Base-line susceptibility of *Busseola fusca* for Bt maize in South Africa

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It all starts here ™

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ABSTRACT

Genetically modified maize that express insecticidal proteins (Bt proteins) have been commercialized in South Africa for the control of *Busseola fusca* (Lepidoptera: Noctuidae). *Busseola fusca* has been reported to be resistant to Bt maize (Cry1Ab protein) at several localities in South Africa. Reports of pest infestation in Cry1Ab Bt maize (MON810) are regularly made in several regions, however resistance has only been confirmed in few controlled laboratory experiments. There is an urgent need to evaluate *B*. *fusca* populations in South Africa for their susceptibility to Bt maize. The aim of the study was to screen different populations of *B. fusca* for resistance to Bt maize and to generate baseline data regarding pest susceptibility for South Africa. Results provided an indication of the resistance status of *B. fusca* populations across the maize production area. Stem borer larvae were collected from 11 different field sites in and around the main maize production area of South Africa. Laboratory feeding studies with maize events expressing Cry1Ab (MON810) and Cry1A.105+Cry2Ab2 (MON89034), were conducted to compare pest fitness to that on non-Bt iso-hybrids as control. Different life-history parameters were monitored during the laboratory feeding bioassays. These were: larval survival and mass, LT50, mortality, larval duration, pupation percentage, male and female pupal mass, male and female pupal duration, sex ratio and male and female moth longevity. Large differences in susceptibility were observed between populations. Larval survival of up to 54.8% on MON810 was observed in two populations and no survival was recorded on the MON89034 event. Larval mass for some populations was significantly higher on the non-Bt iso-hybrid compared to the single-gene event. The LT50 for larvae feeding on the non-Bt maize control treatments ranged between 16-33 days compared to those on MON810 treatments with 6-25 days, and MON89034 with 4-8 days. The corrected percentage mortality for a Venda population (susceptible) was 94.16% compared to the known resistant population from Vaalharts at 0%. Larval development period on non-Bt maize was shorter compared to that on the MON810 treatment. No significant difference was observed between the non-Bt and Bt treatment in terms of the pupal mass, sex ratio or moth longevity. This study documented the levels of resistance of *B. fusca* and will allow us to be able to give early-warning if this pest also evolves resistance to the pyramid events which have been launched in South Africa from 2013 onwards.

Keywords: Bt maize, *Busseola fusca*, fitness, resistance, susceptibility.

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Chapter 1: Introduction and literature review

1.1 Maize production and the adoption of Bt maize in South Africa

Maize is one of the most important grain crops in Africa, produced throughout the continent under various environmental conditions (Du Plessis, 2003) on both small and large scale farms (Odendo *et al.*, 2003). In developing countries such as those in Africa, maize is the staple diet for most of the population (Du Plessis, 2003).

The main maize production provinces in South Africa are the Free State (39%), North-West (23%) and Mpumalanga (21%) provinces where maize is generally grown under rain fed conditions. Approximately 10% of South Africa's maize is produced under irrigation (Department of Agriculture, Forestry and Fisheries, 2012).

Different genetically modified (GM) crops have been commercialized in 19 developing countries (ISAAA, 2013). South Africa was the eighth largest producer of genetically modified crops in the world with its cultivation of 2.9 million hectares of GM maize, cotton and soybeans in 2012 (ISAAA, 2013). Approximately 2.73 million hectares of maize is planted in South Africa, of which 2.4 million hectares are genetically modified maize (James, 2013). It is estimated that 680,342 hectares of the GM maize in South Africa, contain a single Bt gene while 1.3 million hectares are planted with maize that have stacked Bt and herbicide tolerant genes (James, 2013). It is estimated that approximately 70% of maize that is planted in South Africa are genetically modified and 43% of that maize is modified to control maize stem borers (Falck-Zepeda *et al.*, 2013).

Bt maize was introduced into South Africa to control the stem borers species, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) (Van Rensburg, 2007). There are several benefits for farmers to plant Bt maize. The most important benefits are the reduction in insecticide usage, more efficient use of chemicals that may result in a higher biodiversity of insects in crop fields, and also reduced problems with insecticide applications (Huesing & English, 2004; Hellmich & Hellmich, 2012; Bessin, 1995; Mwangi & Ely, 2001). The use of GM crops is a sustainable way to rapidly control insect pests for the duration of a season, without the climate and weather having an effect on the control methods itself. Bt crops are also target-specific which means that it does not harm beneficial insects, and can be combined with the use of natural enemies in an integrated pest management strategy (Chien, 2013).

