e.g. 20% to 50 %) (Andow, 2002). By increasing the refuge sizes, selection pressure is reduced and more susceptible adults are generated for mating purposes. By preventing the transfer of homozygous resistance genes to the next generations, resistance development could be delayed.

Different options for planting refuges are available including (1) perimeter refuges planted at the edges of the field, (2) block refuges planted on one side of the field, or (3) strip refuges planted intermittently inside the field (Cullen *et al.*, 2008). The refuge requirements under the high-dose/refuge IRM strategy (regulated by law) are the same for South African producers as in most parts of the world.

For the high-dose/refuge strategy to be successful, three assumptions are made namely, (1) alleles coding for resistance are initially present at a low frequency, (2) inheritance of such resistance genes is a recessive phenomenon, and (3) resistant and non-resistant individuals mate randomly within the planted area (Bates *et al.*, 2005). When all three assumptions are met, the high-dose/refuge strategy may result in successful control of susceptible pest populations as well as higher margins of profit to producers due to less pesticide application and improved pest control (Tabashnik *et al.,* 2010). However, non-compliance with either one of the above mentioned assumptions or the occurrence of multiple target pests with varying susceptibilities to the same dose of Bt protein, may lead to a decreased efficacy of the high-dose/refuge strategy (Bates *et al.*, 2005).

The level of Bt protein expression has an effect on the dominance of a target pest population's gene characteristics. Consider three genotypes of which two are homozygous (SS, RR) and one is heterozygous (RS) (Fig. 1.1). At a low-dose of Bt protein expression most of the SS insects are killed, but all of the RS individuals survive (Onstad and Guse, 2008). A high-dose of Bt protein kills all SS and RS insects, but most of the RR individuals survive (Onstad and Guse, 2008). Therefore at low-dosages, resistance genes are dominant, whereas resistance genes are recessive at high dosages of the protein (Onstad and Guse, 2008).

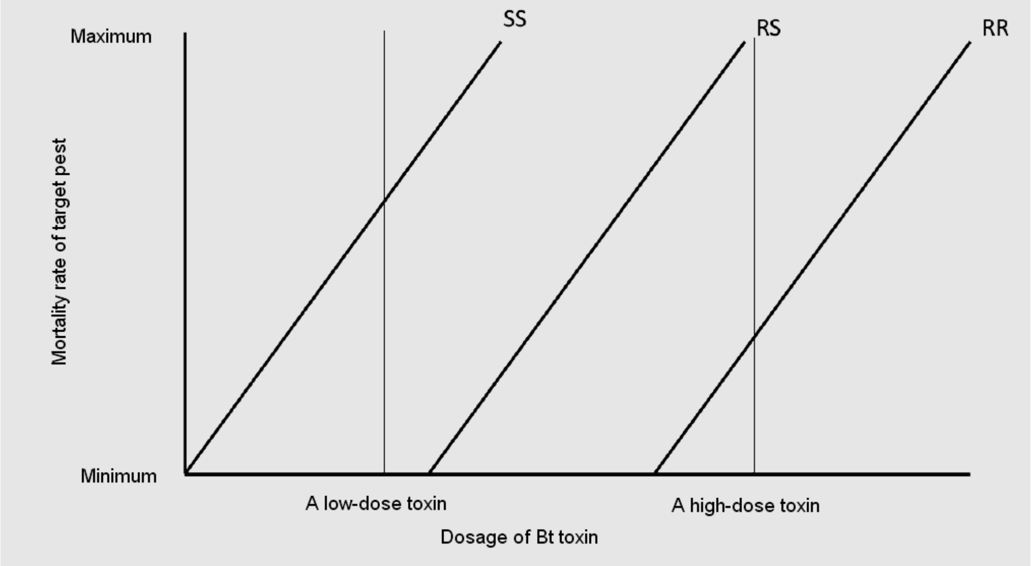

Figure 1.1. The mortality rate of three different genotypes of a pest at various dosages of Bt protein (Onstad and Guse, 2008).

As is the case with any IRM strategy, the various ways in which Bt-proteins affect different target species from different geographical regions needs to be considered. Compliance to refuge requirements is another critical requirement to the success and sustainability of the high-dose/refuge strategy as an IRM tool.

1.2.2 Seed mixtures or "Refuge in a bag" strategy

A more recently developed IRM strategy for Bt crops (specifically maize) has been approved by the EPA in 2010 using a seed mixture concept, also known as "refuge in a bag" (RIB) (Gray, 2010). A single bag of seed contains a predetermined ratio of non-Bt and Bt seed, thereby including a random refuge within the planted area (Onstad *et al*., 2011). A separate non-Bt refuge is therefore not required, simplifying adherence to refuge requirements (Davis and Onstad, 2000).

Benefits linked to the deployment of seed mixtures for IRM includes increased profit from not having to plant separate non-Bt refuges while saving more on chemical control (Davis and Onstad, 2000; Onstad *et al*., 2011). Due to easier use of seed mixture products, producers might even be more willing to plant Bt maize (Onstad *et al*., 2011), making it a clear advantage from a seed manufacturer's point of view. Another benefit of seed mixtures is the emergence of adult insects in close proximity to other suitable adults, thereby enhancing random mating (Davis and Onstad, 2000; Murphy *et al.*, 2010; USEPA, 2011). The use of pyramided gene technology in combination with seed mixtures could enhance its efficacy over time, but also increase purchase costs of RIB products with control mechanisms not necessarily needed for the producer's intended application (Onstad *et al*., 2011).

Movement of larvae between plants within a seed mixture could pose a potential concern regarding resistance evolution. A reduction in numbers of susceptible individuals due to pre-feeding movement from non-Bt to Bt plants could increase the potential for RS larvae to survive and adults to mate, which could in turn lead to an increase in the incidence of homozygous RR individuals in the offspring (Murphy *et al.*, 2010). Development of resistance may also occur when more mature larger larvae move from non-Bt to Bt plants, consequently being exposed to sub-lethal dosages of Bt proteins (Murphy *et al.*, 2010).

Since only first and second instars are controlled by Bt proteins, larvae that developed on non-Bt plants would not be killed when confronted with Bt proteins. This may lead to an increased risk of resistance development over time. A target pest exhibiting more sedentary behaviour might however not be affected in this way. Such insects (for example aphids) would endure longer exposure to Bt proteins throughout its lifecycle (Gould 2000). A scenario where insects move from non-Bt to Bt plants and are killed reduces the number of susceptible adults being generated by the random refuge. This could be compared to a reduction in the practical size of the refuge (Davis and Onstad, 2000).

This implies that feeding and dispersal behaviour of target species may play an important role in the efficacy of seed mixture strategies (Davis and Onstad, 2000). Monitoring of pest status, damage levels and development of resistance may be more difficult to apply when the RIB strategy is used compared to the high-dose/refuge strategy. Identifying the difference between non-Bt and Bt plants in a random refuge poses a difficult and expensive endeavour due to the mixed nature of the planted area.

The ratio of non-Bt to Bt seeds in the RIB strategy may be an important factor regarding long term management of resistance development. Although a 5 % non-Bt mixture may hold yield benefits for the producer over the short term, the selection pressure asserted on the pest populations is increased. A higher ratio of 20 % non-Bt seed could extend resistance development over the long term (Murphy *et al.*, 2010). This is due to an increased number of susceptible adults being generated on non-Bt plants in order to mate with resistant individuals. However, mature larvae migrating between non-Bt and Bt plants could increase exposure to sub-lethal dosages of Bt proteins when high seed ratios is used.

Seed companies currently market two Bt maize products using the RIB concept to control both above and below-ground pests. Both contain a 5 % ratio of non-Bt seeds (Monsanto, 2012), meaning maximum potential yield for the producer due to better pest control. The seed company, Syngenta, received EPA registration for two newly developed RIB products to be commercially introduced during the 2013 growing season (Bloomberg, 2012). RIB products are currently only available in the USA. It would seem that if the RIB strategy is able to provide sufficient control of pest numbers and prolong resistance development, it could be a market success.

There are certain mandatory considerations before seed mixture products may be deployed. A seed mixture product expressing pyramided genes to control both below- and above ground target pests must still be deployed in conjunction with a 20 % separate non-Bt refuge (USEPA, 2011). This is applicable only to regions where cotton is cultivated and significant corn earworm (*Helicoverpa zea*) (Boddie) (Lepidoptera: Noctuidae) infestations occur (USEPA, 2011).

1.2.3 The role of pest behaviour in IRM: feeding and migration between plants

The feeding behaviour and movement of pest insects between plants can determine the efficacy of pest management tactics as well as resistance management strategies (Onstad and Gould, 1998). Movement of pest insects within a seed mixture planting is especially of importance due to the close proximity of non-Bt and Bt plants.

For example, a study conducted by Mallet and Porter (1992) suggested that a seed mixture strategy could actually promote the development of resistance of pests that exhibit large scale movement between plants, even more than a pure Bt field. However, Tabashnik (1994a) suggested that seed mixtures are always better in delaying resistance development over a broad range of conditions compared to a pure Bt setup. Nevertheless it was also concluded that a separate refuge could ensure that the Bt trait lasts as long, if not longer, than in the case of a seed mixture (Tabashnik, 1994a). Pre- and post-migration feeding is also an important consideration, possibly affecting exposure to different levels (or dosages) of Bt proteins (Goldstein *et al*., 2010).

Several studies have been conducted regarding the pest-plant interactions of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). This pest is considered the major pest of maize in the USA, with annual pest management costs reaching US$ 1 billion (Goldstein *et al*., 2010). Dispersal behaviour of *O. nubilalis* larvae have been studied for various types of IRM strategies and could provide insight into the efficacy of the RIB concept. During the first 48 hours after hatching, maximum dispersal of larvae occurs with 50-56 % of individuals attacking the whorl of the host plant and 80-94 % of the larvae remaining within the infested row of plants (Ross and Ostlie, 1990). Ninety per cent of recovered larvae were found on the infested plant as well as the two adjacent plants within the same row (Davis and Onstad, 2000).

The degree to which larvae feed on transgenic plants before dispersal is likely to influence a seed mixture's efficacy. If larvae migrate to a non-Bt host after feeding sufficiently on a primarily infested Bt plant, a poorer survival rate compared to larvae that hatched on non-Bt plants, could result in strong selection pressure (Davis and Onstad, 2000). Dispersal of more mature larvae from non-Bt to Bt plants could also be of concern due to them being potentially exposed to sub-lethal dosages of Bt proteins (Davis and Coleman, 1997). This could increase the risk of resistance development over time when deploying seed mixtures as an IRM strategy (Davis and Onstad, 2000).

A controlled study on dispersal and movement of *O. nubilalis* larvae on Bt and non-Bt maize indicated that larvae were unable to detect Bt proteins in plant tissues during the first 10 minutes after commencement of feeding (Goldstein *et al.,* 2010). Detection of Bt proteins did, however, occur within the first hour, with larvae dispersing from Bt plants earlier than from non-Bt plants (Goldstein *et al.,* 2010). Movement of *O. nubilalis* larvae from Bt to non-Bt plants was identified as a potential source of resistance development and could have adverse implications for a seed mixture strategy (Goldstein *et al*., 2010). During studies conducted by Onstad and Gould (1998) to simulate delayed resistance strategies for *O. nubilalis* populations, results indicated that separate refuges may be more capable in delaying resistance than seed mixtures. Success rate of high-dose expression, pre-dispersal mortality rates and the rate of larval dispersal from Bt plants all affect a seed mixture's ability to delay resistance development (Onstad and Gould, 1998). Onstad and Gould (1998) concluded that a possible increased risk of rapid resistance development may be present when seed mixtures are deployed to manage *O. nubilalis*.

1.3. Deployment of Bt crops as part of insect pest management (IPM)

Integrated pest management can be described as the application of multiple control methods as part of a decision based system in order to manage various pest classes with a minimum of adverse environmental, social and economic consequences (Prokopy and Kogan, 2003). IPM plays a crucial role in advancing the sustainability of the agricultural industry, not only from an economical but also environmental point of view. Bt crop cultivation may lead to reduced application of insecticides and is considered beneficial to the environment due to the specificity of Bt proteins, especially when compared to broad-spectrum insecticides (Kennedy, 2008).

An example is the deployment of Bt cotton as part of IPM strategies in Australia. Since its introduction, cultivation of Bt cotton has led to reduced chemical control (Fitt, 2000). Moving away from pesticide reliance or any single control method is one of the core objectives of IPM. Therefore Bt crops should not be viewed as a single control method only, but as part of an IPM strategy (Fitt, 2000).

1.4. Deployment of GM crops in South Africa

South Africa has taken the lead on the African continent in terms of transgenic crop cultivation. The first transgenic crop cultivated in South Africa was Bt cotton, which was introduced in 1997 (Cooke and Downie, 2010). By 2011 South Africa was the ninth largest producer of GM crops globally, cultivating 2.3 million hectares of transgenic maize, cotton and soybean (James, 2011). Traits expressed by GM crops in South Africa include insecticidal properties (maize, cotton) and herbicide tolerance (maize, cotton and soybean). Bt maize was first deployed in South Africa during the 1998/1999 growing season with use of an event expressing Cry1Ab proteins to manage two Lepidoptera pests, namely *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *B. fusca*. It was originally developed in the USA to manage *O. nubilalis*, the south-western corn borer (*Diatraea grandiosella*) and was also then shown to be effective against the pink borer (*Sesamia cretica*) (Lederer) in Egypt (Monsanto, 2002). Before the introduction of Bt maize in South Africa, studies found that *B. fusca* displayed a higher tolerance to insecticidal proteins from specific Bt events than *C. partellus* (Van Rensburg, 1999).

Field resistance of *B. fusca* to Bt maize expressing Cry1Ab was first reported in the Christiana area during the 2006/2007 growing season (Van Rensburg, 2007), followed by areas in the Vaalharts irrigation scheme (Kruger *et al*., 2009). Resistance development was partly ascribed to non-compliance with refuge requirements as well as increased selection pressure resulting from late planting dates (Kruger *et al*., 2009; Van Rensburg, 2007). Recent information does however show that the assumption of recessive inheritance of resistance by *B. fusca* was not true, and that resistance is inherited in a dominant pattern (Campagne *et al*., 2013). Resistance of *B. fusca* to the Cry1Ab protein has resulted in concern over the future sustainable deployment of Bt maize.

During 2007 second generation Bt maize events utilizing pyramided genes expressing Cry1A.105 and Cry2Ab2 proteins were deployed in the USA (CERA, 2009; USEPA, 2010). It targets Lepidopteran pests and was originally developed to manage *O. nubilalis*, *D. grandiosella*, the southern cornstalk borer (*Diatraea crambidoides*) (Grote), the corn earworm (*H. zea*), the fall armyworm (*S. frugiperda*), the corn stalk borer (*Papaipema nebris*) and the sugarcane borer (*Diatreae saccharalis*) (USEPA, 2010).

Pyramided events expressing Cry1A.105 and Cry2Ab2 proteins was approved for commercial release in South Africa during 2010, being made available to producers for planting during the 2011/12 season to control *C. partellus* as well as resistant *B. fusca* populations (Monsanto, 2010).

1.5. The African stem borer (*Busseola fusca*) and the damage it causes to maize plants

Busseola fusca moths are active at night searching for suitable mates, while being inactive during the day (Harris and Nwanze, 1992). Females lay egg batches (containing between 30 and 100 eggs) behind leaf sheaths of host plants and eggs hatch after approximately three to seven days (Harris and Nwanze, 1992).

After hatching, the larvae migrate to the maize leaf whorl, where it starts to feed on the soft whorl tissue (Seshu Reddy, 1998). When the growth point of a maize plant is destroyed due to feeding by larvae, further development of the plant can be inhibited (Seshu Reddy, 1998). This phenomenon, also known as “dead heart”, may be fatal to the plant or at least cause significant yield loss (Harris and Nwanze, 1992). Dispersal of *B. fusca* larvae from plant whorls seems to occur up to four weeks after egg hatch, after which a decline is observed (Van den Berg and Van Rensburg, 1991). The dispersal of larvae immediately after infestation does not appear to be dependent on the density of larvae within the whorls (Van den Berg and Van Rensburg, 1991). The density of larvae hatching from eggs at the oviposition site could have a greater effect on larval dispersal.

More mature larvae migrate from the whorl to penetrate and feed on stem tissue, creating tunnels as well as cavities to pupate in (Seshu Reddy, 1998) (Fig. 1.2). Severe damage to stems weakens the plant's structural integrity, thereby making the plants more vulnerable to breakage (Harris and Nwanze, 1992). Maize ears can also be damaged by larvae (Harris and Nwanze, 1992), causing reduced quality of yield or, depending on severity of infestation, quantitative yield loss. Maize infested with stem borers may be more susceptible to adverse weather conditions and secondary infections than non-infested crops (Seshu Reddy, 1998).

Figure 1.2. *Busseola fusca* (African stem borer) larva.

Adults emerge from pupae between nine and fourteen days after pupation (Harris and Nwanze, 1992) (Fig. 1.3). The life cycle of *B. fusca*, under optimal conditions, takes approximately seven to eight weeks to complete but may vary with humidity and ambient temperature (Harris and Nwanze, 1992). *Busseola fusca* larvae have the ability to enter diapause in order to survive winter months or dry seasons (Van Rensburg *et al*., 1987a), ensuring a potential source of individuals for the next growing season. When in diapause, larvae in the lower part of the stem (usually just beneath the soil surface) are well protected against external weather conditions as well as natural enemies (Kfir *et al.*, 2002; Van Rensburg *et al*., 1987b). Larvae enter diapause due to dry weather conditions, decreasing nutritional value of host plants (e.g. increase in carbohydrates along with decreased protein and water), decreased ambient temperatures and shorter daylight length (Usua, 1973; Kfir *et al.*, 2002). It would seem that *B. fusca* undergoes facultative diapause (Kfir, 1990). Due to the ability of *B. fusca* to survive adverse periods between growing seasons and having the potential to cause serious economic damage to cereal crops, various control methods have been considered to manage their numbers.

Figure 1.3. *Busseola fusca* moth.

There are various methods that have been developed to control *B. fusca*. Planting date plays an important role in the type and severity of damage caused to maize by *B. fusca*. With emergence of moths from crop residues, commencing during October, this species can have three subsequent flights under optimum conditions (Van Rensburg *et al*., 1985), enhancing damage potential throughout the growing season. Early infestations (first generation) attack immature stages of maize plants, while late first generation as well as second generation infestations damage plants during reproductive growth stages (Van Rensburg *et al*., 1985; Kruger *et al.,* 2012). Pre-flowering plants may be able to sustain more damage compared to their mature counterparts. A study by Van Rensburg *et al.* (1987b) found that moths that deposit eggs three to five weeks after plant emergence form the most important part of the total infestation during the planting season. It was concluded that the degree of infestation decreases towards the middle of November, but increases later on in the planting season (Van Rensburg *et al*., 1987b). By adjusting planting dates in order to avoid the exposure of mature maize plants to periods of high infestation, a reduction in potential yield loss could be achieved.

Tillage as a method to control overwintering *B. fusca* larvae in stem residues, involves burying of infested plant residues in the soil (preventing adult moths escaping from the stem) as well as exposing residues to adverse weather conditions and natural enemies (Van den Berg *et al*., 1998). Although a beneficial and rather inexpensive method to managing pest resurgence, there are certain scenarios where the use of tillage as a control method may be problematic.

Conservation of soil moisture as well as soil erosion, labour and fuel costs are considerations that must be taken into account before tillage is undertaken during winter months (Van den Berg *et al*., 1998). Also, tillage is not recommended as part of conservation agriculture practices (Hobbs *et al*., 2008).

Physical removal of infested plants as is practised with sorghum in Nigeria (Van den Berg *et al*., 1998) can also decrease potential resurgence of *B. fusca* numbers. Removal of maize residues might have the same effect for *B. fusca* control. By removing crop residues, the number of larvae that could give rise to the first-generation moth flight of the next season is reduced. Physical removal of crop residues may be difficult to implement by commercial producers (Van den Berg *et al*., 1998), but works very well when practised in small scale farming.