Bt maize was primarily developed to control *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Ostlie *et al.,* 1997) and *Diatraea grandiosella* (Lepidoptera: Crambidae) (Archer *et al.*, 2001) in North America before it was introduced into South Africa (Van Wyk *et al.,* 2009). Bt maize expressing Cry1Ab proteins (MON810 – single gene) was first commercialized in South Africa in 1998 (Van Rensburg, 1999). In 2009 a combination of two new Bt genes (pyramid) was evaluated, MON89034 expressing the Cry1Ab.105 and Cry2Ab2 proteins (Monsanto, 2009), which was approved for cultivation in 2012. Survival of *B*. *fusca* on Bt maize expressing Cry1Ab (MON810) was reported since 2004 (Van Wyk *et al*., 2008) and field resistance in the Christiana region (27º57'S, 25º05'E) was reported in 2006 (Van Rensburg, 2007). After resistance was reported, the process of getting the pyramid gene event approved was started. Compared to MON810, MON89034 is able to control a wider spectrum of lepidopteran pests and assure the durability of Bt maize expressing these Cry proteins (Monsanto, 2009). The combination of the two Cry proteins in a single plant provides a more effective insect resistance management (IRM) strategy since the mode of action differs between the two Cry proteins, due to these having different binding sites on the midgut of the target lepidopteran species (Monsanto, 2009). These hybrids provide farmers the opportunity to successfully control target pests with toxins produced in all plant parts throughout the season (Gould, 1998; Campagne *et al.*, 2013). Van Rensburg (2007) reported that larvae of *B. fusca* feeding on the silks of Bt maize plants containing the single gene (MON810) may pose a threat to the continuous use of Bt, as the higher water content in silks may lead to reduced concentration of the Bt protein.

1.2 *Busseola fusca* biology

Maize in Africa is attacked by many lepidopteran pests which include the African stem borer, *B. fusca*, the spotted stem borer, *C. partellus,* the pink stem borer, *Sesamia calamistis* (Hampson) and the sugar cane borer, *Eldana saccharina* (Walker) (Mailafiya *et al.*, 2009). The most economically important insect pest on maize for South Africa, is *B. fusca* which can cause yield losses between 10–100% and serious grain quality reduction (Van Wyk *et al.*, 2008). *Busseola fusca* is widely distributed over South Africa and it was first thought that the geographical distribution of this pest on maize and sorghum are generally dependent on elevation and that this stem borer is found at elevations greater than 600m above sea level. Sithole (1987) found that temperature, rainfall and humidity were the aspects responsible for the distribution of this stem borer in certain areas in Africa.

The general biology of *B. fusca* does not differ much from the other stem borer species but its larvae are known to migrate to adjacent plant whorls after hatching, causing the distinctive shot hole damage on young leaves. Older larvae migrate to lower parts of the

plant causing tunneling damage and may also migrate to neighboring plants after flowering, making it difficult to control (Harris and Nwanze, 1992).

Busseola fusca larvae go into an overwintering phase from autumn onwards when larvae migrate to the lower parts of the plant (Van Rensburg, 1985). During spring, moths emerge from crop residues to give rise to the first seasonal moth flight (Van Rensburg, 1985). *Busseola fusca* is known to have a characteristic flight pattern of two to three distinct peaks (Fig. 1.1). The first moth flight occurs between October and December, the second flight during the end of January and mid-February and the third from March to May (Van Rensburg *et al*., 1985). The first and second generation of larvae generally attack maize that is in the pre-flowering stages while the third generation larvae attack maize during reproductive growth stages, which is of insignificant economic importance (Van Rensburg 1985).

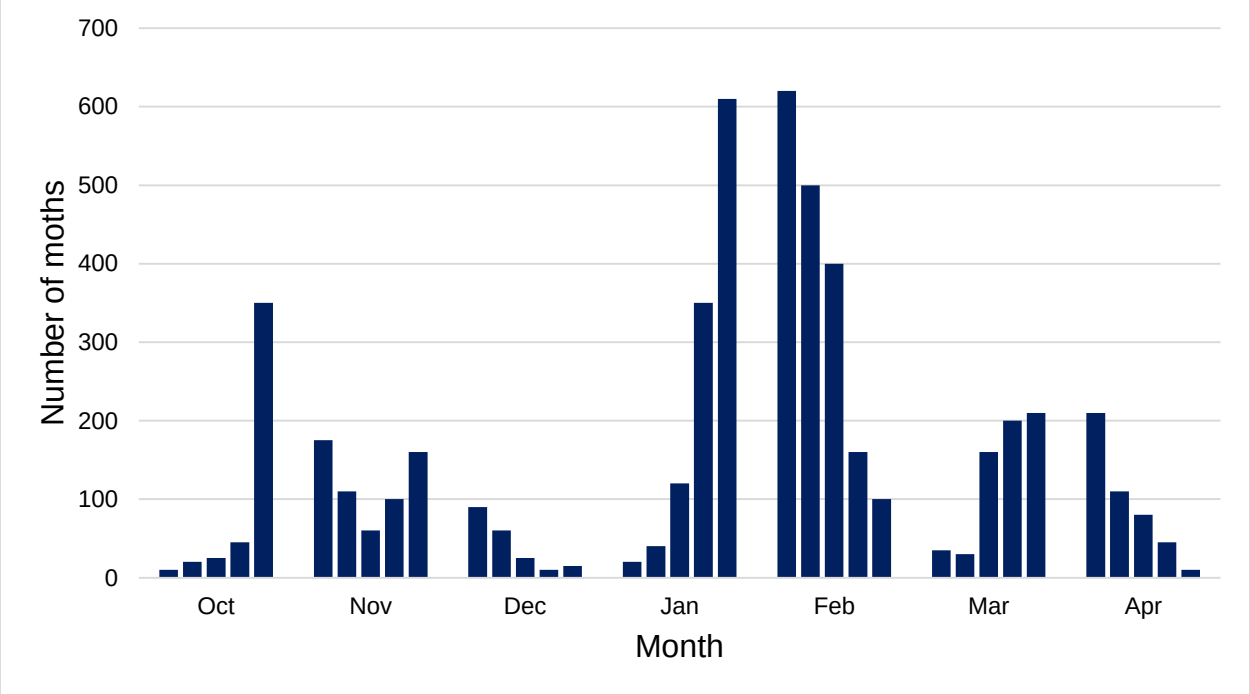

Figure 1.1: Seasonal moth flights of *Busseola fusca* captured in light traps (Based on data obtained from Van Rensburg *et al*., 1985) (each column represents the total number of moths captured).

1.3 Resistance to Bt crops

Insect resistance development to insecticides are common and therefore also a reality for Bt maize. Since the first commercialization of MON810 there were concerns about resistance evolution in target pests (Tabashnik 1994; Gould, 1998). The first field resistance reports were recorded by Van Rensburg (2007) who observed and recorded

survival of *B. fusca* larvae on Bt maize expressing MON810 proteins in the field. These larvae were collected from Bt maize stubble in an irrigated field near Christiana (North-West province) and they also survived in the laboratory on the same single-gene event. However, the definition of the term "field resistance" should be clear in every situation.

The National Research Council (NRC, 1986) defined resistance as a genetically inherited transformation in a population that causes a decline in susceptibility levels of an organism. Tabashnik *et al.* (2009) added to this definition by introducing field-evolved resistance. It was defined as a genetically facilitated decrease in susceptibility of an insect pest population to a specific toxin due to exposure to it, in contrast to field resistance which refers to control/product failure under field conditions caused by field-evolved resistance (Tabashnik *et al.*, 2009). Tabashnik *et al.,* (2009) also stated that it is important to note that inherited low susceptibility and detection of resistance-conferring alleles does not indicate field-evolved resistance. Factors such as resistant allele frequency, increased survival caused by resistance, distribution and density of resistant populations and the pest status of insects will indicate the relationship between field control problems and field-evolved resistance (Tabashnik *et al*., 2009). Sumerford *et al.* (2013) identified several shortcomings in the use of the term "field-evolved resistance" in many definitions as it inadequately states the extent of resistance and changes in Bt product efficacy. A number of factors impact the evolution of resistance in insect pests to Bt maize. The intensity of selection for survival on Bt crops are influenced by contributions from the crop and pest. Variables such as the Bt protein expression and number of different Bt proteins present in the plant as well as the resistance mechanism of the insect, the number of generations exposed to the cry-proteins and genetic make-up of the pest influence evolution of resistance. Ecological aspects such as the size of the refuge, number of alternative hosts, adult movement and refuge compliance must also be taken into consideration (Sumerford *et al*., 2013).

Resistance can also evolve in a laboratory setting and laboratory selected resistance may occur when a heritable decrease in susceptibility to a toxin is observed (Tabashnik *et al.*, 2009). Tabashnik *et al.* (2009) also specified that the process associated with evolution of resistance, arises at population level, where resistance indicate hereditary resistance from the breeding pair and the changes in susceptibility is due to exposure of insect populations to Bt toxins (Sumerford *et al.*, 2013). Unlike Tabashnik *et al.* (2009), Sumerford *et al.* (2013) stated that resistance can be categorized in two forms. The one form of resistance is when deviations are observed in the performance of field collected insects form conventional maize and used in Bt bioassays while the other form of resistance is field collected insects from a Bt crop for bioassays in laboratory setting. The reports associated with the deviations in performance of field collected insects on Bt products are time-based changes in susceptibility of the sampled insects measured in laboratory studies, and provide an early

detection of resistance in a laboratory setting. Sumerford *et al.* (2013) stated that follow-up studies are needed to confirm reports and determine the field relevance of the resistance report. Reports of field collected insects from Bt crops may be seen as an early warning before the occurrence of product failure and needs to be confirmed with field collections.

There are several differences between selection for resistance to commercial insecticides and Bt crops in insect populations. According to Sumerford *et al.* (2013), repeated applications of insecticides with increased doses enable resistance to evolve in insect pests, whereas Bt crops express continuous high doses of Bt toxins, thus making IRM strategies necessary for preserving the technology and its benefits. The term ''product failure'' is not commonly used to describe the phenomenon of resistance but is used by Sumerford *et al.* (2013) to describe control failure observed under field conditions that may lead to the withdrawal of Bt products in some localities (Storer *et al.*, 2010).

According to Carrière *et al.* (2010) there are three conditions necessary for evolution of resistance: 1) there must be variant individuals surviving on Bt crops, 2) inheritance of the resistant genes, and 3) consistent fitness differences in survival on Bt crops.