Another control method known as the “push-pull” system has been implemented by African small-scale farmers to control stem borers attacking their maize and sorghum crops (Khan *et al.,* 2001)*.* The “push-pull” system relies on the simultaneous actions of both repelling and attracting semiochemical stimuli to influence the behaviour of pest insects as well as their natural enemies (Heuskin *et al.,* 2011). The main crop (e.g. maize or sorghum) is intercropped with plants producing repellent semiochemicals, the purpose of which is to mask chemical communication between the main crop and a specific target pest (Hassanali *et al.,* 2008). Alternative host plants (e.g. Napier grass) planted along the perimeter of the field present a more attractive option to the pest species than the main crop. Pest insects are then “pulled” to the alternative host, while natural enemies (predators and parasitoids) are lured by the intercropped plants (Hassanali *et al.,* 2008). These natural enemies then serve as effective biological control agents against remaining pest insects.

Insecticide use for *B. fusca* management may not be as easy to apply as with other pest organisms. Timing and positioning (preferably into the whorls of pre-tassel maize plants) of insecticide applications is crucial (Van den Berg and Van Rensburg, 1996). Once larvae have entered the stems or even maize ears, chemical control becomes a rather impractical method. This doesn’t mean that insecticide application should be discarded as a control method. If applied responsibly and accurately, chemical control can still be used as part of an IPM approach (Harris and Nwanze, 1992).

Biological control agents provide an excellent tool for the management of pest insects (Levins, 2007). However, these natural enemies are usually susceptible to broad spectrum insecticides, thereby greatly reducing their efficacy in managing pest numbers (Van Hamburg and Guest, 1997). Several parasitoid species has been identified that could serve as potential biological control agents for *B. fusca*, but successful establishment after releases has not been made (Harris and Nwanze, 1992).

Many of these control methods have benefits as well as disadvantages. The introduction of Bt maize in South Africa in 1998 proposed an opportunity for easier, more generically applicable stem borer management strategies.

1.6. Objectives of study

Studies on the survival and migration patterns of *B. fusca* in plantings done with mixtures of Bt and non-Bt maize seed have not been done before in South Africa. The objective of the study was therefore to determine whether migrating larvae of *B. fusca* will, over time, be controlled effectively using the seed mixture approach and if there is potential for using this strategy as an IRM strategy for this pest. The need therefore existed to study migration patterns of this pest within maize plantings based on the seed mixture strategy.

The scale of *B. fusca* migration and levels of survival could indicate whether this strategy would be able to control larval damage as well as to evaluate whether the potential deployment of this strategy could be beneficial for managing resistance development of *B. fusca*.

The results of this study are provided in separate chapters with the dissertation as follows:

- Chapter 2 - Migration patterns and survival of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larvae within maize plantings containing different ratios of non-Bt and Bt seed.
- Chapter 3 - Survival of *Busseola fusca* larvae migrating between Bt and non-Bt maize plants: mimicking a seed mixture scenario.
- Chapter 4 - Damage caused by migrating *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larvae, to plants of single- and pyramided gene Bt maize events.

- Chapter 5 - Comparisons between the high-dose/refuge and seed mixture strategies - a review
- Chapter 6 - Conclusions and recommendations

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Chapter 2 - Migration patterns and survival of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larvae within maize plantings containing different ratios of non-Bt and Bt seed

2.1. Abstract

The high-dose/refuge IRM strategy is used globally to manage resistance development in Bt crops, since their first deployment in the 1990's. The "refuge in a bag" (RIB) or seed mixture strategy is also being considered for a number of pest species, especially after several cases of resistance development have been reported. *Busseola fusca* is the primary target stem borer in South Africa and have evolved resistance to Bt maize expressing Cry1Ab proteins. The objective of this study was to determine whether migrating *B. fusca* larvae will be effectively controlled using the RIB strategy. A field study with a single gene event (Cry1Ab) and a "pyramided" gene event (Cry1A.105 + Cry2Ab2) was conducted in which the migration pattern of *B. fusca* larvae inside different treatments was studied. The experiment consisted of five seed mixture ratios (5%, 10%, 15%, 20% non-Bt seed and non-Bt as control), each replicated four to six times. Two field trials (two seasons) were conducted where plots of maize (7 x 5 m) was planted using the mentioned seed mixture ratios. Natural infestation was augmented by artificial inoculation with neonate larvae into the central non-Bt maize plant of each plot. Rate of larval survival and migration, measured in terms of the increase in number of plants that exhibited borer damage over time was recorded at weekly intervals until flowering. The rate of survival and migration was significantly higher in the plots with maize expressing Cry1Ab proteins and control plots than in plots with the pyramid Bt event (Cry1A.105 + Cry2Ab2). Although damage to plants in the Bt treatments did not differ among themselves within each Bt event, the damage was significantly different from the control treatments. Since the increase in number of damaged plants over time is associated with migration of older and larger larvae, the observed tendencies may indicate that the assumed high-dose does not kill larvae above a certain developmental stage. Before the seed mixture strategy is deployed on large scale, its efficacy compared to the high-dose/refuge must be considered.

2.2. Introduction

The use of transgenic crops with insecticidal properties may be considered a significant step forward in pest management. These transgenic crops are genetically modified to express *Bacillus thuringiensis* (Bt) proteins that are able to control specific target insects (Haung *et al*., 2011). Bt *Cry* proteins cause perforations in the mid-gut of target insects, thereby having a lethal effect (Gray, 2010). One of the most important benefits linked to Bt crop cultivation is a potential reduction in insecticide use (Fitt, 2000). Although first-generation Bt crops each produce a single Bt toxin, some second-generation Bt crops produce two distinct Bt toxins that are active against the same pest (Tabashnik *et al*. 2009).

As with any pesticide, the development of resistance of target pests to Bt proteins is a concern. Insect resistance management (IRM) is therefore crucial in delaying resistance development and ensures the sustainability of Bt crop cultivation (Gould, 2000). Several IRM strategies are deployed as part of Bt crop cultivation. The high-dose/refuge strategy is currently the default choice in IRM deployment. This strategy relies on a crop expressing a high dosage of Bt proteins in order to kill as many of the target pest population as possible (Goldstein *et al.,* 2010). Along with this high-dose expression, a separate non-Bt refuge of predetermined size must be planted near the Bt crop (Gould, 2000). The non-Bt refuge serves as a source of susceptible adults in order to mate with the few resistant adults that survive on the Bt crop (Gould, 2000). This susceptible offspring is then controlled by the high-dose of Bt protein expressed in the crop. Refuge compliance has been identified as a possible weakness of the high-dose/refuge strategy (Kruger *et al.,* 2009). If refuge requirements are not adhered to, the risk of resistance development of target species may be elevated.

Another IRM strategy is the use of seed mixtures or ''refuge in a bag'' (RIB) strategy. In this case, a single bag of seed contains a predetermined ratio of non-Bt and Bt seed, thereby eliminating the need to plant separate non-Bt refuges (Onstad *et al*., 2011). A random refuge is therefore included within the cultivated area (Onstad *et al*., 2011). The RIB concept greatly simplifies adherence to refuge requirements, with producers not needing to take responsibility for planting of a separate refuge (Davis and Onstad, 2000). Although seed mixture products are very practical from a planting point of view, there is concern regarding its efficacy in managing resistance development over time (Mallet and Porter, 1992).

The feeding behaviour and migration of target pest insects between plants play an important role in determining the effectiveness of seed mixtures (Davis and Onstad, 2000). Larger larvae that move from non-Bt to Bt plants could be exposed to sub-lethal dosages of Bt proteins, leading to an increased risk of resistance development (Murphy *et al*., 2010). Only first and second instars are successful controlled, with third instars indicating the most migration patterns. A target pest exhibiting reduced migration behaviour between plants would probably not be affected in this way. Apart from the concern about the ability of a seed mixture strategy to delay resistance development, some studies suggest that deploying this strategy is still better than using no refuge at all (Carroll *et al*., 2012).

Studies on the survival and migration patterns of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) within a field planted to a mixture of Bt and non-Bt seed has not been done before. Suitable IRM strategies for managing *B. fusca* resistance development in Africa is needed since this pest was one of the first in the world to develop resistance against the Cry1Ab protein expressed in Bt maize (Van Rensburg, 2007). The objectives of the study were therefore to determine to what extent migrating *B. fusca* larvae are effectively controlled by Bt plants, using the RIB approach, and if there is potential for using this strategy to manage resistance development by *B. fusca*.

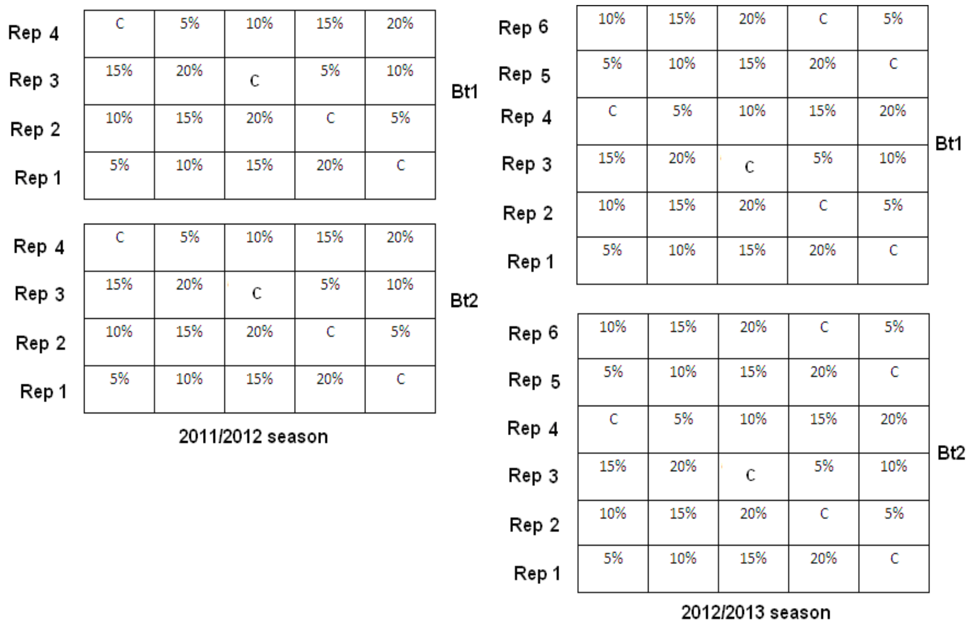
2.3. Materials and methods

A field study was conducted over two consecutive growing seasons (2011/12 and 2012/13), at the Agricultural Research Council - Grain Crops Institute, Potchefstroom, North-West province, South Africa (-26°43'57.46"; +27°4'44.98"). Two Bt maize events commercialised in South Africa were evaluated. These were the single gene (Cry1Ab) event (further referred to as Bt1) and a recently commercialised "pyramid" event (Cry1A.105+Cry2Ab2) (further referred to as Bt2).

2.3.1. Experiment conducted in the 2011/12 growing season

The experimental layout consisted of two main blocks, each with five treatments planted to different ratios of Bt and non-Bt seeds (Fig. 2.1). The two main blocks consisted of the Bt1 and Bt2 events respectively.

The five treatments (plots) in each block were 5 %, 10 %, 15 % and 20 % non-Bt to Bt seed ratios with the control plots containing non-Bt seed only. Each treatment was replicated four times and each plot consisted of a 7 x 5 m area with 5 rows planted with maize seeds at an inter-row spacing of 0.9 m and an intra-row spacing of 0.17 m.



2011/2012 season

Bt1

Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

Bt2

Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

2012/2013 season

Bt1

Rep 6	10%	15%	20%	C	5%
Rep 5	5%	10%	15%	20%	C
Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

Bt2

Rep 6	10%	15%	20%	C	5%
Rep 5	5%	10%	15%	20%	C
Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

Figure 2.1. Schematic diagram of the Bt1 and Bt2 blocks for both growing seasons.

A single non-Bt plant, in the middle row of each treatment, was inoculated with 50 neonate *B. fusca* larvae (Fig. 2.2). The inoculation size of 50 larvae was used to stimulate larval migration behaviour. The larvae used to inoculate plants with were from a Bt-resistant *B. fusca* population collected at Ventersdorp in the North-West province. This population was previously shown to survive on Bt maize, although not at very high levels. Natural infestation also occurred in this experiment. The level of natural infestation was determined on a block of non-Bt maize adjacent to the experiment, six weeks after seedling emergence. Three days after inoculation, the monitoring of visible damage ("pinholes and windows") to furl leaves commenced. These observations were recorded at weekly intervals and continued for 10 weeks, until anthesis. The position of each damaged plant was also noted on map created of each treatment plot (40 plots in total), each with between 120 and 140 plants.

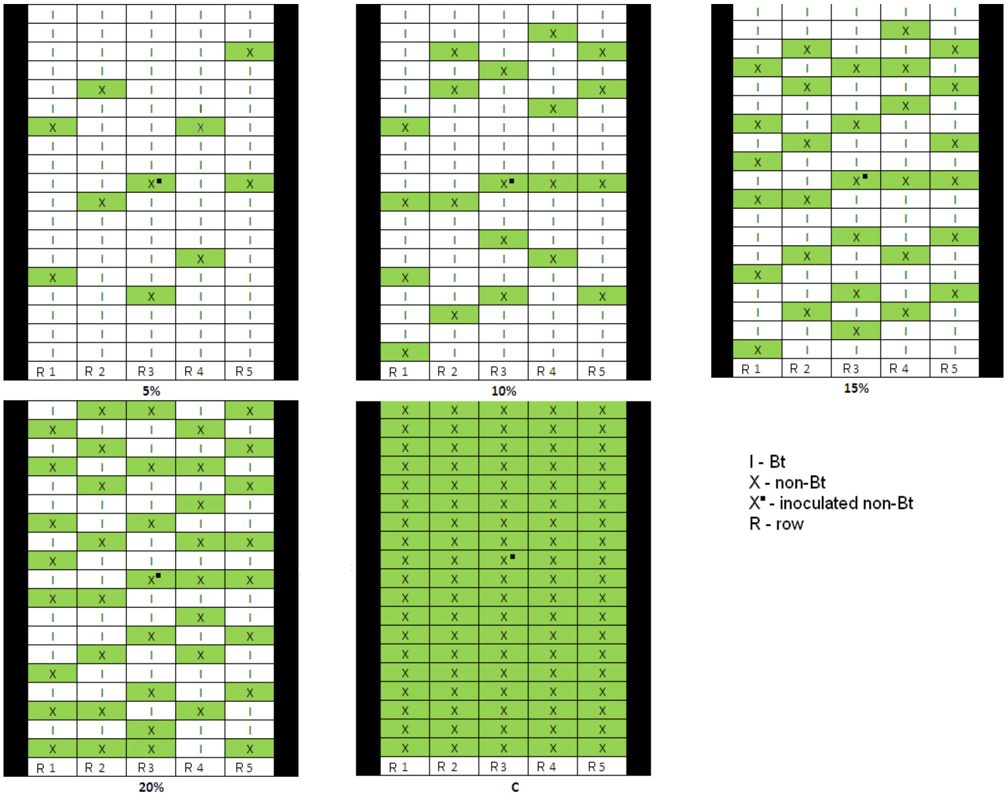


Figure 2.2. Example of the layout of the five treatments within the plots (replicate 1 for both growing seasons). Note that the drawing is not to scale.

In order to assess larval development over time without having to do destructive sampling in the experiment, a reference plot (7 x 30 m) containing 150 non-Bt plants was planted. Plants in this plot were inoculated with 10 neonate *B. fusca* larvae each. Fourteen maize plants were randomly selected from this plot and dissected on a weekly basis to recover larvae. The number of surviving larvae and larval mass per plant was then recorded.

2.3.2. Experiment conducted in the 2012/13 growing season

The above mentioned study, with a few adaptations, was repeated during the 2012/13 growing season. For the 2012/13 growing season, two additional replicates were added, increasing the number of replicates to six (Fig. 2.1). Again both Bt1 and Bt2 were used. A second adaptation was the calculations of the ratios of non-Bt to Bt seed mixtures in the treatment blocks. Since seedling emergence was not 100 %, the exact ratio of non-Bt plants to Bt plants that emerged was calculated for each plot.

The number of plants per treatment plot, however, remained high (between 160-180) due to a higher seeding rate that was used during this season. As with the previous growing season, a reference plot with non-Bt plants were used to monitor larval growth and survival during the study period.

2.4. Data analysis

The mean percentage of plants exhibiting stem borer damage was calculated at weekly intervals in each treatment plot. Larval mass and survival on plants in the reference plots were also determined and graphically presented with the inclusion of standard errors (SE). Data for the mean percentage of plants exhibiting damage over time was statistically analysed using the Table Curve 2D Version 5.01 program. This was done in order to generate sigmoid curves describing the increase in incidence of damage per plot over time. Mean incidence of damaged plants (%) in the different treatments was compared using Tukey tests obtained from multifactor ANOVA's. These calculations were done using Statistica version 11 (StatSoft Inc, 2013).

2.5. Results

The natural infestation during the first season was approximately 5 % and during the second season, 3 %.

2.5.1. Experiment conducted in the 2011/12 growing season

2.5.1.1. Migration patterns of *B. fusca* larvae as a function of observed plant damage:

Sigmoid curves were generated for all the treatments for both the Bt maize events (Bt1 and Bt2) (Fig. 2.3 A and B). The mean percentage stem borer damaged plants for the different treatments in Bt1 was relatively similar. The incidence of damaged plants (%) was approximately the same for the 15 % and 20 % as well as for the 5 % and 10 % treatments, respectively. The incidence of damaged plants in the seed-mixture treatments were similar to that in the control treatment, with steeper slopes observed compared to treatments of Bt2. The percentage stem borer damage in the control treatment for Bt2 was significantly higher than that of the different seed mixture treatments ($F_{(4,15)}$ = 20.645; P = 0.00001). A steeper slope and shorter time period was observed before the increase in damaged plants levelled off in the plots with only non-Bt maize, compared to the treatments with Bt2.

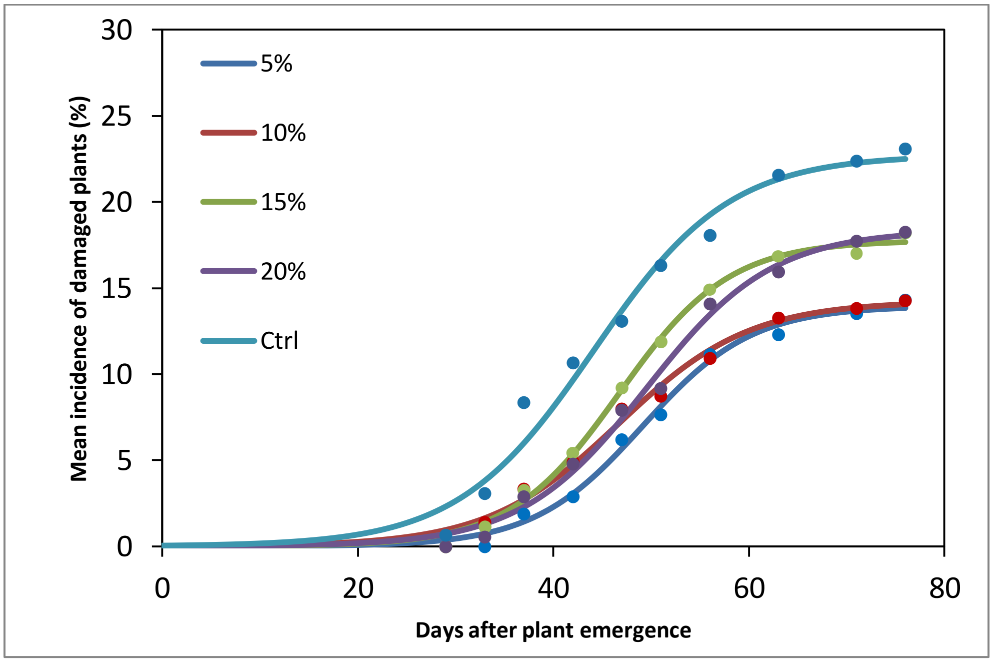


Figure 2.3. (**A**) Mean incidence of damaged plants at different times after infestation by *Busseola fusca* of maize plants in plots planted to different ratios of Bt1 and non-Bt maize seed (2011/12 growing season).