According to Gould (1998) there are five distinct points in the Bt toxicity pathway inside an insect that could decrease the efficacy of Bt on an insect pest. Firstly, reduced solubilisation of the Bt protein crystal together with a decreased division (split) of the Bt protein into an active fragment may have an influence on the affectivity of Bt. Furthermore, a higher proteolytic digestion of the active fragment, impaired binding of active fragments to the midgut and reduced functional pore formation may all be factors that contribute to the infectivity of Bt on pests. Understanding the physiological changes and processes that take place in the insect pest may provide important information about the possibility of adaptations that could occur in a single gene and whether that gene is dominant or recessively inherited (Gould, 1998). The likelihood of cross-resistance to other Bt toxins should also be investigated as well as whether this characteristic will have an associated fitness cost (Gould, 1998).

Resistance to Bt crops is already a problem in some areas in South Africa where farmers need to use pesticides to control *B. fusca* (Van Rensburg, 2007; Kruger *et* al., 2009; Van den Berg *et al*., 2013) on Bt maize. In cases where field resistance is confirmed in an area, the most feasible solution is to reduce selection pressure by withdrawal of the technology or to implement a high-dose/refuge strategy.

Susceptibility of insect pests can be measured by means of bioassays, collecting field populations to measure resistance as a larval response after the exposure to Bt proteins. Observing different life history parameters such as larval survival and mass, development rate, sex ratio, fecundity and fertility will give an indication of the susceptibility level of the pest. The fitness of a susceptible target insect is influenced by the Bt proteins expressed by the maize plant (Kruger *et al*., 2012). A study done by Kruger *et al.* (2011b) indicated that the mass and development time of resistant larvae that fed on Bt maize (MON810) were negatively affected. Effective resistance management approaches can be developed when there is more knowledge about the fitness of resistant insect pests (Kruger *et al*., 2012).

A lack of refuge compliance and use of a high-dose Bt hybrid may have contributed to field resistance for *B. fusca* (Van Rensburg, 2007; Campagne *et al.*, 2013). A study done by Campagne *et al.* (2013) confirmed the expectation that the assumption of non-recessive resistance was not met in the high dose standards of the MON810 event in South Africa. Furthermore, Campagne *et al.* (2013) indicated that functionally non-recessive resistance will result in a higher density of resistant phenotypes in an insect population and that it will reduce the efficacy of the refuge strategy. The primary goal of resistance monitoring is to detect field-evolved resistance early enough to implement a management strategy before control/product failure occurs (Tabashnik *et al*., 2009).

In this study field resistance is defined as a genetically mediated increase in the ability of a target pest to feed and complete development on a commercial Bt crop under field conditions (Tabashnik *et al*., 2009). Therefore, for the purpose of this study, the term resistance levels will be used to report on the resistance status of different *B*. *fusca* populations collected from the field.

1.4 Delaying insect resistance development

In North America the most successful IRM strategy for sustaining susceptibility in pest populations is the “high dose/refuge” strategy (Tabashnik *et al*., 2013). This strategy was only considered in 1991 when Monsanto had the technology to produce plants with high toxin expressions to kill all susceptible genotypes in a population (Gould, 1998). Four simple strategies were used to delay insect resistance adaptations: 1) a refuge approach with Bt and non-Bt hybrids, 2) stacking and pyramiding of toxins in a single plant, 3) making use of natural enemies in combination with low doses of toxins and 4) expression of toxins in different parts of the plant over different times (Gould, 1998).

Using a population genetic based theory, susceptible individuals in the refuge will mate with resistant individuals that survive on Bt maize, diluting the alleles with resistance in a population (Gould, 2000). The Bt and refuge areas should be appropriately spaced to ensure interaction between resistant and susceptible individuals. According to Gould (2000) an extreme measure is to plant a mixture of Bt and non-Bt seed with a possible result of larvae moving from a non-Bt plant to a Bt plant, reducing the number of larvae that would have escaped the selection impacts of the toxin. A concern with this strategy is that migrating larvae between the Bt and non-Bt plants may lead to resistance evolution (Murphy *et al*., 2010). The fact that *B. fusca* larvae migrate throughout their life cycle to different parts of the host plant and may migrate to neighboring plants (Calatayud *et al*., 2014) makes this strategy inadequate for this specific pest species.

The high dose refers to a high enough Bt dose expressed by the plant, that is sufficient to control approximately 95% of the offspring from the susceptible and resistant individuals that mated and only a few resistant individuals will develop on the Bt plants itself (EPA, 1998). A very important factor essential to this strategy is that recessive resistance is accomplished with the increase in the dose of a Bt toxin (Carrière *et al*., 2010). The objective of this high-dose / refuge strategy is to delay the evolution of resistance by maintaining a susceptible insect population in the refuges on non-Bt plants to mate with resistant insects in the population (Carrière *et al*., 2010). Inter-mating of resistant individuals will result in transmitting resistant genes to future generations causing resistance to spread (Sumerford *et al.,* 2013).