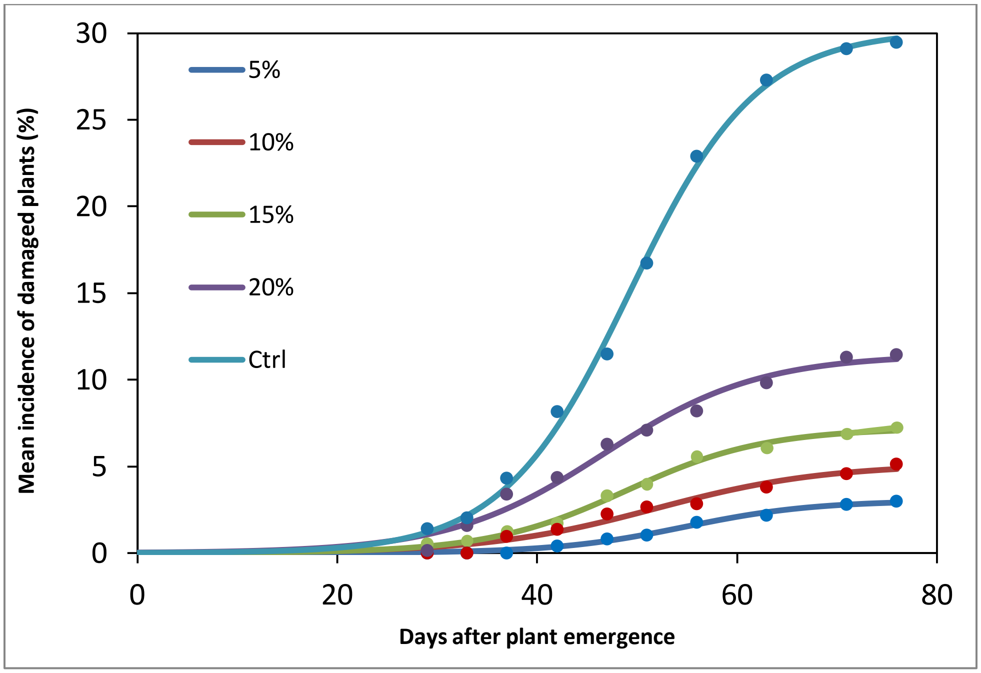


Figure 2.3. (**B**) Mean incidence of damaged plants at different times after infestation by *Busseola fusca* of maize plants in plots planted to different ratios of Bt2 and non-Bt maize seed (2011/12 growing season).

2.5.1.2. Mean damage (%) over time on Bt1 and Bt2 blocks

The mean incidence of damaged plants did not differ significantly between any of the four seed mixture treatments and non-Bt control treatments in the block planted with Bt1 seed ($F_{(4,15)}$ = 1.6242; P = 0.21979) (Fig. 2.4). Percentage damage in all seed mixture treatments except for the 20 % ratio, were higher than the proportion of non-Bt seeds present in the respective treatments. Mean damage for all the seed mixture treatments in the Bt2 block did not differ significantly but were significantly higher in the control treatment ($F_{(4,15)}$ = 20.645; P = 0.00001). Percentage damage in none of the seed mixture treatments exceeded that of the proportion of non-Bt plants present in the treatment.

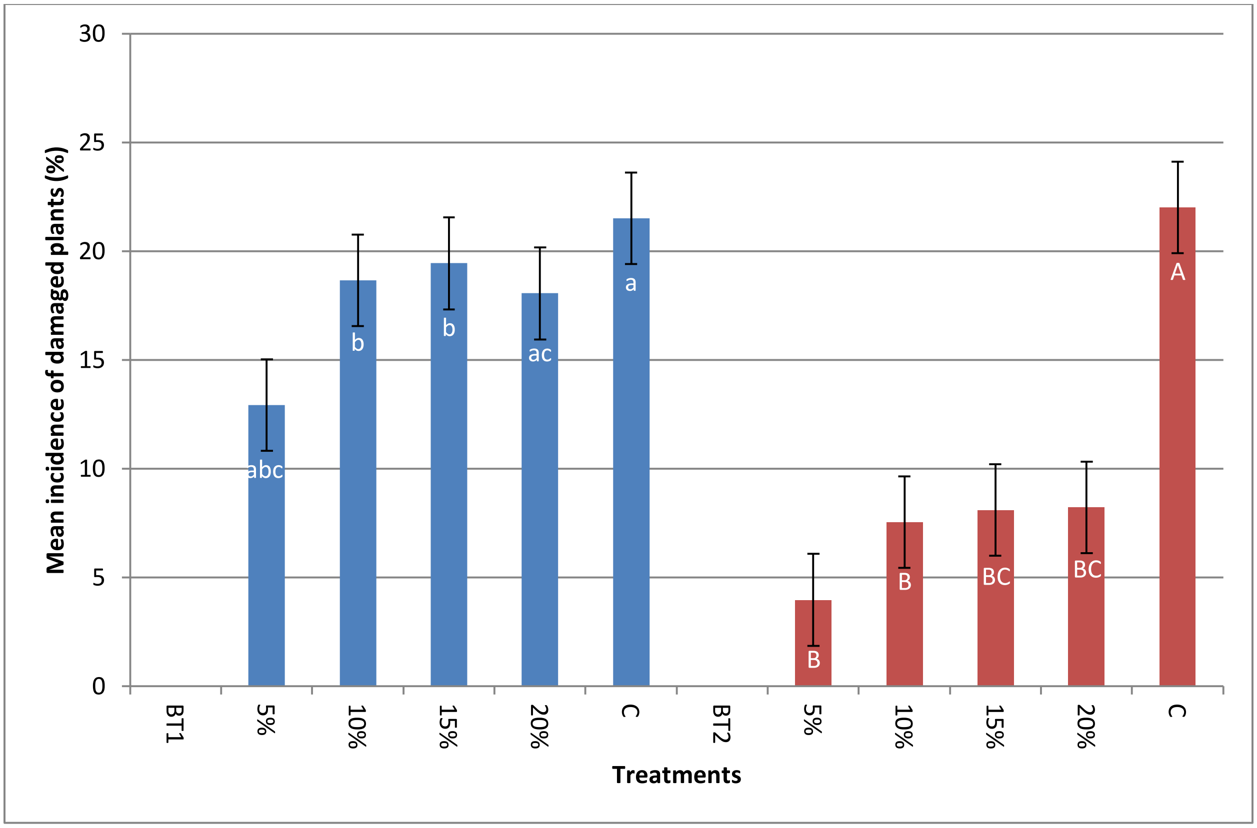


Figure 2.4. Mean incidence of damaged plants (%) caused by *Busseola fusca*, to Bt1 and Bt2 maize plants 11 weeks after plant emergence (2011/12 growing season). Bars with the same letters do not differ significantly for incidence of damaged plants (%). Blue bars indicate Bt1 and red bars Bt2. Bars shown are standard errors (SE).

Significant differences in the mean incidence of damaged plants in the treatments between the two Bt events were observed ($F_{(4,30)} = 2.8209$; $P = 0.04243$). Mean damage in all the treatments except the control was significantly higher in the Bt1 treatments when compared to Bt2.

No significant difference in mean damage for the two control plots was observed between the two events. The mean incidence of damaged plants reached 18.11 % for Bt1 and 9.96 % for Bt2, 11 weeks after seedling emergence.

2.5.1.3. Larval survival and mass in the reference plot

The mean percentage larvae recovered decreased during the time period 27 to 55 days after plant emergence and is presented in Figure 2.5. A rapid decline was observed in larval survival per plant during the first six days with an increase in the mean larval mass over time. Pre-pupae started to form at 35 days after inoculation (data not shown).

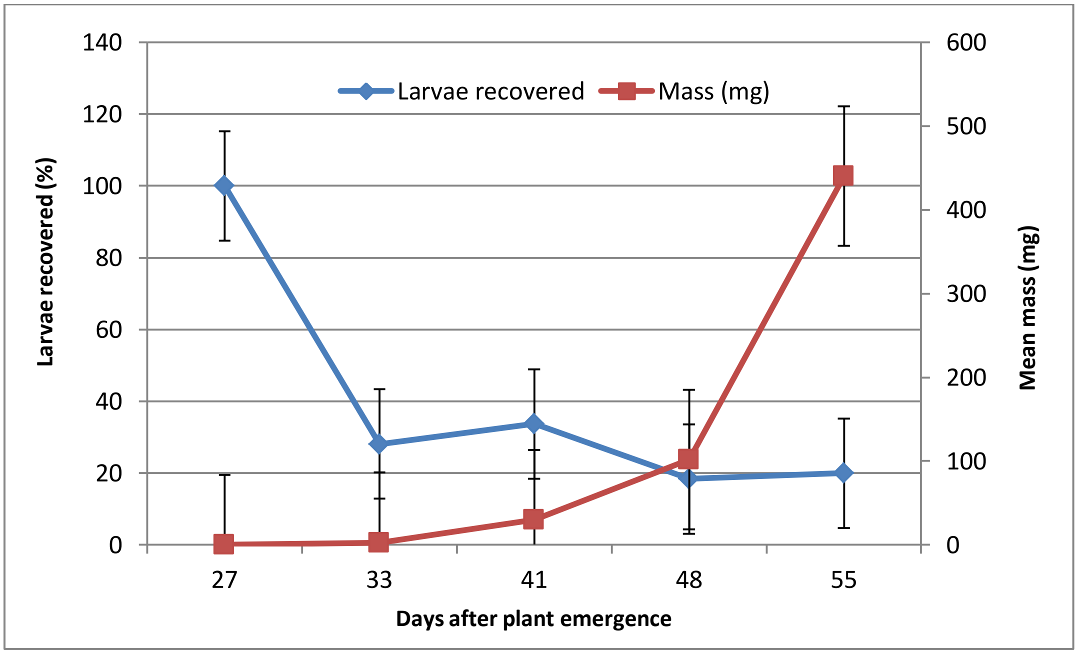


Figure 2.5. The mean percentage larvae recovered per plant from the reference plot as well as mean larval mass over time (2011/12 growing season). Bars represent standard errors (SE).

2.5.2. Experiment conducted in the 2011/13 growing season

2.5.2.1 Migration patterns of *B. fusca* larvae as a function of observed plant damage

Sigmoid curves were generated for all the treatments as well as the control plots for both Bt1 and Bt2 (Fig. 2.6 A and B).

The mean percentage stem borer damage between the different treatments in the Bt1 block was similar. The incidence of damaged plants (%) was highest for the 5 % treatment and lowest for the 10 % treatment. The 15 % and 20 % treatments indicated incidence of damaged plants (%) intermediate to the 5 % and 10 % ratios. The incidence of damaged plants in treatment plots in the Bt1 block were also similar to the control treatment, generating steeper slopes when compared to treatments in Bt2. Incidence of damaged plants (%) in the control treatment increased more rapidly over time compared to the treatments in Bt2.

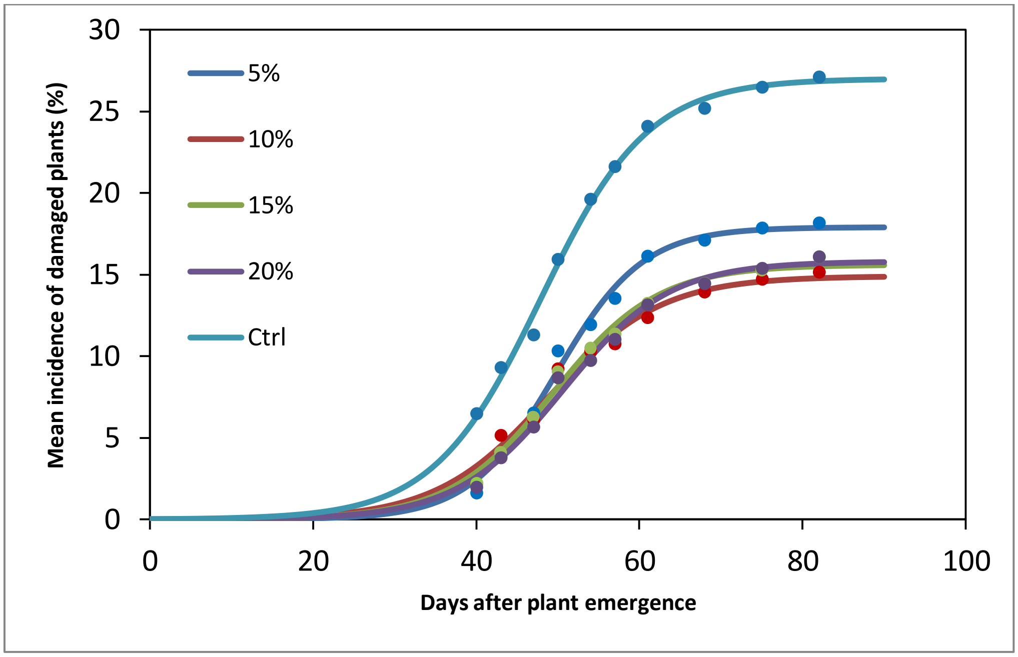


Figure 2.6. (**A**) Mean incidence of damaged plants at different times after infestation by *Busseola fusca* of maize plants in plots planted to different ratios of Bt1 and non-Bt maize seed (2012/13 growing season).

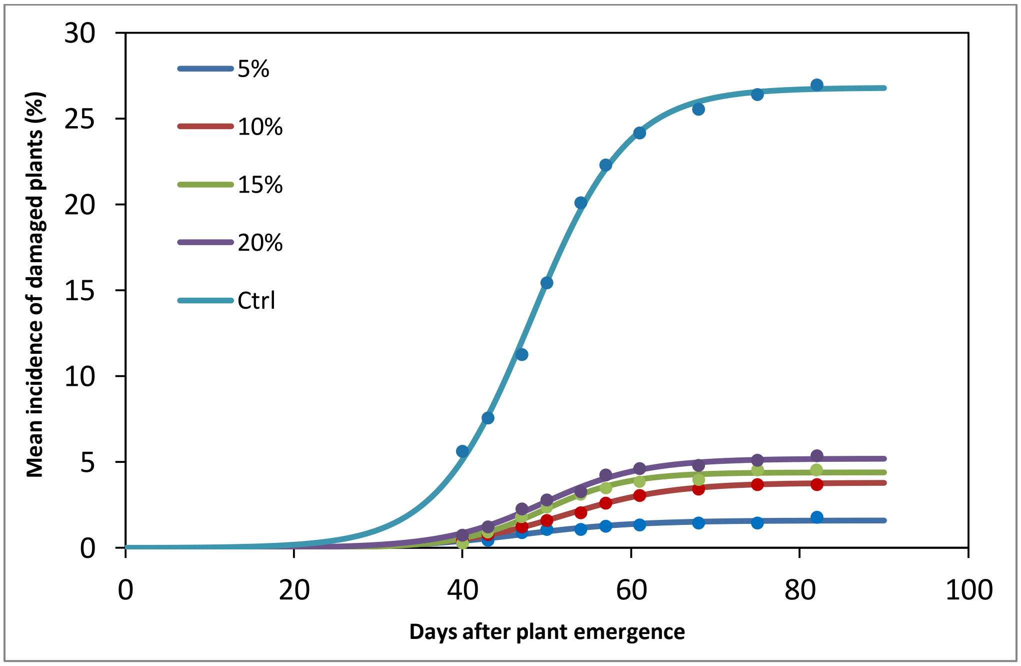


Figure 2.6. (**B**) Mean incidence of damaged plants at different times after infestation by *Busseola fusca* of maize plant sin plots planted to different ratios of Bt2 and non-Bt maize seed (2012/13 growing season).

2.5.2.2 Mean damage (%) of the five treatments between Bt1 and Bt2

Mean damage (%) for seed mixture treatments with Bt1 did not differ significantly (Fig. 2.7). However, the damage in the seed mixture treatments with Bt1 differed significantly from the control ($F_{(4,25)}$ = 7.6560; P = 0.00036). As with the previous growing season the percentage damage in all Bt1 treatments were higher than the proportion of non-Bt plants present in each treatment, except for the 20 % treatment.

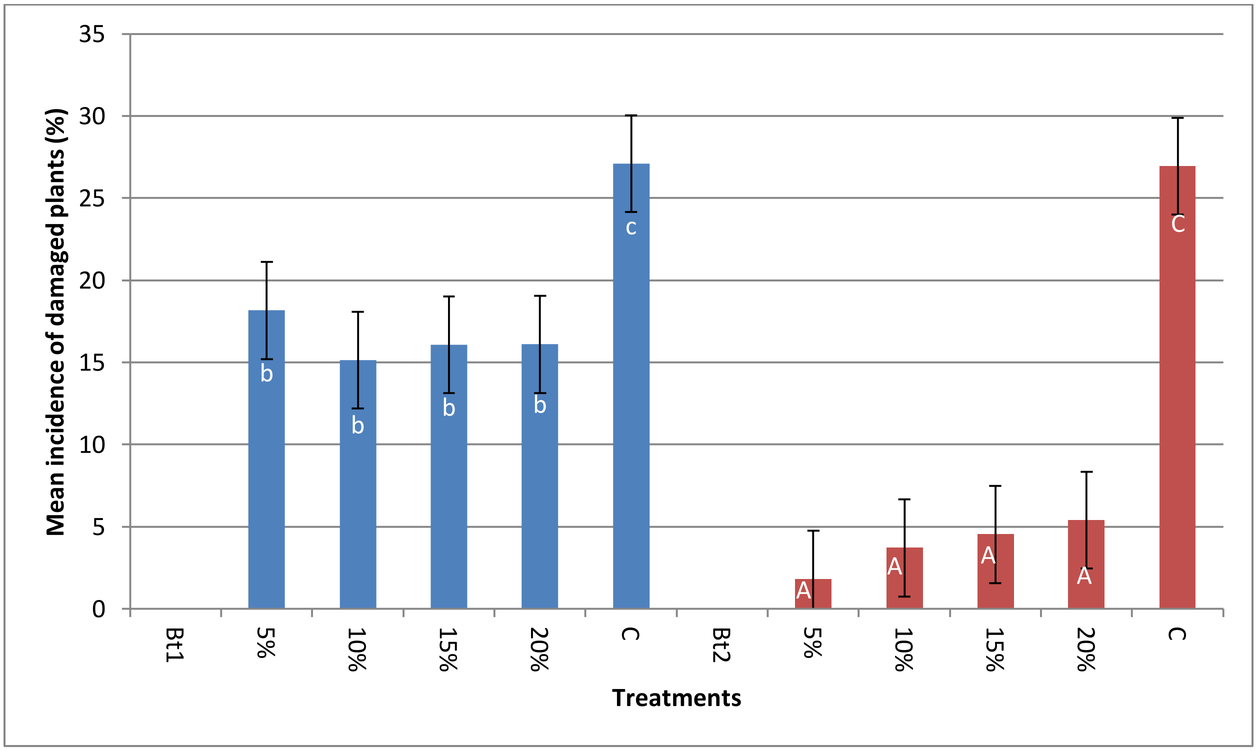


Figure 2.7 Mean incidence of damaged plants (%) caused by *Busseola fusca*, to Bt1 and Bt2 maize plants 11 weeks after plant emergence (2012/13 growing season). Bars with the same letters do not differ significantly for incidence of damaged plants (%). Blue bars indicate Bt1 and red bars Bt2. Bars shown are standard errors (SE)

Mean damage for all the seed mixture treatments in the Bt2 block differed significantly from the control treatments ($F_{(4,25)}$ = 90.661; P = 0.00000). In none of the seed mixture treatments within Bt2 block did the incidence of damaged plants exceed the proportion of non-Bt plants in the treatment. A significant difference in mean damage (%) between the various Bt1 and Bt2 treatments was obtained ($F_{(4,50)}$ = 8.1426 ; P = 0.00004) 11 weeks after seedling emergence, with the mean incidence of damaged plants being 18.51 % for Bt1 and 8.48 % for Bt2.

2.5.2.3. Larval survival and mass on the reference plot

The mean percentage larvae recovered decreased rapidly between the time interval 35 to 42 days after plant emergence (Fig. 2.8). After 49 days after plant emergence, a more gradual decrease in larval recovery was observed. Mean mass of recovered larvae increased up to 63 days after plant emergence.

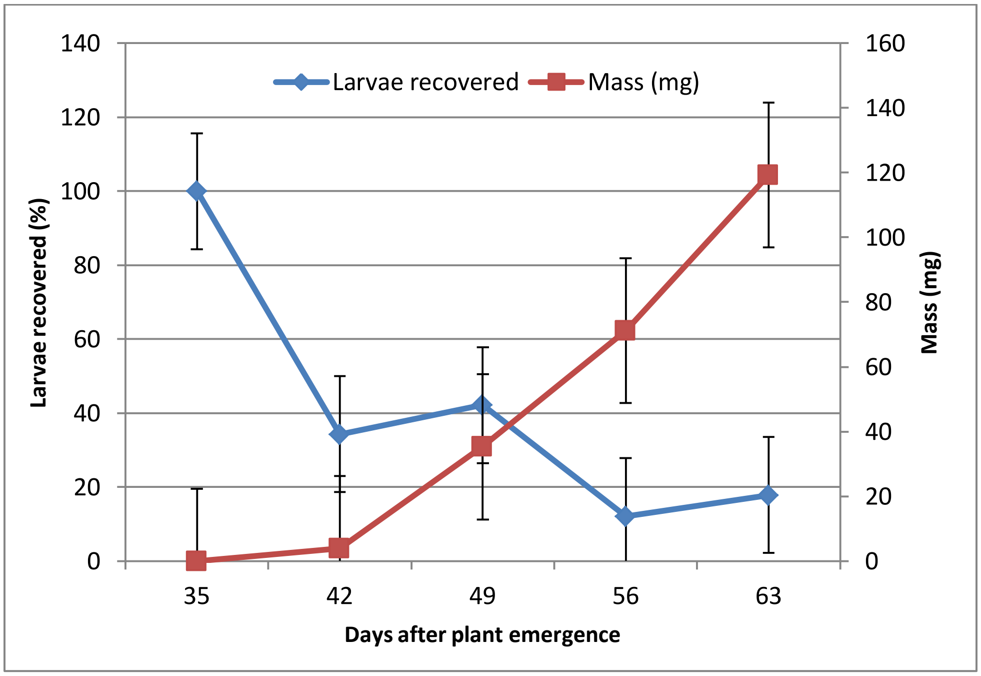


Figure 2.8. The mean percentage larvae recovered per plant from the reference plot as well as mean larval mass over time (2012/13 growing season). Bars represent standard errors (SE).

2.6. Discussion

Results obtained during both seasons indicated large scale larval migration within all the treatments. This was especially evident in control plots and treatments in which Bt1 was used. The increased incidence of damaged Bt1 plants and control plots was to be expected considering *B. fusca* resistance to Cry1Ab protein (Van Rensburg, 2007) and control plots contained only non-Bt plants.

The proportion of stem borer damaged plants within all the treatments of Bt1, except for the 20 % seed mixture, exceeded the proportion of non-Bt seed. This indicated that several Bt plants were also damaged suggesting significant larval migration throughout the plots. The high level of resistance of *B. fusca* to the Cry1Ab protein is evident from the fact that the incidence of damaged plants was similar in all treatments, including the control. Seed mixture treatments indicated a high degree of larval migration onto Bt1 plants. Stem borer damage did, however, differ significantly between Bt1 and Bt2 treatments, with damage in plots expressing Cry1Ab proteins being higher. The Bt1 event was therefore not able to control migrating larvae as was the case with Bt2 plants.

Results from the Bt2 event showed that it was more effective in controlling migrating larvae. None of the treatments exhibited a higher incidence of damaged plants than the proportion of non-Bt plants that were present inside the respective plots. No significant differences in stem borer damage between the various treatments existed, but significant differences in damage compared to the control treatments did occur. This again would suggest enhanced efficacy of the Bt2 in controlling migrating larvae. The 5 % treatments exhibited the least amount of stem borer damage compared to other treatments and could therefore be the most effective to control migrating larvae. Most damage and therefore most larval migration (within the seed mixture treatments) was found in the 20 % treatment, which is to be expected considering it had the highest proportion of non-Bt plants.

A possible explanation for the sigmoid shape of the migration figures could be intra-specific competition between larvae. During approximately the first 30 days after plant emergence, smaller larvae induce minimal competition (for resources) on each other. However, as larvae grow and the need for food resources increases, movement to surrounding plants occurred. Post-tassel migration also increased due to larvae being forced out of the reducing maize whorl. This is represented by the maximum slope of the curves. Maximum distribution of larvae within the field as well as the forming of pupae instigates a minimum intra-specific competition level, thereby possibly explaining the plateau being reached.

The decrease in mean percentage larvae recovered from non-Bt plants in the reference plot could be ascribed to larval mortality during migration to other plants as well as to predation or parasitism by natural enemies. The increase in mean mass of larvae recovered from dissected plants in the reference plot suggests growth on non-Bt plants which could influence the efficacy of Bt proteins, should larvae migrate to Bt plants. This could lead to exposure to sub-lethal dosages of Bt protein, thereby increasing potential risk of resistance development over time.

2.7. Conclusion

The level of larval migration will depend on whether initial infestation occurs on non-Bt or Bt plants. Initial infestation on a non-Bt plant would benefit larval growth, which could in turn increase the risk of exposure to sub-lethal dosages of Bt proteins if the larvae were to migrate to a Bt plant.

Initial infestation on Bt plants could control pre-migrating larvae due to high dosages of Bt toxin, but the period of time a larva spends feeding on a Bt plant before migrating to a non-Bt host could reduce the efficacy of the Bt proteins (Davis and Onstad, 2000). Five percent seed mixtures seems to be the most efficient in managing damage caused by migrating larvae, while mixtures with non-Bt seed at higher ratios could benefit larval survival and movement. A 5 % mixture could, however, increase the selection pressure of Bt proteins on larvae, while potentially exposing a greater number of resistant adults to each other during the mate-finding and mating process. Although the 5 % ratio provided the best control of migrating larvae, fewer susceptible adults may be generated by the random refuge of this size. If there are no statistically significant differences in incidence of plant damage between 5 % and 20 % seed mixtures, then a 20 % ratio might be the better option to reduce selection pressure and generate more susceptible adults. The disadvantage of increased larval survival in 20 % treatments could be offset by more susceptible adults in order to mate with resistant individuals (Davis and Onstad, 2000; Murphy *et al.,* 2010). A concern, however, with a 20 % seed mixture is the potential exposure of migrating larvae to sub-lethal dosages of Bt proteins. The large scale migration of *B. fusca* would suggest that the RIB strategy might not be an appropriate method in order to manage resistance development. A RIB strategy could be a more fitting method in managing pest insects having reduced migration patterns.

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Chapter 3 - Survival of *Busseola fusca* larvae migrating between Bt and non-Bt maize plants: mimicking a seed mixture scenario

3.1. Abstract

The development of resistance of target pest insects to insecticidal proteins expressed by Bt crops is a concern. A recently deployed insect resistance management (IRM) strategy is based on the seed mixture concept also known as "refuge in a bag" (RIB). Non-Bt and Bt seed are mixed in a predetermined ratio within a single bag in order to ensure a random refuge is included within the field. A possible disadvantage with deployment of the RIB strategy is migration of pest insects between non-Bt and Bt plants. This could lead to exposure to sub-lethal dosages of Bt proteins. The objective of the study was to determine the efficacy of Bt maize to control *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larvae of different growth stages that migrate from non-Bt to Bt plants. A laboratory study was conducted in which Cry1Ab-resistant *B. fusca* larvae were reared on various treatments of maize (Non-Bt, Cry1Ab and Cry1A.105 + Cry2Ab2) in order to reach predetermined developmental life stages (three, nine and twenty one days of age). The study involved the feeding of *B. fusca* larvae on maize whorls as well as maize ears. At each larval developmental stage, five larvae were removed from rearing containers where they were reared on the various treatments of maize, weighed and placed on various feeding treatments (also non-Bt, Cry1Ab, Cry1A.105 + Cry2Ab2). This was replicated at least ten times depending on larval availability. Larvae were then allowed to feed for seven days, after which larval survival and mass were determined. Results indicated that older larvae were able to survive when moved from non-Bt to Bt maize. The "pyramid" gene event was more effective in controlling larvae than the single gene event, especially very young larvae. Results indicated that the chance of survival of larvae migrating from Bt to non-Bt plants increased as larvae increased in age.

3.2. Introduction

Use of transgenic crops expressing insecticidal proteins is a valuable tool in insect pest management. However, as with any pesticide, the development of resistance of target pest insects to proteins expressed by Bt crops is a concern (Cohen *et al*., 2000). In order to prolong the development of resistance, insect resistance management (IRM) is needed (Gould, 2000).

The default IRM strategy deployed globally at the moment is the high-dose/refuge strategy. High-dose Bt expression events control most of the target pest insects with only a few resistant individuals surviving (Goldstein *et al.,* 2010). A separate refuge of non-Bt plants must be planted alongside the Bt crop in order to ensure a supply of susceptible adults (Gould, 2000). Mating between susceptible and resistant adults dilutes the homozygous resistance genes which can then be managed by the high-dose Bt expression (Gould, 2000; Kruger *et al*., 2009). A possible weakness of the high-dose/refuge strategy has been identified with regard to refuge compliance by producers. An example is the development of resistance of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) to the Cry1Ab protein in parts of South Africa (Van Rensburg, 2007). Non-compliance to refuge requirements most likely contributed significantly to resistance development of this pest (Kruger *et al*., 2009).

A solution to non-compliance to refuge requirements could be the deployment of seed mixture refuges as part of IRM programmes. A seed mixture, also known as “refuge in a bag” (RIB), contains a predetermined ratio of non-Bt to Bt seed and a random refuge is therefore included within the field (Onstad *et al*., 2011). A benefit of the latter strategy over the high-dose/refuge strategy is that refuge requirements are automatically adhered to when a seed mixture product is used (Davis and Onstad, 2000). However, due to the random arrangement of non-Bt and Bt plants resistance monitoring in a RIB strategy may be exceedingly more difficult than in a high-dose/refuge strategy.

The migration patterns of a target pest species have been suggested as a possible weakness to a seed mixture’s ability in delaying resistance development. The concern is that larvae that develop on non-Bt plants could move to Bt plants, thereby possibly being exposed to sub-lethal dosages of Bt proteins (Gould, 2000; Murphy *et al.*, 2010).

The scenario could be repeated if neonate larvae migrate from Bt to non-Bt plants before sufficient dosages of Bt proteins are ingested. In both cases the risk of resistance development is enhanced. The feeding and migration behaviour between plants of target pest species is therefore an important consideration (Davis and Onstad, 2000). Studies have suggested that a seed mixture strategy could benefit the development of resistance of pest insects that exhibit large scale movement between plants, even more than a pure Bt field (Mallet and Porter, 1992). However, some studies suggest that a seed mixture is still better than having no refuge at all but that a separate refuge is generally better in prolonging resistance development (Tabashnik, 1994; Carrol *et al.*, 2012). Pre- and post-migration feeding is also an important consideration, possibly affecting exposure to different levels (or dosages) of Bt proteins (Goldstein *et al.,* 2010).

Studies conducted with the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) indicated that the deployment of a separate refuge could provide enhanced management of resistance development than seed mixtures (Onstad and Gould, 1998). Factors including the high dosage of Bt expression, pre-dispersal mortality rates and the level of larval dispersal from Bt plants could affect a seed mixture’s efficacy as an IRM strategy (Onstad and Gould, 1998).

The objective of the study was to determine the efficacy of Bt maize to control *B. fusca* larvae when migration between non-Bt and Bt plants occurs, during various larval growth stages. If growth and survival of larvae occur it could give an indication of the possible efficacy of seed mixture refuges as an IRM tool.

3.3. Material and methods

A laboratory study was conducted at the Agricultural Research Council - Grain Crops Institute in Potchefstroom, South Africa (-26^{o}43’57.46”; +27^{o}4’44.98”). The study involved inoculation of maize whorls and ears with *B*. *fusca* larvae. Movement of larvae was simulated between two commercialised Bt maize events. These events were: a single gene (Cry1Ab) (further referred to as Bt1) and a “pyramid” gene (Cry1A.105+Cry2Ab2) (further referred to as Bt2). Bt-resistant *B. fusca* larvae collected from the Ventersdorp area were reared in the laboratory on three different treatments (non-Bt, Bt1 and Bt2) for periods of three, nine and 21 days.

Five larvae were removed from rearing containers when they reached the desired age, weighed and placed on various feeding treatments (also non-Bt, Bt1 and Bt2) in plastic containers. This was replicated at least 10 times depending on larval availability from rearing containers. Larvae were then allowed to feed for seven days, after which larvae were removed. Larval survival and mass were determined.

3.3.1. Rearing of **B. fusca** *larvae on maize whorls*

Maize was planted in a commercial greenhouse augmented by plants from the field. Non-Bt, Bt1 and Bt2 maize plants were inoculated with 20 first instar larvae into plant whorls. At three, nine and 21 days after inoculation larvae were removed from the maize whorls and used in a laboratory experiment where they were placed on the respective treatments.

3.3.2. Rearing **of B. fusca** *larvae on various maize ears*

The same procedure described above was followed in the experiment with maize ears except that maize ear tips were inoculated in plastic containers to rear larvae. Therefore to generate larval populations for the ear study, larvae were not reared in the field but in the laboratory.

Twenty cylindrical plastic containers (100 ml), with stainless steel mesh lids, was placed into nine large plastic containers (530 mm x 350 mm x 110 mm). Three large containers were used for each maize treatment, one each for the 3, 9 and 21-day old larvae. The purpose was to rear *B. fusca* larvae on the different maize treatments for later use during the study of larval survival on maize ears. The maize treatments consisted of Bt1, Bt2 and a non-Bt (control). Tips of maize ears collected from plants grown in the field were added to each small cylindrical container after which approximately twenty neonate *B. fusca* larvae were added. Along with the tips of maize ears, pieces of filter paper were placed in each cylindrical container to absorb excess moisture.

All rearing containers were placed in rearing rooms at a constant temperature of 27 °C and humidity of between 40 % and 70 %. Larvae were then reared on the maize treatments until the predetermined age (3, 9 and 21 days) was reached. Timers in the breeding room were programmed to turn lights on at 5h00 in the morning and turn it off again at 19h00 the evening.

3.3.3. Evaluating survival and growth when simulating migration of **B. fusca** *larvae between non-Bt and Bt plants*

Larval mass was recorded when larvae were removed from the rearing containers before they were put onto the different maize treatments. Whorls and ear tips were inoculated with five larvae and placed in the small cylindrical plastic containers (Fig. 3.1, 3.2).

Figure 3.1. Sections of the bases of maize whorls placed in containers before larvae were added.

Figure 3.2. Tips of maize ears placed in containers before larvae were added.

The different treatments implied moving larvae between Bt and non-Bt maize treatments as follows:

- Non-Bt to non-Bt
- Non-Bt to Bt1
- Non-Bt to Bt2
- Bt1 to non-Bt
- Bt1 to Bt1
- Bt1 to Bt2
- Bt2 to non-Bt
- Bt2 to Bt1
- Bt2 to Bt2

Each large plastic container contained at least ten small containers with five larvae inside each. The number of replicates depended on the number of larvae that could be recovered from the respective rearing colonies.

For example, the migration of larvae that hatched on a non-Bt plant was simulated as follows: larvae from the non-Bt group reared on whorls or ears were removed to inoculate whorls or ears with five larvae per container (100ml cylindrical plastic container). Larval survival and mass were then determined after seven days of feeding on the non-Bt to non-Bt, non-Bt to Bt1 and non-Bt to Bt2-treatments. The same procedure was repeated for the larvae reared on Bt1 and Bt2.

3.4. Data analysis

Larval survival and mass for all developmental stages were statistically analysed by conducting Tukey tests calculated from ANOVA's. The Statistica version 11 software was used to perform the statistically analysis (StatSoft Inc, 2013).

3.5. Results

3.5.1. Growth and survival on maize whorl tissue

3.5.1.1. Three day-old larvae growth and survival on maize whorls

Larvae transferred from non-Bt plants to non-Bt and Bt1 maize whorls did not differ significantly in survival and mass, but did, however, differ significantly from other treatments ($F_{(8,90)}$ = 16.211; P = 0.000) (Fig. 3.3). Very few larvae survived after three days in the containers on the Bt2 maize which limited the numbers that could be used in the experiment. Seventy eight per cent of larvae from the Bt1 group survived after seven days when placed on non-Bt treatments, but highest mortality was observed when they were transferred to Bt2 maize. When Bt1 larvae were transferred to the Bt1 treatment, survival and mass gain was lower than larvae transferred to non-Bt, but higher than for larvae on Bt2 treatments. Larvae from the Bt2 group showed 50.0 % survival when placed on non-Bt and Bt1, with low survival recorded when transferred to Bt2. Larval mass gain from all groups was the greatest on non-Bt treatments, and lowest on Bt2 maize whorls ($F_{(8.90)}$ = 10.357; P = 0.000).

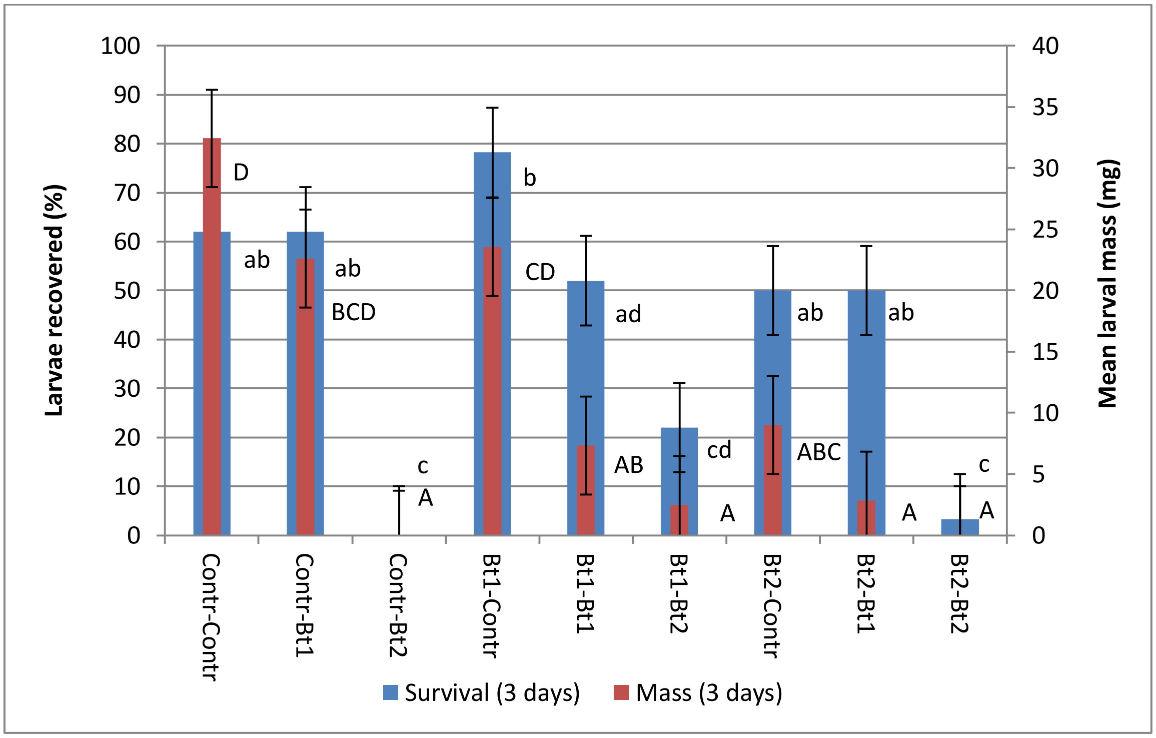

Figure 3.3. Survival and mass of three day-old larvae transferred to whorls of different maize treatments for a 7-day period. Bars with the same higher and lower case letters do not differ significantly for mass and survival. Error bars indicate standard errors (SE).

3.5.1.2. Nine day-old larvae growth and survival on maize whorls

Larvae reared on non-Bt maize had the highest survival when transferred to Bt1 (80.0 %), followed by non-Bt (70.9 %) and Bt2 (20.0 %) respectively ($F_{(8.93)}$ = 49.167; P = 0.000) (Fig. 3.4). Most Bt1 larvae were recovered from non-Bt treatments with only a few surviving the transfer to Bt2. None of the larvae reared on Bt2 survived over the nine-day period and no nine and 21-day evaluations could therefore be done. Mass gain of larvae was greatest when feeding on non-Bt whorls, and least on Bt2 ($F_{(8.93)}$ = 10.41; P = 0.000).