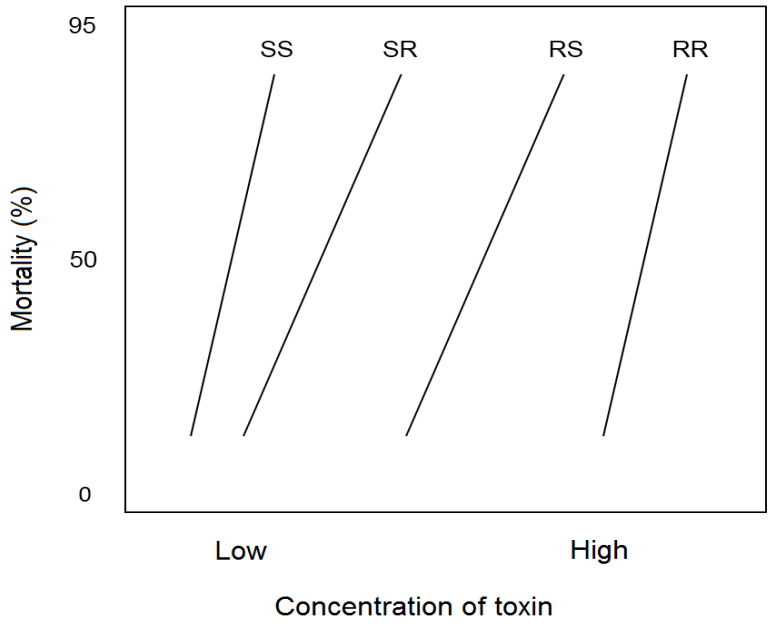

Figure 1.2: The effects of Cry1Ab toxin concentration on different homozygous and heterozygous genotypes of *Busseola fusca* larvae (S: susceptible allele, R: resistant allele) (Onstad & Guse, 2008).

The effect that different concentrations of Bt has on different genotypes of *B. fusca* larvae is indicated in Figure 1.2. The most susceptible (SS) individuals are killed by a low toxin dose. None of the heterozygotes (SR & RS) are killed by low doses of toxin if the resistance is dominant. At high doses of the toxin, SS, SR and RS individuals will be killed and resistance is considered recessive (Onstad & Guse, 2008).

For the high dose/refuge strategy to be successful in South Africa there are essential requirements to be met to maintain effective recessiveness of resistance:

- resistant individuals should mate exclusively with susceptible individuals as random mating could compromise this strategy (Gould, 1998; Carrière *et al*., 2010).
- development time of resistant and susceptible individuals should not differ significantly. It could be possible that resistant individuals emerge later when susceptible individuals have already mated and produced eggs (Gould, 1998). It has also been shown in other studies with *Heliothis virescens* (Lepidoptera: Noctuidae) that resistant individuals may appear less attractive to susceptible individuals due to their size although mating is still possible (Gould, 1998).
- the toxin expressed by the plant must be at a level that results in a functional recessiveness of the resistant trait (Campagne *et al.*, 2013). Generally, it is expected to have approximately three percent of commercial seed that does not express Bt toxins and could compromise the high dose strategy (Gould, 1998).
- according to Gould (1998) the efficacy of the high dose strategy could be compromised when crops have multiple pests that feed on different crops, for instance when an insect is a secondary pest on a crop and ingests an intermediate dose of toxin because it is not the target pest, cross-resistance could develop to the related toxins.

Carrière *et al.* (2010) stated that in the past 14 years only five pests were recorded to have field-evolved resistance to Bt events which indicate that the refuge strategy has successfully delayed resistance.

1.4.1 Pyramid strategies

Pyramiding is one of two main strategies used to delay resistance and reduce genetic variation of resistance in populations (Carrière *et al*., 2010). It entails the use of two or more genes expressed in a single plant for the control of a target pest (Carrière *et al*., 2010; Storer *et al*., 2013). Gould *et al.* (2006) stated that for this strategy to be successful, resistance to the Bt genes need to be recessive, the presence of fitness costs and refuges

are required and that cross-resistance is not caused by the selection of one Bt toxin to another (Carrière *et al*., 2010).

1.4.2 Bt crops as part of integrated pest management

Integrated pest management (IPM) focuses on management strategies which include biological, chemical and cultural control (Kogan, 1998), and also includes plant resistance. These methods are used in a variety of combinations to fit a farmer's profile as a large scale or subsistence farmer to maintain pest population levels below economic injury levels. In an IPM system, Bt maize can be incorporated as plant resistance, the inherent ability of the crop to restrict pest infestations (Dent, 2000). Using Bt maize as the only control agent is not recommended as resistance is already a known problem in certain areas.

1.5 Aims and objectives

The aim of the study was to develop a base-line data set on the level of resistance of different populations of *B. fusca* to Bt maize in South Africa since no data on this is available. The objectives were to screen different populations of *B. fusca* for resistance to Bt maize events commercialized in South Africa and to assess the resistance status of *B. fusca* populations in maize production areas of South Africa.

Resistance can be measured as a response after exposure of the pest to Bt proteins. This response can be described by measuring the following life history parameters:

- larval survival (mortality) and mean mass
- LT_{50} (lethal toxin to kill 50% of the population)
- corrected percentage mortality
- period to pupal development
- pupation percentage per population
- male/female pupal mass
- sex ratio
- moth longevity
- fecundity
- fertility

This dissertation reports on these objectives in different chapters as follows:

- Chapter 2 – The status of resistance of different *Busseola fusca* populations to single-gene and pyramid Bt maize in South Africa.

- Chapter 3 – Conclusion.

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Chapter 2: The status of resistance of different *Busseola fusca* populations to single-gene and pyramid Bt maize in South Africa

Abstract

Transgenic maize expressing Cry proteins have been commercialized in South Africa for the control of *Busseola fusca* (Lepidoptera: Noctuidae). *Busseola fusca* has been reported to be resistant to Bt maize (Cry1Ab protein) at several localities in South Africa and reports of borer infestation in Cry1Ab Bt maize (MON810) are regularly made in several regions. However, resistance has been confirmed with larvae collected from a few of these regions. There is an urgent need to evaluate *B*. *fusca* populations in South Africa for their susceptibility to Bt, both the first-generation single-gene events and the new stacked events. The aim of this study was to screen different populations of *B. fusca* for resistance to Bt maize and to generate baseline data regarding pest susceptibility. Stem borer larvae were collected from 12 different sites in the maize production region of South Africa. Feeding studies in which *B. fusca* larvae were reared on plant tissue of maize events expressing Cry1Ab and Cry1A.105+Cry2Ab2 proteins (pyramid, MON89034), were conducted to compare pest fitness of *B. fusca* larvae reared on non-Bt iso-hybrids. Resistance levels were observed between the populations screened. Larval survival of up to 54.8% was recorded on MON810 plant tissue while no survival was recorded for larvae fed on tissue of the MON89034 event. The number of days until 50% mortality (LT50) recorded for the different populations on non-Bt maize ranged between 16-33 days compared to 6-25 days on MON810 maize and 4-8 days on MON89034 maize. This study provide baseline information on pest susceptibility that can be used in other African countries where Bt maize will be introduced in future.

2.1 Introduction

Transgenic Bt crops that express insecticidal toxins (Cry proteins) are important tools in the management of crop pests. Bt crops have the potential to reduce the use of chemical pesticides (Gould, 1998) but if pests evolve resistance to this technology, the benefits associated with Bt crops will be lost. From the first commercialization of Bt maize, there have been concerns about resistance evolution in target pests such as *Busseola fusca* (Lepidoptera: Noctuidae) (Tabashnik, 1994; Gould, 1998). The first report of *B. fusca* resistance to Bt maize (MON810) came from the Christiana area in South Africa (North-West province) during 2006 (Van Rensburg, 2007). There has however not yet been any reports of *B. fusca* larvae surviving on plants of the stacked event (MON89034) which produces both Cry1Ab and Cry105+Cry2Ab2 proteins.

Resistance is defined as a genetic inherited adaptation in a population that cause lower susceptibility levels in individual pest insects (NRC, 1986). Resistance is measured as a larval response after exposure to Bt proteins and life parameters such as larval survival, mass and development rate are used as indicators of resistance status. Biotic and abiotic factors may affect various aspects of fitness of larval and adult stage individuals and may therefor influence the interpretation of results that could indicate resistance. Life history parameters may be indicative of the adversarial effects caused by biotic and abiotic factors (Kruger *et al.*, 2014). Life history parameters that can be monitored include the following: larval survival, mass, development time, percentage pupation, pupal mass, duration of pupal period, sex ratio, moth longevity, fecundity and fertility.

Horner *et al.* (2003), who studied the effects of Bt maize on life history parameters of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) indicated that it is important to focus on the group of larvae that survive and complete their lifecycles on the Bt crop. Studying and monitoring these resistant individuals that pass resistance genes on to the next generation provide information that can provide insights to resistance risks and proper management systems.

In a study done by Jakka *et al.* (2014) on *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), different life history parameters where monitored and data demonstrated that there were no fitness costs associated with field-evolved resistance in the pest. The only life history parameter that was significantly affected in resistant *S. frugiperda* larvae was larval development time which was prolonged, which resulted in emergence asynchrony between the resistant and susceptible individuals. Crespo *et al.* (2010) also did a fitness study on *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) and observed that resistant individuals had increased development times, reduced pupal mass, lower fertility and a higher number of unsuccessful matings. However, there were still no fitness costs associated with the resistant strain of *O. nubilalis.* Evaluation of the resistance levels of *B. fusca* to MON810 maize has been done for a limited number of localities, before stacked maize became available in South Africa. Kruger *et al.* (2012) compared life history parameters of different resistant and susceptible *B. fusca* populations and determined that Bt maize expressing Cry1Ab proteins had an adverse effect on pupal mass, longevity and reduced fecundity observed in the resistant populations. Further studies done by Kruger *et al*. (2014) determined that for the Vaalharts population collected in that region during 2011, there were no fitness costs associated with resistance when different life history parameters were compared with that of a susceptible population.