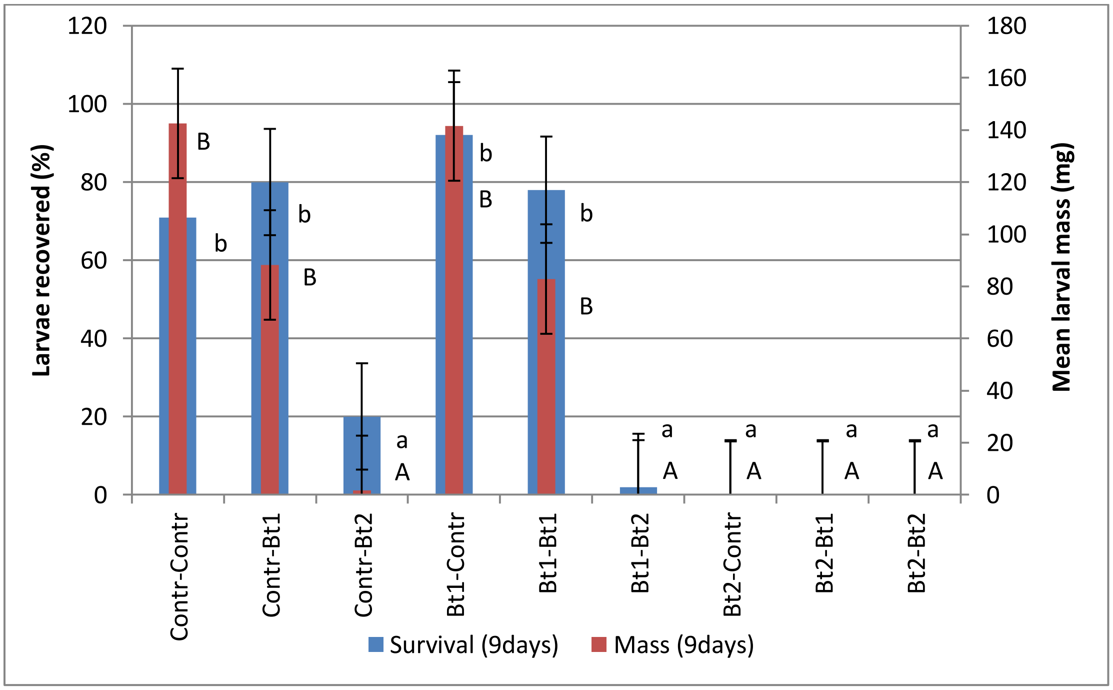

Figure 3.4. Survival and mass of nine day-old larvae on maize whorl treatments. Bars with the same higher and lower case letters do not differ significantly for mass and survival. Error bars indicate standard errors (SE).

3.5.1.2. Twenty one day-old larvae growth and survival on maize whorls

Larvae reared on non-Bt maize showed no significant difference in survival when placed onto non-Bt, Bt1 and Bt2 treatments. However, there was significant differences with other treatments ($F_{(8.93)}$ = 66.487; P = 0.000) (Fig. 3.5). Most larvae reared on Bt1 were recovered during the study from non-Bt treatments (50.0 %), followed by Bt1 (30.0 %) and Bt2 (20.0 %) treatments. Again no larvae reared on Bt2 were available to be included in this part of the study. Larvae from non-Bt whorls feeding on non-Bt and Bt1 whorls showed no significant difference in mass gain, but did differ from larvae that were feeding on Bt2 ($F_{(8.93)}$ = 48.203; P = 0.000). There was no significant difference in mass gain of Bt1 larvae placed on non-Bt and Bt1. Larvae reared on Bt1 and placed on Bt2 had a slight increase in mean mass from nine day-old larvae but was still low (13.65 mg) in comparison with other 21-day old larval treatments.

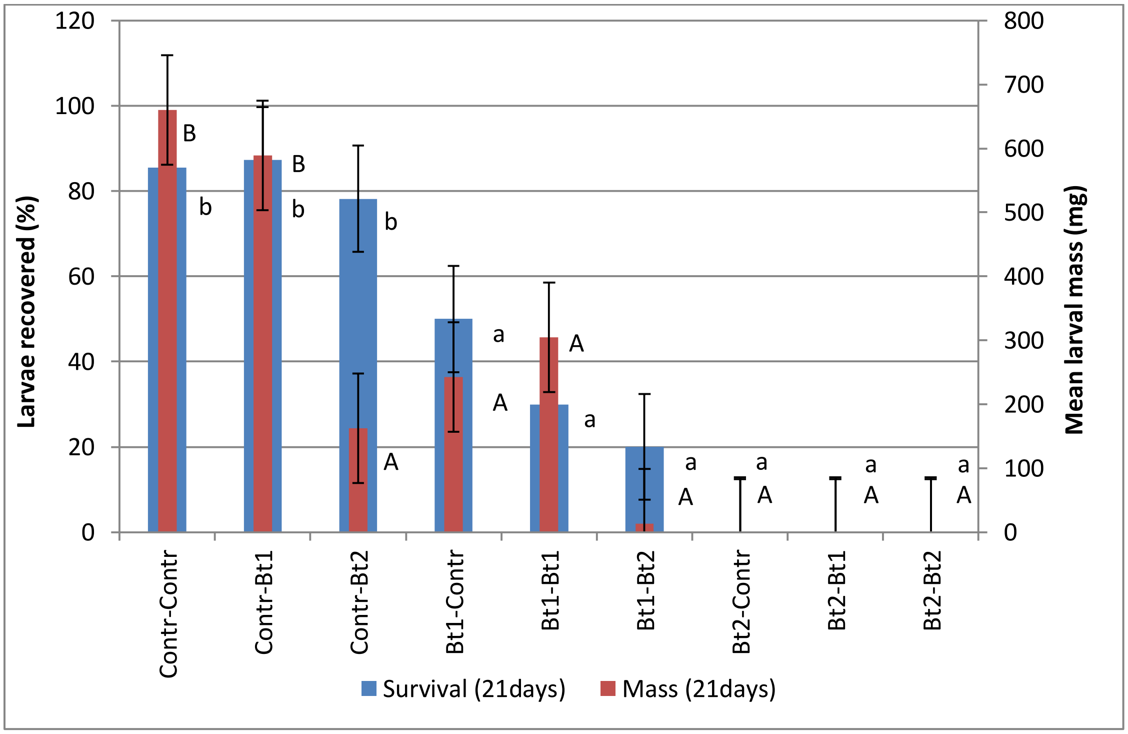

Figure 3.5. Survival and mass of twenty one day-old larvae on maize whorl treatments. Bars with the same higher and lower case letters do not differ significantly for mass and survival. Error bars indicate standard errors (SE).

3.5.2. Growth and survival on maize ear tissue

3.5.2.1. Three day-old larvae growth and survival

Three day-old larvae reared on non-Bt maize ears had the highest survival on non-Bt followed by Bt1 and Bt2 ($F_{(8.54)}$ = 7.747; P = 0.000) (Fig. 3.6). Larvae reared on Bt1 and transferred to non-Bt ear tips had the highest survival. Survival of Bt2-feeding larvae transferred to non-Bt was 52.0 %, and did not differ significantly from non-Bt larvae transferred to non-Bt treatments (53.3 %). Twenty five per cent of Bt2-feeding larvae survived after being transferred to Bt1 treatments. None of the larvae reared on Bt2 survived on Bt2 treatments seven days after inoculation. Mass gain of larvae was highest when placed on non-Bt and Bt1 treatments, with larvae placed on Bt2 indicating the lowest mass gain. There was a significant difference between treatments in terms of mass gained after three days of feeding ($F_{(8.54)}$ = 6.176; P = 0.00001).

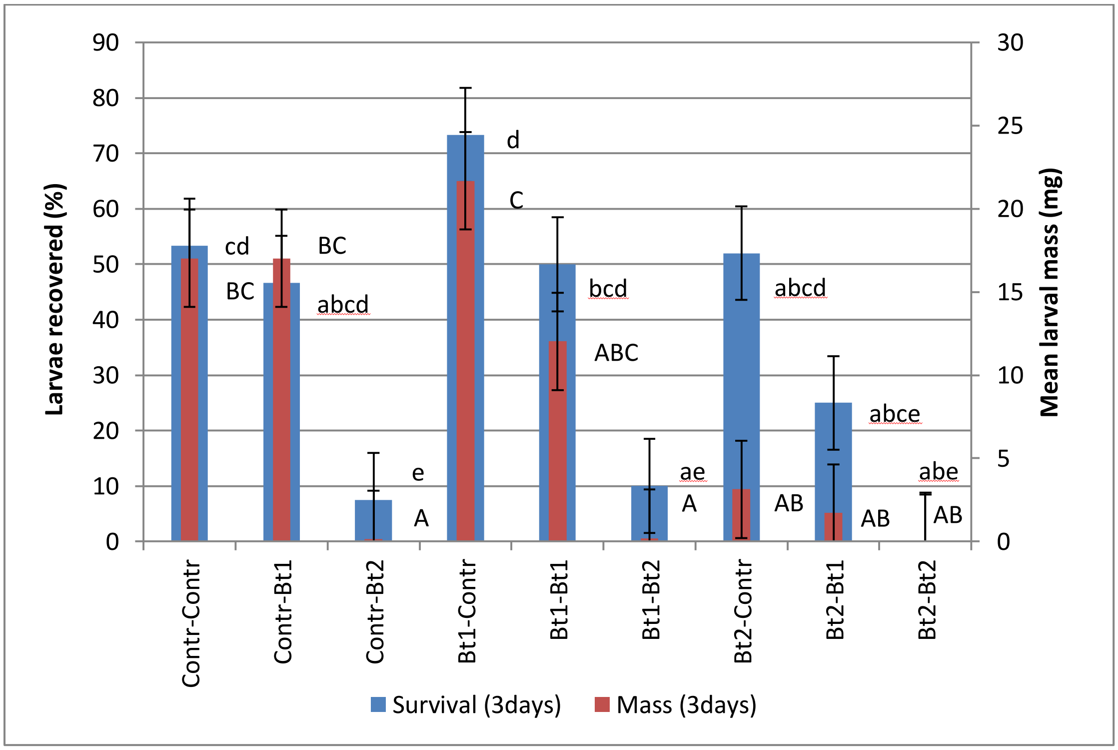

Figure 3.6. Survival and mass of three day-old larvae on maize ear treatments. Bars with the same higher and lower case letters do not differ significantly for mass and survival. Error bars indicate standard errors (SE).

3.5.2.2. Nine day-old larvae growth and survival

Survival of nine day-old larvae reared on non-Bt maize ears was higher than that of the three day-old larvae when feeding on non-Bt (98.3 %), Bt1 (90.9 %) and Bt2 (80.0 %) treatments. There was significant differences between the various treatments ($F_{(8.61)}$ = 12.120; P = 0.000) (Fig. 3.7). Larvae from the Bt1 group transferred to non-Bt treatments had the highest survival, while larvae on Bt2 showed the highest mortality. No nine day-old larvae survived from Bt2 rearing containers to be used in the study. Mass gain of larvae was the highest on non-Bt treatments and lowest on Bt2 treatments ($F_{(8.61)}$ = 20.356; P = 0.000).

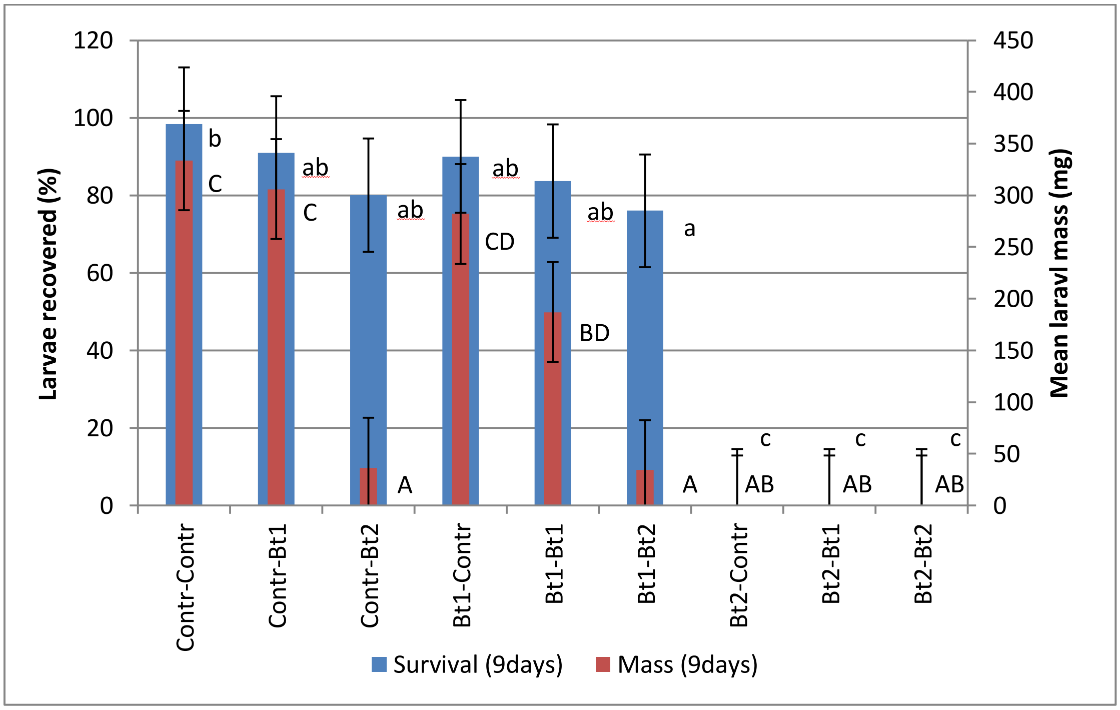

Figure 3.7. Survival and mass of nine day-old larvae on maize ear treatments. Bars with the same higher and lower case letters do not differ significantly for mass and survival. Error bars indicate standard errors (SE).

3.5.2.3. Twenty one day-old larvae growth and survival

Larval survival of the non-Bt group was very high when transferred to non-Bt and Bt1 treatments ($F_{(8.54)}$ = 29.915; P = 0.000) (Fig. 3.8). Larvae reared on non-Bt maize and transferred to Bt2 had a high percentage survival (86.0 %). There was no significant difference between survival of Bt1-reared larvae which was transferred to non-Bt and Bt1 treatments. Larvae from Bt1 groups placed on Bt2, showed 94.0 % survival after the seven day feeding period. None of the larvae reared on Bt2 survived up to 21 days to be included in the study. Gain in larval mass was observed in all larval populations with significant difference between the treatments ($F_{(8.54)}$ = 5.701; P = 0.0003). Mass gain of 21-day old larvae transferred to Bt2 treatments was especially evident when compared to younger larval stages.

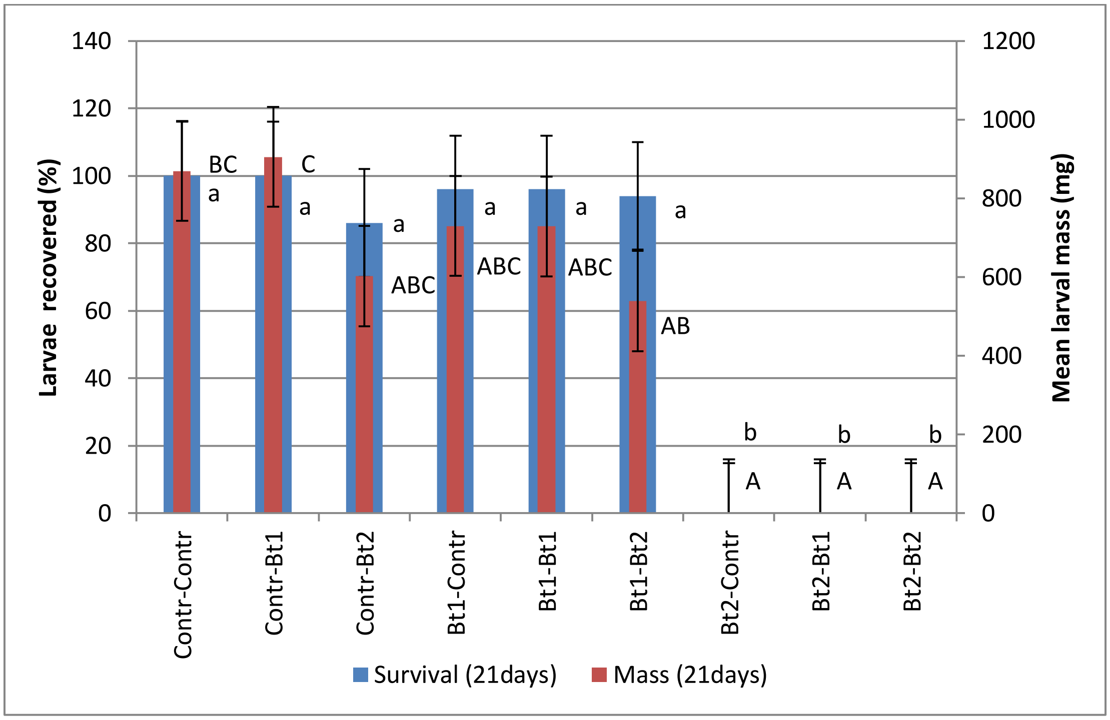

Figure 3.8. Survival and mass of twenty one day-old larvae on maize ear treatments. Bars with the same higher and lower case letters do not differ significantly for mass and survival. Error bars indicate standard errors (SE).

3.6. Discussion

Survival and mass of larvae at all developmental stages were higher in non-Bt and Bt1 treatments than in Bt2 treatments. Younger larvae fared poorer than their older counterparts in growth and survival when feeding on Bt2. Older larvae were generally less affected by exposure to Bt proteins, even in the case of the “pyramid” event (Bt2). Although older larvae survived better on Bt2 than the younger larvae, mass gain was much lower compared to older larvae on non-Bt treatments.

Three day-old larvae showed highest survival and mass gain on non-Bt treatments, and lowest on the Bt2 maize treatment. Three day-old larvae reared on non-Bt maize whorls showed a high mortality when transferred to Bt2. Some three day-old Bt2 larvae were able to survive for the seven day period on Bt2 treatments. However, mass gain by these Bt2 larvae was much lower compared to larvae on non-Bt treatments and they would most probably not have completed their life cycle.

Nine day-old larvae from non-Bt and Bt1 populations survived better, in terms of greater numbers, when placed on non-Bt or Bt1 treatments than on Bt2. Larval mortality was the highest on Bt2. However, nine day-old non-Bt reared larvae transferred to Bt2 maize whorls showed higher survival compared to three day-old larvae.

Larvae reared on non-Bt maize whorls for 21 days survived the best on all treatments. Larval survival of 78.2 % was recorded for non-Bt larvae that fed on Bt2. Also Bt1 larvae placed on Bt2 indicated improved survival compared to younger larvae placed on the same treatment. The same tendencies were recorded for survival and mass of larvae that fed on maize whorl and ear tissue. However, larvae were controlled to a lesser extent on Bt maize ears than on Bt whorls. This could lead to larval exposure to sub-lethal dosages of Bt proteins due to possible differences in levels of gene expression in various plant parts. Bt1 larvae placed on Bt2 treatments of maize whorls and ears showed better survival and mass gain than did Bt2 larvae feeding on Bt2 treatments. This could indicate that larvae developing on Bt1 tissue were less susceptible to Bt2 when exposed to these plants as older larvae.

Survival and mass gain of larvae from all development stages were highest when feeding on non-Bt treatments. Bt1 had a reduced effect on larval survival and mass gain of larvae of all ages compared to Bt2. This was to be expected as the *B. fusca* population used in this study is known to be resistant to Bt1. Three day-old larvae were better controlled by Bt2 compared to 21 day-old larvae. The inability of larvae to survive up to nine days after transfer to Bt2 maize suggests that this event is capable of controlling younger *B. fusca* larvae. Survival of older larvae was affected by Bt2 to a lesser extent than younger larvae. Although survival of older larvae was higher, larval mass gain was lower in comparison to non-Bt and Bt1 treatments. This was the case for both whorl and maize ear tissue.

Walker *et al.* (2000) suggested that Bt events that controlled third instar *O. nubilalis* larvae could be considered a high-dose whereas if fourth instar larvae are controlled, the Bt event could be regarded as a ultra-high dose. It could be of importance to determine whether Bt events commercialised in South Africa has a level of high-dose expression. The ability of older *B. fusca* larvae to survive Bt proteins could identify a potential risk of sub-lethal Bt expression, thereby affecting the efficacy of not only Bt crops but also the IRM strategies deployed.

Whether initial infestation occurs on a Bt or a non-Bt plant in a seed mixture planting, as well as pre-migration feeding behaviour will determine larval growth and survival on these plants. Younger larvae that migrate from Bt to non-Bt plants before sufficient exposure to Bt proteins occurred, could result in larval recovery. Older larvae that developed on non-Bt plants could be exposed to sub-lethal dosages of Bt proteins when migration to a Bt plant occurs. This could also benefit larval survival. A study by Razze and Mason (2012) found that neonate *O. nubilalis* dispersal is significantly greater from Bt plants than non-Bt plants and that this could influence the efficacy of a seed mixture. For *B. fusca*, pre-dispersal feeding time could play an important role in the ability of a seed mixture to prolong resistance development.