It is important to monitor life history parameters because of concerns about evolution of resistance due to the extensive cultivation of Bt maize. Monitoring the fitness of a pest to survive and reproduce provides valuable information that can be used in the management

of insect resistance evolution. *Busseola fusca* is already known to be resistant to Bt maize that express Cry1Ab proteins (Van Ransburg, 2007; Kruger *et al*., 2014). It is therefore important to know if there is any fitness cost present in resistant populations. Fitness costs could possibly play a role in resistance management strategies as it may select against resistance (Carriére & Tabashnik, 2001). The aim of this study was to develop a baseline data set on the level of resistance of different *B. fusca* populations to Bt maize in South Africa. This was done by evaluating larval fitness on different Bt and non-Bt varieties as mentioned in chapter 1.

2.2 Material and methods

All evaluations were done under laboratory conditions, using bioassays in which larvae were reared on tissue of Bt and non-Bt maize plants grown under field conditions. The life history parameters of the different populations of *B. fusca* from different localities across South Africa were compared between localities as well as between individuals from the same population feeding on either Bt or non-Bt maize.

Most of the populations were collected from sites located inside the main maize production area of South Africa (Fig. 2.1). Populations outside of the main maize production area were included since it was assumed that these would be comparatively more susceptible to Bt maize due to reduced selection pressure of resistance evolutions in these areas. The reason for suspecting high levels of susceptibility in areas outside of the main maize production region, is based on the fact that small farmers in these regions do either not plant Bt maize or have not done so for a long time.

2.2.1 Collection and rearing of different *Busseola fusca* populations

Populations of *B. fusca* were collected in non-Bt maize fields in the districts of Potchefstroom (26º30'S; 27º14'E), Grootpan (26º6'S; 26º17'E), Petrusburg (29º7'S; 25º26'E), Venda (23º3'S; 30º3'E), Ventersdorp (26º15'S; 26º47'E), Bothaville (27º24'S; 26º37'E), Bethlehem (28º14'S; 28º18'E), Douglas (29º3'S; 23º53'E), Ficksburg (28º50'S; 27º52'E), Lichtenburg (26º4'S; 25º58'E), Vaalharts 2013 (27º53'S; 24º50'E) and Vaalharts 2014 (27º49'S; 24º49'E) in non-Bt maize fields (Fig. 2.1). These sites are located in and around the main maize production area of South Africa.

The larvae from the Venda region were collected from plants during the growing season while larvae of the other populations were collected as diapause larvae in harvested fields. This was done by uprooting and dissecting the lower parts of the plant.

Approximately 500-1000 diapause larvae were collected during the winter months (2013/2014) from non-Bt maize stubble at each collection site. The larvae were placed in 25 ℓ containers with dry maize leaves and transported to the laboratory. Larvae were stored in rearing chambers maintained between 10-12 °C until spring when maize could be planted in the field to serve as food for larvae in the bioassays.