3.7. Conclusions

Results suggest that if larvae develop for a certain period on non-Bt maize before migrating to neighbouring Bt plants, there is a high risk of larval survival. This is an important consideration when deploying seed mixture refuges. Movement of older larvae from non-Bt to Bt plants could expose larvae to sub-lethal dosages of Bt proteins, thereby increasing the risk of resistance development. If a "pyramided" Bt event is used and larvae are controlled within a short period of time before migration to non-Bt plants occur, effective control may be obtained. Migration increases during the third instar (21 days). Bt2 would therefore not be able to control such larvae originating from non-Bt plants. RIB poses a threat. In the long term it can be expected that larvae may become resistant to the stacked gene too. It would seem therefore that to avoid exposure to sub-lethal Bt expression, a seed mixture strategy should control pest insects before development to adulthood is achieved and mating occurs. However, the main objective of a non-Bt refuge is to generate sufficient numbers of susceptible adults. Therefore a refuge strategy should generate susceptible adults without increasing the risk of pest insects being exposed to sub-lethal Bt expression. Although older larvae that developed on Bt1 maize survived relatively well on Bt2 treatments, further studies should be conducted since larvae surviving Bt2 was fairly stunted in growth.

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Chapter 4 - Damage caused by migrating *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larvae, to plants of single- and pyramided gene Bt maize events

4.1. Abstract

Busseola fusca (Fuller) (Lepidoptera: Noctuidae) is one of the most important Lepidopteran insect pests of maize in sub-Saharan Africa. Transgenic Bt maize with insecticidal properties have been deployed in South Africa in order to manage *B. fusca* infestations. To delay the development of resistance to *Cry* proteins expressed by Bt maize, insect resistance management (IRM) strategies are needed. The high-dose/refuge strategy is the default IRM strategy used globally, but recently, an alternative seed mixture concept has been suggested. The objective of this study was to determine the actual degree of damage caused by migrating larvae to Bt and non-Bt maize plants in plantings done with seed mixtures. Two field experiments were conducted over two growing seasons. Each experiment consisted of five seed mixture ratios (5 %, 10 %, 15 %, 20 % non-Bt seed) and non-Bt as control. Both a single gene- (Cry1Ab) and a “pyramided” gene event (Cry1A.105 + Cry2Ab2) were used in the experiment in which neonate *B. fusca* larvae were inoculated onto the central non-Bt plant in each plot. At harvest the incidence of damaged internodes per stem, number of damaged ears and yield were determined. Results showed that the two Bt events responded similarly over the two seasons to borer infestation and that there were no significant differences between the incidence of damaged ears, numbers of damaged internodes per stem and yield of the different treatments within each of the two Bt events. However, in one season, the incidence of damaged stems within the single gene event (in the 15 % and 20 % seed mixture treatments) was similar to the control treatments.

4.2. Introduction

Busseola fusca (Fuller) (Lepidoptera: Noctuidae), the African stem borer is native to the African continent and is widely distributed throughout sub-Saharan Africa. This stem borer is considered a major pest on maize and sorghum and to a lesser extent sugarcane and some wild grasses (Harris and Nwanze, 1992). *Busseola fusca* larvae damage host plants by feeding in plant whorls and stems, disrupting nutrient and water movement in plants. Not only are host plants damaged by the direct feeding of larvae, larval infestations also increase the risk of secondary infections due to plant pathogens (Seshu Reddy, 1998).

Maize is considered a staple food in South Africa and its sustained production is therefore of great importance. In South Africa maize yield loss due to *B. fusca* may annually reach high levels (Van Rensburg and Flett, 2010). Production of transgenic Bt maize with insecticidal properties presents a new form of pest management with the potential addition of environmental benefits. Transgenic maize expressing insecticidal proteins have been deployed in South Africa since 1998 and was used with success until the appearance of resistance to Bt maize in 2006 (Van Rensburg, 2007).

Insect resistance management (IRM) strategies are used to prolong resistance development by target pest species (Gould, 2000). The high-dose/refuge strategy is the default IRM application used in South Africa. However *B. fusca* developed resistance to the Cry1Ab protein in several areas of the country (Kruger *et al*., 2012; 2013). Resistance development was initially largely ascribed to incorrect management (spatial distribution) of refugia (Van Rensburg, 2007) and non-compliance to refuge requirements (Kruger *et al*., 2009), and more recently, also to dominant inheritance of resistance (Campagne *et al*., 2013).

A new IRM strategy based on seed mixtures is being deployed in the United States of America (USA) in order to delay resistance development of target pest insects. This strategy is also known as “refuge in a bag” (RIB) (Gray, 2010), which implies that a bag of seed contains a predetermined ratio of non-Bt and Bt seed (Onstad *et al*., 2011). The producer is therefore benefited in that a random refuge is automatically included within the planted area (Davis and Onstad, 2000). Although refuge requirements are easily adhered to when this strategy is deployed, there is concern about its long-term efficacy in delaying resistance evolution.

This concern is mainly due to pest insects migrating between plants and being exposed to sub-lethal dosages of Bt proteins, thereby elevating risk of resistance development over time (Mallet and Porter, 1992; Murphy *et al.*, 2010).

Previous studies on interactions between Bt maize and *B. fusca* in South Africa investigated the levels and spread of resistance (Kruger *et al*., 2009, 2012), survival of larvae on different plant parts (Van Rensburg, 1999) and fitness costs associated with resistance (Kruger *et al*., 2013). No information exists on the actual degree of damage and yield losses caused by Bt-resistant *B. fusca* larvae that infest Bt maize, and how this is affected in plantings done with seed mixtures.

The objective of the study therefore was to determine the incidence and degree of stem and ear damage caused by migrating *B. fusca* larvae to maize plants in plantings done with mixtures of Bt and non-Bt seed. The study may also provide new information on the efficacy of RIB as an IRM strategy against *B. fusca,* give insight on late-instar migration and damage to plants in the post-flowering period which is not easily perceived.

4.3. Materials and methods

The experimental design was described in Chapter 2 of this dissertation. The field study was conducted over two growing seasons (2011/12 and 2012/13) (Fig. 4.1, 4.2). Two Bt maize events commercialised in South Africa was used in this study. These were a single gene (Cry1Ab) (further referred to as Bt1) and a “pyramid” gene (Cry1A.105 + Cry2Ab2) event (further referred to as Bt2). The field trails were subject to natural infestation complemented by artificial inoculation.

Bt1

Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

Bt2

Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

2011/2012 season

Bt1

Rep 6	10%	15%	20%	C	5%
Rep 5	5%	10%	15%	20%	C
Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

Bt2

Rep 6	10%	15%	20%	C	5%
Rep 5	5%	10%	15%	20%	C
Rep 4	C	5%	10%	15%	20%
Rep 3	15%	20%	C	5%	10%
Rep 2	10%	15%	20%	C	5%
Rep 1	5%	10%	15%	20%	C

2012/2013 season

Figure 4.1. Schematic diagram of the Bt1 and Bt2 blocks for both growing seasons.

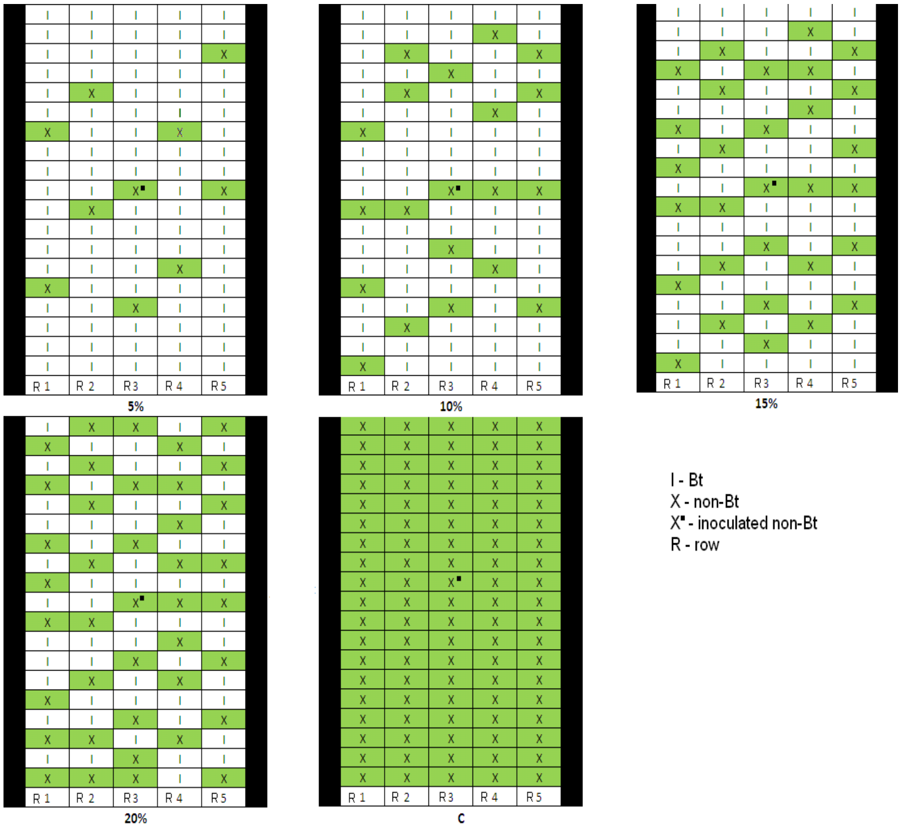

Figure 4.2. Example of the layout of the five treatments within the plots (replicate 1 for both growing seasons). Note that the drawing is not to scale.

All plants within the three central rows of each treatment block were dissected and the number of damaged internodes per stem recorded, 21 weeks after plant emergence. All maize ears were also harvested from these three rows and placed in marked bags according to the specific treatment and replicate. Maize ear damage due to *B. fusca* larvae was assessed using an index, i.e., the reciprocal index of attack (RIA), as described by Hanuss *et al*. (1968). This index value was calculated to determine the degree of stem borer damage to ears. The RIA was calculated using the following equation:

$$\mathrm{RIA} = \frac{f1 + (0.8 \times f2) + (0.5 \times f3) + (0.2 \times f4)}{f1 + f2 + f3 + f4}$$

where f1 to f4 was the frequency of ears in damage-classes one to four respectively. The classes of damage were as follows: f1 = ears exhibiting no damage, f2 = ears with mild levels of damage, f3 = ears with moderate levels of damage, and f4 = ears with severe borer damage. If RIA = 1, no damage occurred whereas lower values indicate more damage to maize ears.

4.4. Statistical analysis

The percentage of damaged ears, percentage of stems with internode damage and mean internode damage per plant was calculated for each treatment plot. Factorial analysis was used to determine possible interactions between seasons, Bt events and treatments. ANOVA’s was used to separate the means between treatments, followed by the Tukey post hoc test. The statistical program used was Statistica version 11 (StatSoft Inc, 2013).

4.5. Results

From factorial analysis done between the treatments from both Bt events across both growing seasons, no significant interactions could be determined. Results therefore indicate that the patterns in stem- and ear damage, and yield were similar over the two seasons.

The mean number of damaged internodes per plant did not differ significantly between Bt1 and Bt2 treatments in both the 2011/12 ($F_{(1,30)} = 3.027$; $P = 0.092$) and 2012/13 ($F_{(1,50)} = 0.267$; $P = 0.607$) growing seasons.

The percentage damaged stems also did not differ significantly between the two Bt events in both the 2011/12 ($F_{(1,30)}$ = 1.848; P = 0.184) and 2012/13 ($F_{(1,50)}$ = 0.411; P = 0.524) growing seasons.

The percentage of damaged maize ears in the seed mixture treatments differed significantly between Bt1 and Bt2, with Bt2 treatments having lower incidences of ear damage. Similar results were obtained in both the 2011/12 ($F_{(1,30)}$ = 9.135; P = 0.005) and 2012/13 ($F_{(1,50)}$ = 6.236; P = 0.015) growing seasons, with seed mixture treatments from Bt2 having no ear damage during the latter season. No significant difference was observed in yield between the two Bt varieties in the 2011/12 growing season ($F_{(1,30)}$ = 3.107; P = 0.088). In the 2012/13 growing season the yield of Bt2 plants was significantly higher than that of Bt1 plants ($F_{(1,50)}$ = 5.103; P = 0.028).

4.5.1. Damage to maize ears

During the 2011/2012 growing season, the incidence of damaged ears ranged between 2.3 and 4.5 % in the Bt1 treatment plots, and between 0.48 and 2.4 % in the Bt2 treatment plots. The percentage of ears damaged in the Bt1 event did not differ significantly between the various seed mixture treatments ($F_{(4,15)}$ = 2.222; P = 0.115) (Fig 4.3 A) (table 4.1).

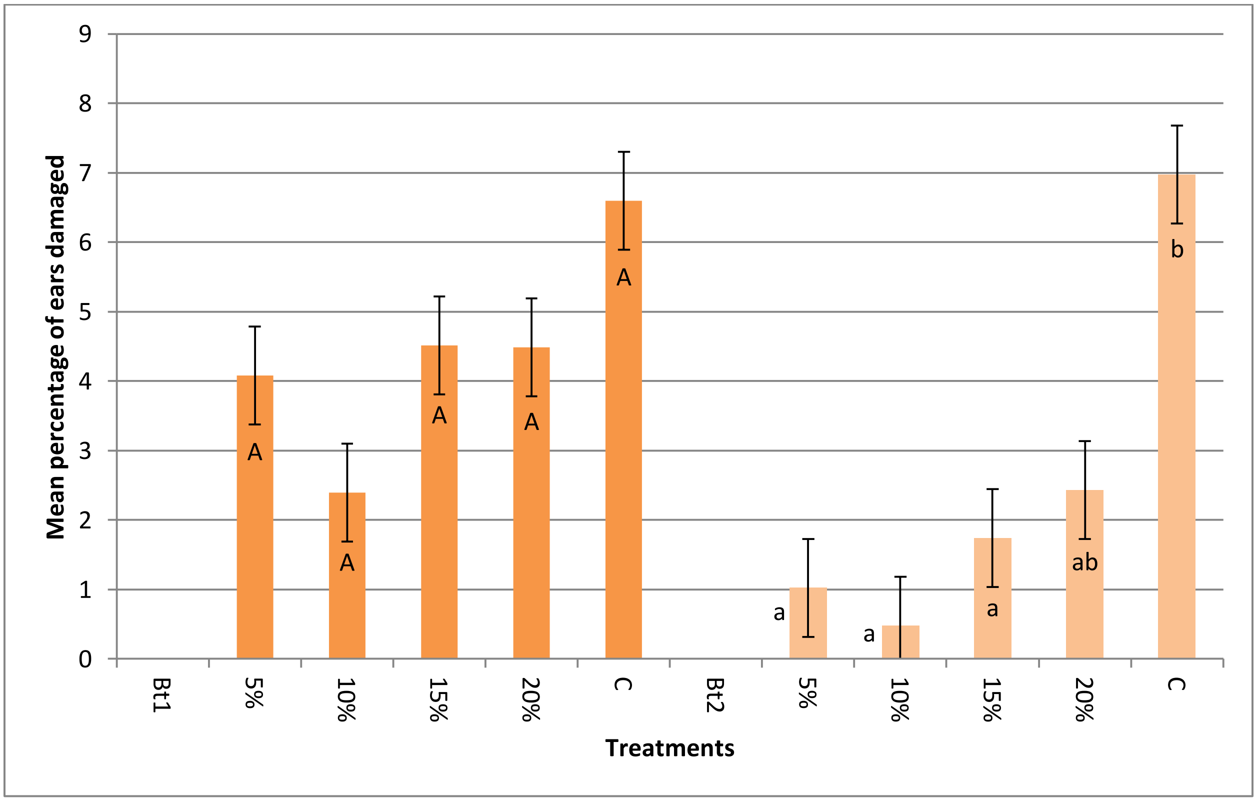

Figure 4.3. (**A**) Mean number of maize ears with stem borer damage (%) (2011/12 season). Bars with the same letters do not differ significantly for incidence of damaged ears (%). Dark orange bars indicate Bt1 and light orange bars Bt2. Bars shown are standard errors (SE).

There was, however, a tendency that the number of damaged ears was highest in the control treatment. The incidence of ear damage did not differ significantly between the various Bt2 treatments but was significantly lower than that in the control treatment ($F_{(4,15)} = 6.264$; $P = 0.003$). The incidence of damaged ears in the Bt2 treatment with the 20 % non-Bt seed, did not differ significantly from the control treatment. The degree of ear damage, indicated by the RIA-values, was very low during the 2011/2012 growing season. The RIA-values were similar in all Bt1 treatments and close to 1.0 (Table 4.1). Control treatments had the lowest RIA value (0.97), which indicates very low degrees of damage on even the control plots. Ear damage on Bt2 plants was even lower and was also similar between treatments. No significant differences existed in ear mass or yield, between the various treatments of both Bt1 and Bt2 plants in the 2011/2012 season (Table 4.1). There was a tendency that Bt1 treatments had higher yields compared to Bt2 for the 2011/12 growing season.

During the 2012/2013 growing season, the incidence of damaged ears ranged between 1.7 and 3.2 % in the Bt1 treatment plots, with no damage occurring on maize ears in the Bt2 treatment plots. No significant differences were observed in the incidence of damaged ears in the different Bt1 seed mixture treatments (Fig 4.3 B) (Table 4.2). The incidence of damage in the Bt1 10 % treatment was, however, significantly lower than in the control ($F_{(4,25)}$ = 3.717; P = 0.016). The control blocks had the highest damage levels of all treatments.

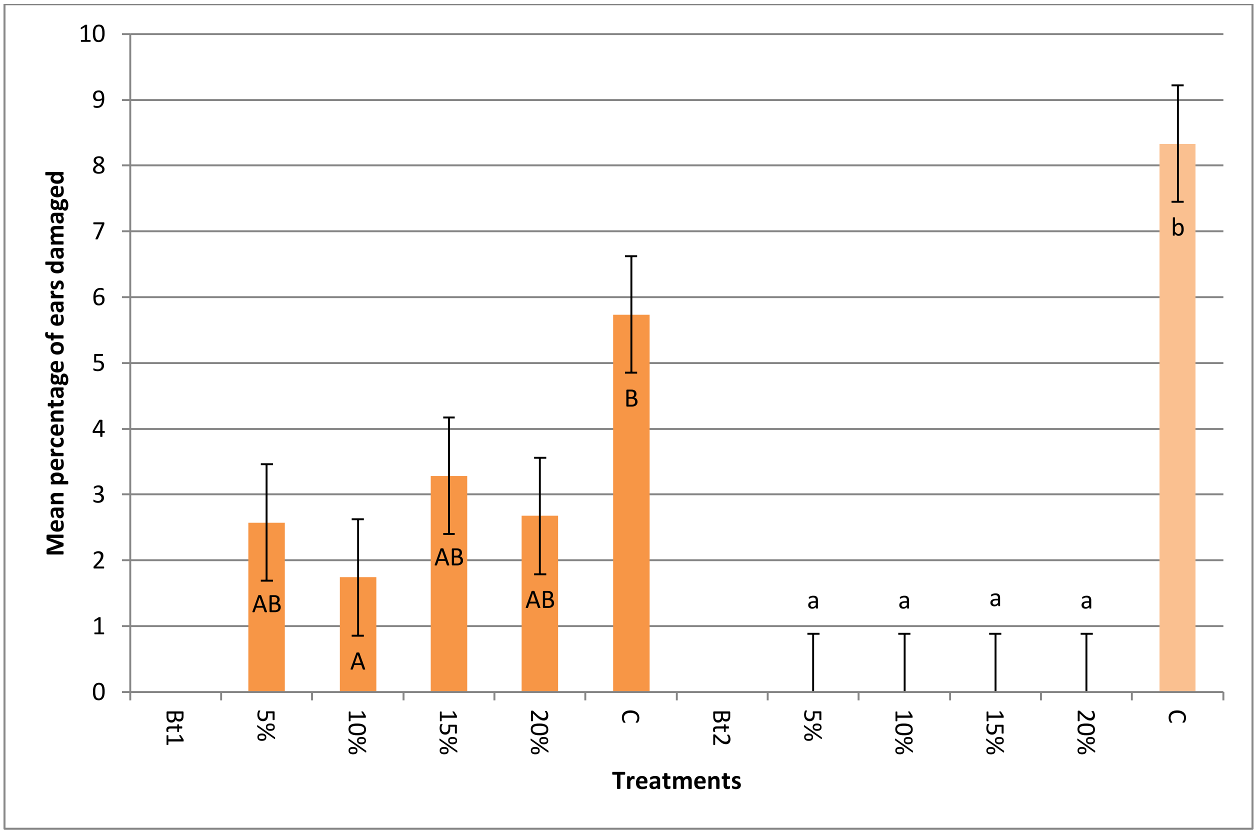

Figure 4.3. (**B**) Mean number of maize ears with stem borer damage (%) (2012/13 season). Bars with the same letters do not differ significantly for incidence of damaged ears (%). Dark orange bars indicate Bt1 and light orange bars Bt2. Bars shown are standard errors (SE).

Similar to the 2011/12 season, the RIA values in the 2012/13 season showed that the degree of damage to ears was very low on plants of Bt1 and no damage occurred on ears of Bt2 plants (Table 4.2). Ears of plants in control plots also suffered very low degrees of damage. Ear mass and yield did not differ significantly between the treatments in both Bt1 and Bt2 treatments (Table 4.2). The yield of Bt2 treatments tended to be higher than that of Bt1 treatments.

4.5.3. Stem damage

The mean number of damaged stems was higher in the first than in the second season. The incidence of damage ranged between 13.5 % and 15.8 % for Bt1 treatments with 26.7 % of stems damaged in the control treatment in the 2011/12 season. In the Bt2 treatments the incidence of damaged stems was between 7.5 % and 11.4 % with the control treatment having 30.4 % damaged plants (Table 4.1).

In the 2012/13 season, the incidence of damaged stems was below 4.2 % for all treatments of both Bt varieties. During both seasons the incidence of damaged stems did not differ significantly between the seed mixture treatments of each of the Bt events (Table 4.1). The mean number of damaged stems was higher in Bt1 than Bt2 treatments. No significant differences were observed in the mean number of damaged internodes per plant for any of the seed mixture treatments of both Bt varieties (Table 4.2). Internode damage was, however, significantly different between the seed mixture and control treatments.

Table 4.1. Damage and yield parameters of Bt1 and Bt2 maize plants infested by *Busseola fusca* larvae (2011/2012 season).

2011/2012 Season													
Bt event	**Treatment**	**Damaged ears (%)**		**Degree of ear damage (RIA)***		**Stems with internode damage (%)**		**Mean number of damaged internodes /plant**		**Ear mass (kg)**		**Grain mass (kg)**	
Bt1	5%	4.08		0.98		13.54 a		0.27 a		15.150		13.100	
Bt1	10%	2.39		0.99		13.98 a		0.25 a		16.210		13.350	
Bt1	15%	4.51		0.98		15.61 ab		0.32 a		13.810		11.415	
Bt1	20%	4.48		0.98		15.87 ab		0.28 a		14.455		11.955	
Bt1	Control	6.60		0.97		26.77 b		0.56 b		13.960		11.560	
F and p-values		**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
		2.222	0.115	/	/	3.786	0.025	6.804	0.002	0.713	0.595	0.746	0.575
Bt event	**Treatment**	**Damaged ears (%)**		**Degree of ear damage (RIA)**		**Stems with internode damage (%)**		**Mean number of damaged internodes /plant**		**Ear mass (kg)**		**Grain mass (kg)**	
Bt2	5%	1.02 a		0.99		7.52 a		0.13 a		15.140		12.285	
Bt2	10%	0.48 a		0.99		9.90 ab		0.15 a		14.025		12.095	
Bt2	15%	1.73 a		0.99		9.12 ab		0.13 a		12.700		10.510	
Bt2	20%	2.43 ab		0.99		11.49 ab		0.18 a		12.645		10.240	
Bt2	Control	6.97 b		0.98		30.46 b		0.63 b		12.885		10.720	
F and p-values		**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
		6.264	0.003	/	/	3.676	0.028	3.368	0.0371	0.942	0.466	1.000	0.437

* Less damage the closer to unity.

Table 4.2. Damage and yield parameters of Bt1 and Bt2 maize plants infested by *Busseola fusca* larvae (2012/2013 season).

2012/2013 Season													
Bt event	**Treatment**	**Damaged ears (%)**		**Degree of ear damage (RIA)***		**Stems with internode damage (%)**		**Mean number of damaged internodes /plant**		**Ear mass (kg)**		**Grain mass (kg)**	
Bt1	5%	2.57 ab		0.99		3.81 a		0.06 a		7.790		6.303	
	10%	1.74 a		0.99		4.29 a		0.05 a		7.800		6.217	
	15%	3.28 ab		0.98		3.78 a		0.05 a		7.630		6.257	
	20%	2.67 ab		0.99		3.43 a		0.05 a		7.847		6.300	
	Control	5.73 b		0.98		14.93 b		0.24 b		6.713		5.547	
F and p-values		**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
		3.717	0.016	/	/	12.341	0.00001	10.549	0.00004	0.253	0.904	0.172	0.950
Bt event	**Treatment**	**Damaged ears (%)**		**Degree of ear damage (RIA)**		**Stems with internode damage (%)**		**Mean number of damaged internodes /plant**		**Ear mass (kg)**		**Grain mass (kg)**	
Bt2	5%	0.00 a		1.00		3.18 a		0.04 a		9.813		7.903	
	10%	0.00 a		1.00		2.07 a		0.02 a		9.943		7.877	
	15%	0.00 a		1.00		3.31 a		0.04 a		8.857		7.113	
	20%	0.00 a		1.00		2.93 a		0.03 a		8.420		6.742	
	Control	8.33 b		0.97		15.64 b		0.28 b		8.753		6.990	
F and p-values		**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
		14.962	0.00000	/	/	12.318	0.00001	18.289	0.00000	0.389	0.814	0.355	0.837

* Less damage the closer to unity.

4.6. Discussion

Although the incidence of damaged ears in this study was low, it was influenced by the Bt variety used. During both growing seasons Bt1 had a higher incidence of damaged ears than Bt2 plants. In the second season, no ear damage occurred on Bt2 plants. Some significant differences in the incidence of damaged ears were observed between the seed mixture treatments when compared between the Bt events. For example during both growing seasons the incidence of damaged ears was significantly higher for Bt1 than Bt2 treatments. Although the percentage of damaged ears was always significantly higher in the control plots, it never exceeded 9.0 %. This indicates a relatively low level of stem borer infestation on maize ears during the study period. RIA values suggested that ears in control plots suffered a negligible level of attack by stem borer larvae.

The percentage of stems with internode damage was relatively lower for the 2012/13 season. This may be attributed to lower overall stem borer infestation during the 2012/13 season. Control treatments had the highest incidence of damaged stems. Within the seed mixture treatments for both Bt events, Bt2 always had fewer damaged stems than Bt1 in both growing seasons. Bt2 plants displayed more damaged stems than the incidence of plants with whorl damage (see chapter 2), suggesting that older larvae migrated onto Bt plants later in the season. It may also suggest the migration of larvae from non-Bt plants onto Bt plants where they continued feeding. Of interest is that during the 2012/13 growing season, the levels of internode damage between the two Bt events were relatively similar. This may also be attributed to increased ambient temperatures and lower rainfall possibly affecting *B. fusca* infestation.

Ear mass and yield (as a function of the grain mass) did not differ significantly between the various treatments, although control plots always had the lowest yield. This was the case for both Bt events during both growing seasons. The only difference is that Bt1 provided a higher yield than Bt2 during the 2011/12 season, whereas Bt2 had the highest yield in the 2012/13 season. This may be attributed to seasonal differences in the response of the two varieties to climatic conditions. Yield loss due to stem borer feeding was not ascertained, but can be assumed to be minimal. The RIA values which were very close to 1.0, showed negligible degrees of ear damage.

4.7. Conclusion

Continued research may also provide better information on possible relationships that exist between whorl (Chapter 2) and stem damage and yield loss of Bt maize varieties. This could in future be used to revise the economic threshold concept for *B. fusca* on maize in South Africa. Very few significant differences in late season damage levels (internodes and maize ears) suggest that the various ratios of seed mixtures successfully controlled migrating larvae. In the case of Bt2, the lack of whorl damage to plants and presence of internode damage later in the season could suggest migration of larvae from non-Bt to Bt plants. It could also be attributed to a reduction in the high-dose efficacy of older Bt plants later in the season. Successful migration and feeding of older larvae on Bt stems may indicate that the RIB concept is not the most appropriate IRM strategy to manage resistance development of *B. fusca* in South Africa. Although yield loss was not determined in this study, it can be accepted, based on the extremely low levels of ear damage, that minimal losses would have occurred. Future studies could improve on this aspect and yield loss comparisons made to plantings done according to the high-dose/refuge strategy.

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Chapter 5: Comparisons between the high-dose/refuge and seed mixture strategies - a review

5.1. Abstract

The introduction of genetically modified crops with insecticidal properties provided an alternative to total reliance on pesticide use. By expressing proteins derived from the soil bacterium, *Bacillus thuringiensis* (Bt), these transgenic crops are more selective in the arthropods they control and also provide a more environmentally beneficial control strategy than large scale use of pesticides. Due to concerns about the development of resistance of target pest insects to Bt proteins, insect resistance management (IRM) strategies are deployed to delay resistance development. The high-dose/refuge strategy deploys a high-dose Bt protein expression in conjunction with a separate refuge of non-Bt plants. The "refuge in a bag" (RIB) strategy, on the other hand, is based on the seed mixture concept, meaning that a bag of seed contains a certain ratio of non-Bt and Bt seed. A random refuge is therefore included within the planted area. Similarities between these two IRM strategies include the use of a high-dose of Bt protein expression, multiple gene "stacking" and provision of non-Bt plants for sustaining sufficient numbers of susceptible pest individuals. Differences do however also exist, for example, the spatial arrangement of Bt and non-Bt plants which may affect how migrating pest insects are influenced by Bt proteins expressed in the plants they encounter at different stages during their life cycle. There are also practical considerations that determine the use of both strategies. Taking into account the differences, benefits and concerns regarding both strategies, the high-dose/refuge strategy may be superior in prolonging resistance development. The seed mixture concept could be more appropriate when used to manage pest insects with sedentary behaviour or low levels of migration activity between plants. As with any other pest control concept, Bt crops should be deployed as part of a variety of decision based control methods, regardless of which refuge-based strategy is more suitable.

5.2. Introduction

Since the mid-20th century, the use of pesticides has dominated in the management of insect pests on agricultural crops. This resulted in concern among various communities as to the damage of widespread pesticide applications on the environment and those whom live in it (Kogan, 1998). The development and introduction of genetically modified crops expressing insecticidal proteins started during the late 20th century (Stone, 2010). By promising effective control of specific target pests and a reduction on pesticide reliance (Federici, 1998), the use of these transgenic crops showed potential for inclusion in future insect pest management (IPM) strategies.

Transgenic crops with insecticidal properties express proteins, also referred to as endo-toxins derived from *Bacillus thuringiensis* (Bt) (Cohen *et al.,* 2000; Haung *et al*., 2011). Upon feeding on a Bt host plant, these proteins bind to the mid-gut of the insect. Perforations to the mid-gut are induced which fatally affect the pest insect (Gray, 2010). Deployment of Bt crops to manage insect pests are target specific and may reduce the use of chemical control. Higher yields may also be achieved due to enhanced pest control. The use of Bt crops has therefore been a market success since its global introduction during the 1990's (James, 2011).

However, as with any pesticide, the development of resistance of target pest insects to Bt proteins is a reality (Cohen *et al*., 2000). In order to delay the development of resistance to Bt proteins, insect resistance management (IRM) strategies are deployed (Gould, 2000). Various IRM strategies have been developed. Among these is the high-dose/refuge strategy, currently the default refuge-based IRM strategy deployed globally. This strategy consists of two components. First, a high-dose Bt protein expression is used to control as many of the susceptible pest individuals as possible (Haung *et al*., 2011). Secondly, a separate refuge of non-Bt plants must be planted along with the Bt crop area (Gould, 2000). Its purpose is to sustain a population of susceptible adults in order to mate with the few resistant individuals surviving exposure to the Bt crop (Gould, 2000; Kruger *et al*., 2009). These resistance genes are then diluted and can then be effectively managed by the high-dose Bt protein expression (Cohen *et al*., 2000).

A more recently introduced refuge-based IRM strategy, based on the seed mixture concept, is the "refuge in a bag" strategy (Gray, 2010). In this case, a single bag of seed contains a predetermined ratio of non-Bt and Bt seed (Onstad *et al*., 2011).

A random refuge is therefore included in the planted area and there is no need to plant a separate refuge of non-Bt plants (Davis and Onstad, 2000). Both of these strategies seem to have strengths and weaknesses, with much debate on which is the best to delay resistance development in the long term.

An IRM strategy deploying a high-dose of Bt proteins functions on the basis of the assumed recessive inheritance of the resistance gene. Pest populations are considered to include three genotypes of which two are homozygous (SS, RR) and one is heterozygous (RS) in terms of resistance and susceptible genes. At a low-dose of Bt protein expression most of the SS insects are controlled, but all of the RS individuals survive (Onstad and Guse, 2008). A high-dose of Bt protein controls all SS and RS insects, but most of the RR individuals survive (Onstad and Guse, 2008). The purpose of a non-Bt refuge is to sustain large numbers of SS moths to mate with RR moths, thereby generating RS offspring. The purpose of the high-dose is to control the RS individuals. Therefore at low-dosages, resistance genes are dominant, whereas resistance genes are recessive at high-dosages of Bt expression (Onstad and Guse, 2008). It is therefore important to ensure that high-dose Bt events are deployed to control target pest species.

The objective of this review is to compare and discuss differences between the high-dose/refuge and seed mixture strategies. This will be done in terms of benefits and concerns associated with each of the strategies, from a pest management as well as a practical point of view.

5.3. Differences and similarities associated with both strategies

When evaluating which IRM strategy might be the optimum to deploy in order to delay resistance development, a clear understanding of the differences and similarities between the concepts are needed (Fig.5.1). Starting with the similarities:

- Both strategies are based on the refuge concept, meaning non-Bt plants are planted in close proximity to Bt plants.
- Both strategies deploy a high-dose protein expression and should be able to control susceptible pest individuals.
- In both strategies, “stacked/pyramided” or multiple gene expression may be included, providing improved strategies for management of resistance evolution. Also, in both cases, Bt proteins are selective in controlling target pests only.

- Both strategies include a 5 % non-Bt refuge. A separate refuge can be increased to 20 % or larger (Gould, 2000; Haung *et al*., 2011).
- The deployment of both strategies prohibits the use of pesticides, except when a 20 % or larger separate refuge is planted (Gould, 2000).
- Both strategies are available as products to manage both above and below ground pest insects. Some products include multiple genes (pyramid) to protect against different types of insects (e.g. lepidopteran and coleopteran pests).

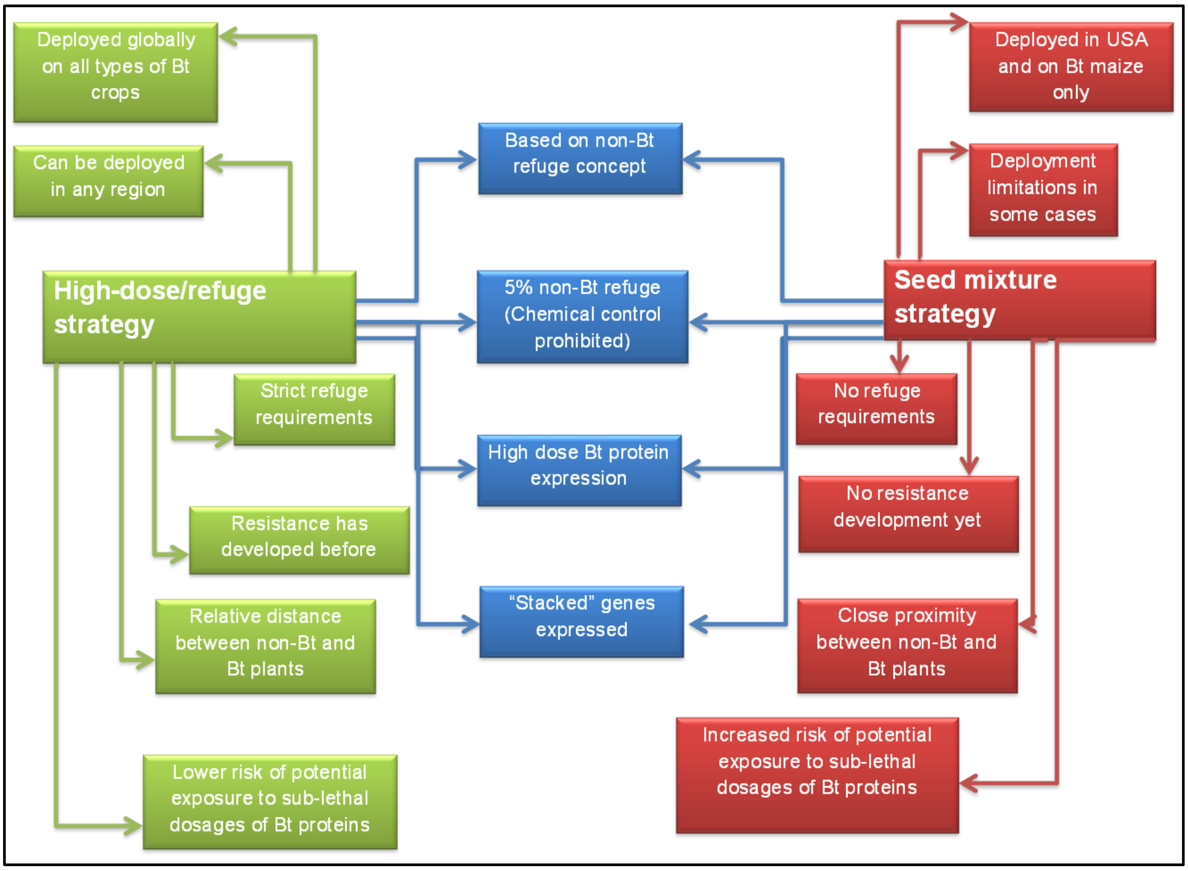

Figure 5.1. Schematic diagram of differences and similarities between the high-dose/refuge and the seed mixture strategies.

Several differences also exist between the high-dose/refuge and seed mixture strategies:

- In the case of a high-dose/refuge, non-Bt refuges are planted separate from the Bt plants, implying that there is a significant distance (up to 400 m) between Bt and non-Bt plantings. These refuges can be planted as perimeter, inter-row or block refuges (Cullen *et al*., 2008). In a seed mixture, non-Bt plants occur randomly within the planted area, meaning that non-Bt and Bt plants are much closer in proximity to each other compared to a separate refuge planting (Fig. 5.2).

It would be a common occurrence for leaves from non-Bt and Bt plants planted as a seed mixture, to overlap and touch when plants are approximately four weeks or older.

- Within a seed mixture, sub-lethal exposure of pests to Bt proteins could in some cases be of greater concern than is the case with a separate refuge. This is due to the different proximities between non-Bt and Bt plants within each strategy and the migratory behaviour of the pest insect.
- There are strict refuge requirements that must be adhered to when separate refuges are deployed (Kruger *et al*., 2009). If these refuge requirements are not complied to, development of resistance of target pests may result (Kruger *et al*., 2009). In the case of the seed mixture strategy, the producer has no refuge requirements to adhere to since the random refuge is already included as part of the RIB strategy.
- Separate refuges can be deployed as an IRM strategy for nearly all types of Bt crops (e.g. maize, rice, cotton, potatoes), whereas seed mixture products are currently only used for Bt maize. RIB products are currently only employed by producers in the United States of America (USA) (Monsanto, 2012) while the high-dose/refuge strategy is used on a global scale.
- The high-dose/refuge strategy can be deployed in any locality whereas RIB products have some limitations. For example, maize RIB products must be deployed with a separate 20 % non-Bt refuge in cotton growing regions where *Helicoverpa zea* (Lepidoptera: Noctuidae) is a pest of concern (USEPA, 2011).
- Resistance to Bt proteins has developed with use of the high-dose/refuge strategy (Van Rensburg, 2007). Although there are as yet no reports of field resistance where the RIB strategy has been deployed, its use is still at an early stage and on small scale compared to that of separate refuges.

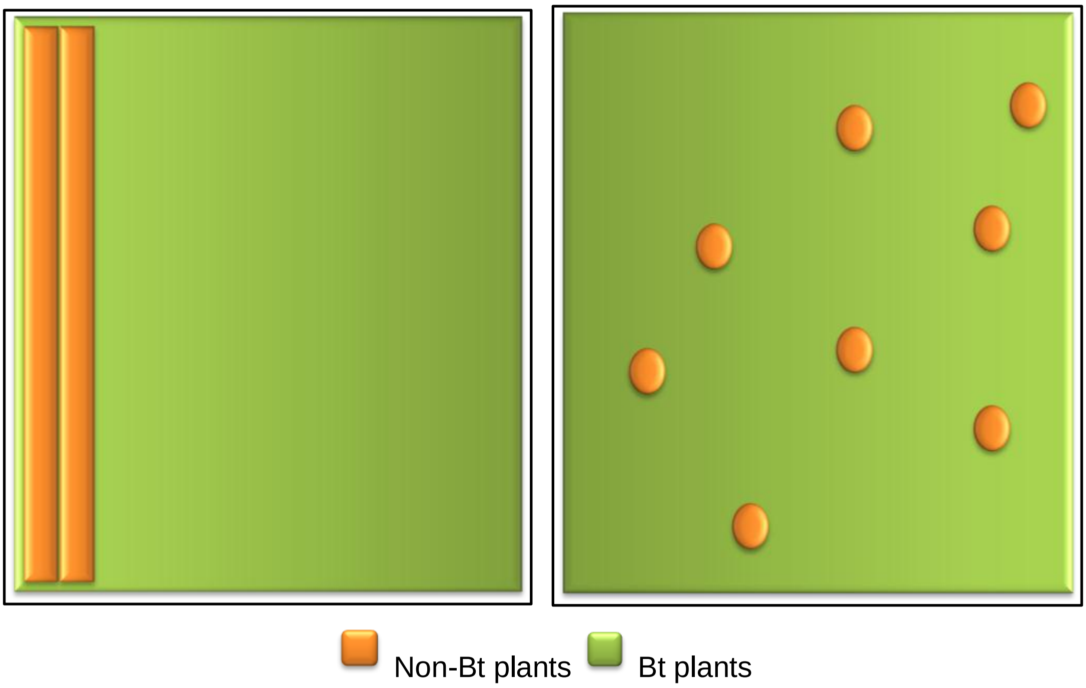

Figure 5.2. Illustrations of a separate non-Bt refuge (high-dose/refuge strategy) and a random non-Bt refuge (seed mixture strategy). Note the difference in distance and proximity between non-Bt and Bt plants in each strategy.

Table 5.1 Benefits and disadvantages associated with both IRM strategies according to the Environmental Protection Agency (EPA) (USEPA, 2011).

High-dose/refuge strategy	**Seed mixture strategy**
Benefits	*Benefits*
• Higher durability of refuges, meaning efficacy of IRM could last longer. • Higher numbers of susceptible insects are produced.	• No refuge requirements needed. • No compliance monitoring needed.
• Refuges can be managed in order to sustain yield.	• No need for a separate refuge. • Ease of RIB product deployment.
Disadvantages	*Disadvantages*
• Reduced random mating depending on the level of insect migration.	• Potential lower durability of random refuges. • Movement of pest insects between plants could increase the risk of resistance evolution.
• Separate refuges must be planted according to strict requirements. Refuge compliance must be monitored.	• Effective decrease in refuge size due to fewer susceptible adults being generated.
• Non-compliance to refuge requirements enhances the risk of resistance evolution.	• Difficulty associated with detecting resistance development and monitoring of pest damage.

5.4. Differences in spatial properties between non-Bt and Bt plants

Although both IRM strategies are based on the concept of providing a non-Bt refuge of theoretically the same size, application of these strategies could lead to alternative outcomes. Due to the close proximity of non-Bt and Bt plants in fields where seed mixtures are planted, movement of pest insects (e.g. migrating stem borer larvae) between these plants could increase the risk of exposure to sub-lethal dosages of Bt proteins (Mallet and Porter, 1992; Gould, 2000; Murphy *et al*., 2010). In many instances, leaves of plants within and between rows overlap as plants grow, thereby providing an easy means for insects to migrate between plants. This could escalate the development of resistance of migratory insects over time. More sedentary insects (e.g. aphids) would probably not be affected in this manner since exposure to the plant on which hatching occurred would continue for a substantial period of its life cycle (Gould 2000). However, some insect pests display a high degree of migration between plants. This would especially induce a high measure of risk if neonate insects migrate away from a Bt onto a non-Bt plant before sufficient feeding had occurred on the former. Another scenario is that immature stages of the pest may develop to advanced stages on a non-Bt plant before migrating to a Bt plant. If these insects then migrate to a Bt plant, the insecticidal proteins may not be able to control such individuals anymore. In both such cases the survival of the pest insect could be benefitted. A study by Crowder and Carrière (2009) suggested that the efficacy of IRM strategies will be enhanced if survival of susceptible insects is promoted and the level of fitness costs is increased. This statement could support a separate refuge, but not necessarily a seed mixture due to the concern that pest insects would migrate between plants. Too many non-Bt plants in a random refuge, could pose a problem if older insects migrate and survive exposure to Bt proteins. Movement of pest insects from non-Bt to Bt plants within a seed mixture could reduce the number of susceptible adults available for mating with resistant individuals that survived exposure to Bt protein, thereby reducing the efficacy of the random refuge (USEPA, 2011). In such a scenario the practical size of the random refuge is reduced (Davis and Onstad, 2000).

A benefit of the close proximity of non-Bt and Bt plants within a seed mixture is that it could enhance random mating between resistant and susceptible adults (Davis and Onstad, 2000). This will contribute to the dilution of resistant genes in the population. In a separate refuge setup there is a degree of spatial separation between non-Bt and Bt plants. This spatial distance is small enough to allow for emerging adults to migrate between the non-Bt and Bt-planted areas, but large enough to prohibit migration between Bt and non-Bt plants.

Therefore, if eggs are laid and hatching occurs on a Bt plant surrounded by several other Bt plants (as is the case with a separate refuge), exposure to Bt proteins should be adequate to kill all initial instars even if migration does occur. Due to the spatial arrangement between non-Bt and Bt plants in a high-dose/refuge strategy, enhanced production of susceptible adults may be achieved if pesticide use on the refuge is excluded (USEPA, 2011).

Monitoring of damage levels could be more difficult in plantings where seed mixtures have been used since easy identification of non-Bt and Bt plants is impossible without the use of Bt test strips. To distinguish between non-Bt and Bt plants when fields have been planted according to the high-dose/refuge strategy is easily facilitated due to the refuge being planted on a specific, separate and usually demarcated area. Monitoring for pest damage in order to identify possible resistance development is therefore easier when separate refuges are planted. In such cases, any damage inside Bt crop fields would be of concern and could point to initial stages of resistance development.

Davis and Onstad (2000) suggested that when seed mixtures are assessed as an IRM strategy for the European corn borer (*Ostrinia nubilalis*) (Lepidoptera: Crambidae), three characteristics of the pest insect must be known. Firstly, the dispersal rate of larvae from plants where hatching occurs should be known, since this indicates the measure of migration that takes place from the infested plant of origin. Secondly, the level of survival of larvae that are exposed to Bt plants before they disperse must be known. This information would indicate the efficacy of the Bt proteins to control neonate larvae before emigration from a Bt plant takes place. The third parameter is the survival rate of neonate larvae that are exposed to Bt proteins. In the case of a separate refuge only one of these three parameters is of importance, i.e. the survival rate of neonate insects on Bt plants. Therefore, in the case of separate refuges, the expression level of Bt proteins inside the plant plays a more important role than the migratory and dispersal behaviour of the pest insect.

The above mentioned discussion could support the assumption that a separate refuge might be the better IRM option for deployment against pests with a high degree of migration between plants. A study by Goldstein *et al.* (2010) found that neonate *O. nubilalis* larvae were able to detect the presence of Bt proteins and dispersal from Bt plants subsequently occurred within a quicker timeframe compared to larvae that hatched on non-Bt plants. Other pest insect species may display similar behaviour when exposed to Bt proteins (Goldstein *et al.*, 2010).

Such preference behaviour exposed by early-instar larvae could have a detrimental effect on the efficacy of seed mixtures to delay resistance development, especially if the target species also have an inherent tendency of large scale migration away from the oviposition site. Within a separate refuge the effect of detection of the presence of Bt proteins in a plant and subsequent increased larval migration could be of lesser importance as contributing factor in resistance development than in seed mixtures. The separation of non-Bt and Bt plants within a high-dose/refuge strategy would still control pest insects migrating in the Bt planted area. Larvae migrating within the Bt-planted area in a high-dose/refuge strategy will still be surrounded by Bt plants, thereby probably being controlled. The most appropriate spatial arrangement of the non-Bt refuge planted as part of an IRM strategy will therefore depend on the biology and behaviour of the target pest (Cohen *et al*., 2000).

5.5. Size of the non-Bt refuge

In both IRM strategies, the size of the refuge plays an important role. Both strategies are deployed with a 5 % non-Bt refuge, with the high-dose/refuge strategy also providing the option of planting a 20 % separate refuge onto which chemical control of the target pest is allowed (Gould, 2000; Haung *et al*., 2011). Several factors may, however, adversely affect the size of a refuge, thereby potentially influencing its contribution to an effective IRM strategy.

Biotic as well as abiotic factors such as drought stress in cases where refuges are planted on marginal land, or herbivory on non-Bt seeds or plants by other animals could reduce the size of a refuge even further. Separate refuges that are larger than 5 % could provide a margin of safety if some of the refuge plants are lost due to unexpected factors. Furthermore, with a 20 % separate refuge, chemical control may be applied if needed, but at the cost of sustaining sufficient numbers of susceptible adults required to mate with resistant adults emerging from the Bt crop. Due to the spatial nature of seed mixtures, higher ratios of non-Bt seed might not be an appropriate option due to practical implications. Chemical control of the target pest on plantings with higher non-Bt seed ratios is not allowed as in the case with larger separate non-Bt refuges (USEPA, 2011). Therefore, since seed mixtures has limited flexibility due to the predetermined ratio of Bt to non-Bt seed in a single bag, a separate refuge might provide more options to a producer in order to sustain sufficient refuge sizes. If non-Bt seed in a seed mixture is lost after planting, replacing the lost refuge area could be a challenging endeavour.

5.6. Practical and financial considerations

With a separate refuge, adhering to strict refuge requirements is central to the sustainability of the high-dose/refuge strategy. Non-compliance to refuge requirements could increase the risk of resistance development to Bt proteins (Kruger *et al*., 2009). This was most likely the case with *Busseola fusca* (Lepidoptera: Noctuidae) resistance development to Cry1Ab protein in regions of South Africa (Van Rensburg, 2007; Kruger *et al*., 2009). To ensure compliance to refuge requirements, a compliance monitoring program should be implemented (USEPA, 2011) and should be regulated by an appropriate authority. This could mean that additional resources and costs are needed to ensure the sustainability of the high-dose/refuge strategy. In the case of a RIB product, refuge compliance is automatically adhered to. With a random refuge being included as part of the seed mixture, a producer need not plant a separate non-Bt refuge (Davis and Onstad, 2000). Therefore, with a seed mixture, savings in terms of cost and resources could be achieved due to the fact that no refuge compliance monitoring systems is needed (USEPA, 2011). Planting a separate refuge implies increased managerial input, potentially more labour and additional costs, whereas RIB products would most likely be more economical to deploy. However, some of these financial benefits could be offset by a higher purchase price associated with RIB products (Onstad *et al.*, 2011).

Due to the resources, equipment and general capabilities that commercial farming provides, planting of separate refuges should not be too challenging. Therefore commercial farming systems could be more suited to the high-dose/refuge strategy compared to small-scale farming systems. The RIB concept may provide a pest management option for small scale farmers. By having no refuge requirements to adhere to and the ease of which seed mixtures can be deployed means that the RIB concept might be well suited for the needs of small-scale farming. One limitation for small-scale farmers could however be the purchase cost of RIB products and the condition that whole bags of seed should be planted as units. Once bags of seed are opened, there should be no seed stored for the next season.

5.7. Comparing the high-dose/refuge and seed mixture

There is much debate over which IRM strategy is the most appropriate to delay resistance development of target pests. Some studies suggest that a separate refuge might be better in prolonging resistance development, especially if larger refuge sizes are used (e.g. 20 % - 50 %) (Tabashnik, 1994; Onstad and Gould, 1998).

There is, however, concern over the long-term efficacy of seed mixtures in delaying resistance development (Mallet and Porter, 1992; Cohen *et al*., 2000; Onstad and Gould, 1998). However, studies have suggested that deploying a seed mixture is still a better alternative than using no refuge at all (Carrol *et al*., 2012). Although Mallet and Porter (1992) suggested that resistance development could occur faster in a seed mixture than in a Bt only setup, the studies described below suggest otherwise.

Tabashnik (1994) suggested that seed mixtures are always better in delaying resistance development over a broad range of conditions compared to a pure Bt setup. Nevertheless, it was also concluded that a separate refuge could last as long, if not longer, than a seed mixture (Tabashnik, 1994). The reasoning behind this statement is that a separate refuge reduces selection pressure but does not change dominance for resistance (Mallet and Porter, 1992). Also, the proportion of insects feeding on non-Bt plants is equal to, or greater in a separate refuge compared to a seed mixture (Tabashnik, 1994). It was concluded by Tabashnik (1994), that separate refuges are generally better IRM strategies than seed mixtures, depending on the variables associated with pest management and pest biology interactions.

A study conducted by Carrol *et al.* (2013) indicated that a seed mixture deploying a “pyramided gene” (Cry1A.105 + Cry2Ab2) in a 5 % seed ratio was as sufficient as a separate refuge strategy against the Southwestern corn borer (*Diatraea grandiosella*) (Dyer) (Lepidoptera: Crambidae). According to this study, the seed mix refuge could endure the migration of larvae between plants due to *D. grandiosella* being highly susceptible to the proteins expressed in “pyramided” gene hybrids. Due to this effective control, resistance development in cases where seed mixtures are used, could be prolonged in spite of a wide variety of pest movement and refuge assumptions (Carrol *et al.,* 2013). The study also stated that a seed mixture refuge might delay resistance development equally, if not longer, than a comparable size separate refuge if producer non-compliance is taken into account. It is therefore possible to achieve better results in the case of a seed mixture if refuge compliance is not adhered to in a high-dose/refuge strategy.

5.8. Conclusion

Although there are some similarities between the high-dose/refuge and the seed mixture strategies, the differences between them influence the outcomes of their application.

The general consensus seems to be that the use of a separate refuge may prolong the development of resistance of target pests compared to seed mixture strategies. High-dose/refuge strategies can be deployed with relative ease and with all types of Bt crops in any agricultural region in commercial level farming systems. However there is one crucial aspect that affects the sustainability of the high-dose/refuge strategy. It is of utmost importance that refuge requirements be adhered to in order to avoid potential, rapid resistance development. Another important aspect is that monitoring for resistance development is important to identify potential problems as soon as possible. Although seed mixtures are much easier to deploy and refuge requirements are automatically adhered to, the behaviour of pests in terms of migration between plants is of concern. RIB products may be more appropriate for management of pests with reduced migration behaviour or sedentary tendencies. A guaranteed way to ensure the sustainability of Bt crops is to apply it in conjunction with various other IPM strategies (Tabashnik, 1994). As with any other pest control concept Bt crops should be deployed as part of a variety of decision based control methods, regardless of which refuge based strategy is more suitable.

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Chapter 6: Conclusions and recommendations

The study provided new insight into the possible efficacy of the seed mixture strategy to delay resistance development of *Busseola fusca* to Bt maize. Large scale migration was observed in all plantings of maize with different ratios of non-Bt and Bt seed, especially in the experiments in which single-gene Bt maize were studied. Under field conditions, the level of larval survival in plantings done with seed mixtures will depend on whether initial infestation occurs on non-Bt or Bt plants. If infestation occurs on a Bt plant, the pre-migration feeding of larvae plays an important role. Should the larvae ingest sufficient dosages of Bt proteins, effective control should occur. Larvae that disperse off Bt plants and onto non-Bt plants before sufficient quantities of Bt proteins is ingested, could survive and develop into adults.

Initial infestation on non-Bt plants could benefit larval survival. These larvae will be the source of the susceptible adults to mate with the few resistant individuals (Gould, 2000). However, due to the close proximity of non-Bt and Bt plants in seed mixture plantings, movement of larvae could pose two problems. First, if larvae develop to more mature stages on non-Bt plants and then migrate to Bt plants, sub-lethal exposure to Bt proteins could occur (Murphy *et al*., 2010). As was described in chapter 3, larvae were able to survive on plants of a “pyramided” Bt event after they reached a certain age on non-Bt food. If a “pyramided” Bt event is used and feeding larvae are killed within a short period of time before migration to non-Bt plants occur, effective control may be obtained. The second problem would be if larvae migrate from non-Bt on to Bt plants and are then effectively controlled. There is then a reduction in the number of susceptible adults being generated by the random refuge. If this happens, the refuge size of the seed mixture is therefore effectively reduced (Davis and Onstad, 2000).

In this study, seed mixtures of the 5 % ratio were observed to be most efficient in controlling migrating larvae compared to higher percentages. However, at low proportions of non-Bt seed an increased level of selection pressure could be expected, especially if migrating larvae induce a reduced practical refuge size. Increasing the ratio of non-Bt seed in a mixture could decrease the selection pressure and provide larger numbers of susceptible adults, but at the cost of potentially benefitting the survival of migrating larvae. It would seem that in a seed mixture strategy the aim should be to prevent older and larger larvae from being exposed to sub-lethal dosages of Bt proteins.

During the laboratory experiment older larvae that were reared on non-Bt plants and then placed on “pyramided” Bt plants were recovered in greater numbers than expected, although the number of individuals reaching maturity should be further investigated. Especially since larvae surviving on “pyramided” Bt plants was fairly stunted in growth compared to their control and single gene-treatment counterparts. Studying growth and survival of larvae when migration between plants is simulated, under field conditions, could also present new insight into seed mixture efficacy.

Due to the lack of interactions between the seed mixture treatments and the growing seasons, further studies into possible factors influencing levels of stem borer damage to maize plants could be enlightening. Continued research may also provide better information on possible relationships between early and late season damage, in order to revisit the concept of economic threshold levels for *B. fusca* on maize in South Africa.

The late season damage (internodes and maize ears) observed on maize plants was generally not statistically different between the single gene and “pyramid gene” Bt events, however, the latter always had increased tendencies in controlling *B. fusca* numbers and dispersal. This suggests that deployment of “pyramided” gene hybrids could provide enhanced control of *B. fusca* in South Africa. The similarity in damage levels (both early and late season) suggests that various ratios of seed mixtures of between 5 % and 20 % result in similar levels of mortality of migrating larvae. A five percent seed ratio may be the best option in order to control early-season infestation and damage before a proportion of larvae could migrate and cause damage to mature plants.

In the case of the “pyramid” gene event, the absence of whorl damage (chapter 2) to plants and presence of internode damage later in the season (chapter 4) could suggest migration of larvae from non-Bt to Bt plants. It could also be attributed to a reduction in the high-dose efficacy of older Bt plants later in the season. Future studies could investigate possible comparisons in yield loss due to *B. fusca* larvae when the high-dose/refuge and the RIB strategies are deployed with a “pyramid” gene event.

Although there are some similarities between the high-dose/refuge and the seed mixture strategies, the differences between them influence the outcome of their application. The general consensus seems to be that the use of a separate refuge may prolong the development of resistance of target pests compared to seed mixture strategies.

High-dose/refuge strategies can be deployed with relative ease and with all types of Bt crops in any agricultural region in commercial-level farming systems. However, there is one crucial aspect that affects the sustainability of the high-dose/refuge strategy. It is of utmost importance that refuge requirements be adhered to in order to avoid potential, rapid resistance development. Another important aspect is that monitoring for resistance development is important to identify potential problems as soon as possible. Although seed mixtures are much easier to deploy and refuge requirements are automatically adhered to, the behaviour of pest insects in terms of migration between plants is of concern. RIB products may be more appropriate for management of pests with reduced migration behaviour or sedentary tendencies. A guaranteed way to ensure the sustainability of Bt crops and the insecticidal proteins expressed, is to apply it in conjunction with various other IPM strategies (Tabashnik, 1994). Therefore, transgenic crops with insecticidal properties should not be regarded as a single pest management method to be deployed, regardless of which refuge based strategy is better.

The large scale migration of *B. fusca* larvae between plants suggests that the RIB strategy might not be an appropriate method in order to manage resistance development. If refuge requirements are adhered to and proper damage monitoring is applied, the high-dose/refuge strategy may still be the better IRM option against *B. fusca* in South Africa.

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