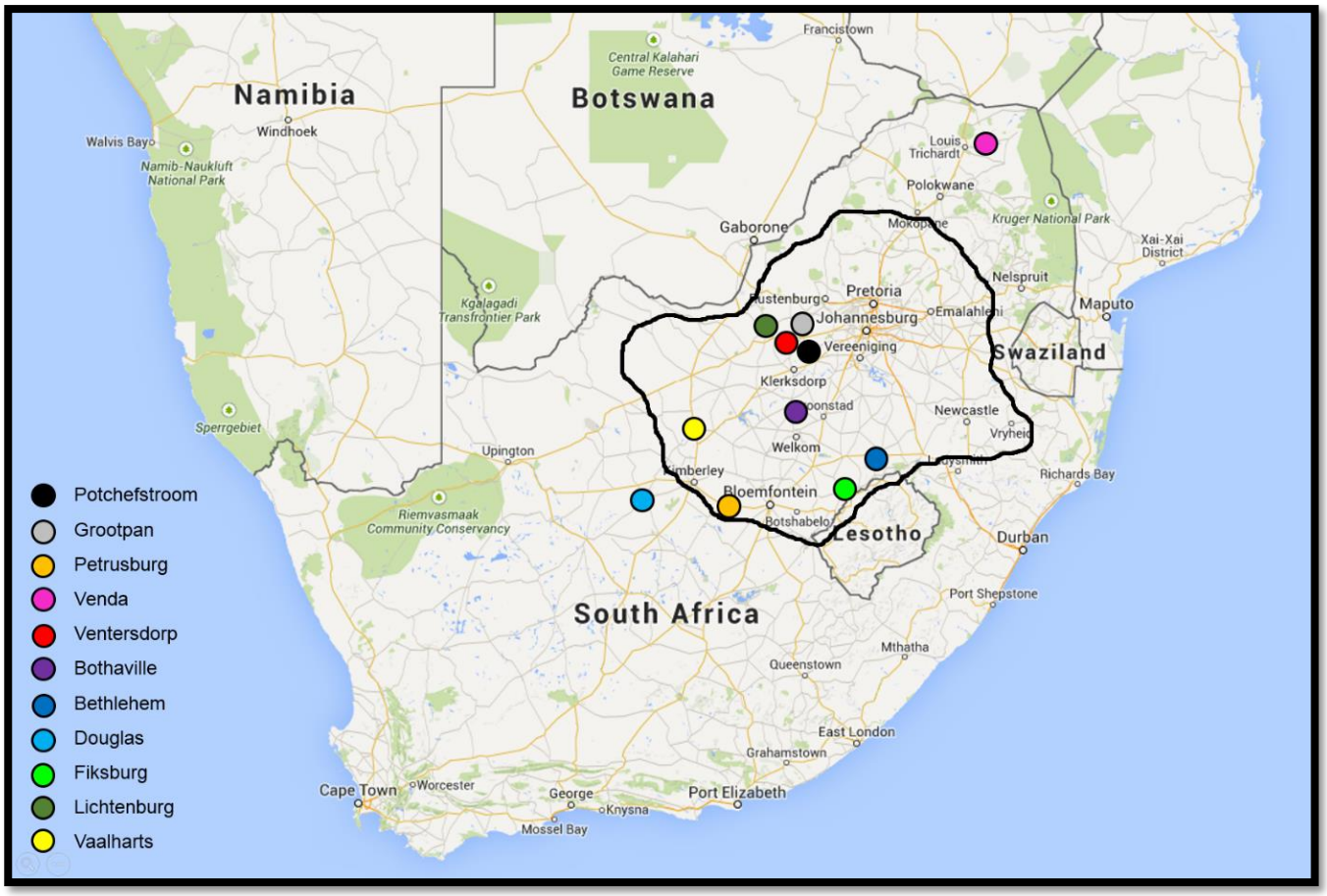

Figure 2.1: Localities where *Busseola fusca* populations where collected in and around the main maize production area of South Africa.

The diapause phase of larvae was terminated following the technique developed by Van Rensburg & Van Rensburg (1993). This involved placing the containers of larvae into a temperature controlled rearing room maintained at an average temperature of 25°C, humidity 60% and a photoperiod of L14:D10. The larvae were sprayed with a fine water mist to imitate the first rainfall in spring and to initiate pupation. Pupae were placed in containers until moths emerged and male and female moths were paired in 2 ℓ plastic bottles to mate and lay eggs. The offspring of 20 breeding pairs of each population was used in the bioassay in which larvae were reared on maize. This was done to ensure that a genetic diverse population of the larvae was used in the study and not only the offspring of a limited number of females.

2.2.2 Feeding bioassay

Larvae were reared on plant tissue of different maize hybrids in the laboratory. These were a non-Bt maize hybrid (control treatment) and the two Bt maize events expressing Cry1Ab protein (MON810) and Cry1A.105+Cry2Ab2 proteins (MON89034). Each treatment was replicated 5 times, with each replicate consisting of 10 containers (100 ml) with 5 larvae per container. These five neonate larvae (F1 generation) were placed on soft maize stem tissue inside plastic aerated containers (Fig. 2.2) and data recorded at regular intervals. Larval survival and mass were determined twice a week over a period of 26 days, when prepupae started to form. Larvae were however provided with maize tissue until pupation and the number of days till pupation recorded. The pupae of the different populations were weighed and sex determined based on their external appearances. Pupae were placed individually in 25 ml containers until moths emerged. Duration of the pupal period was determined from the day pupation commenced until emergence of the moth. Male and female moths were paired in aerated 2 ℓ plastic bottles. Moth longevity was determined form emergence until death.

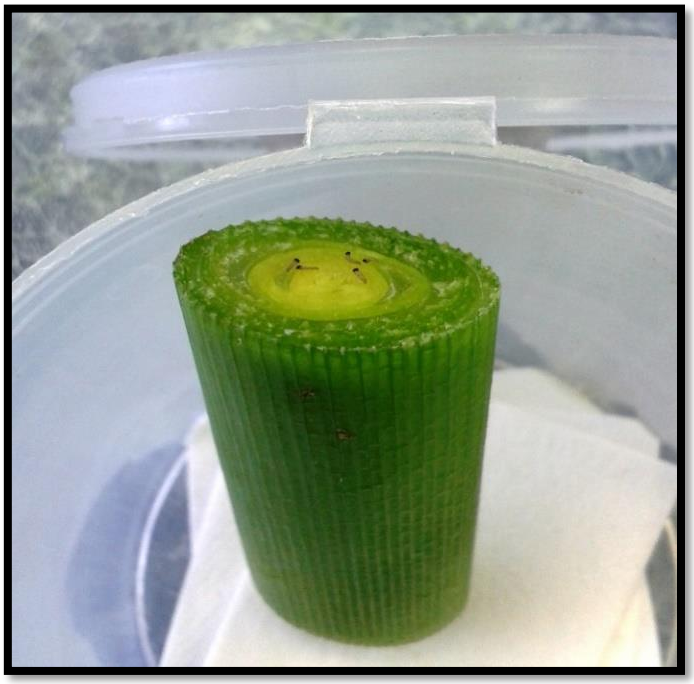

Figure 2.2: Neonate larvae inoculated onto maize stem tissue in a 100 ml container.

Seed of each of the three maize hybrids was planted at 2-weekly intervals throughout the trial period to ensure availability of plant tissue of the same age. These hybrids were planted in blocks under field conditions and tested for the specific Bt protein by means of strip tests (Fig. 2.3) before it was used for the feeding study.

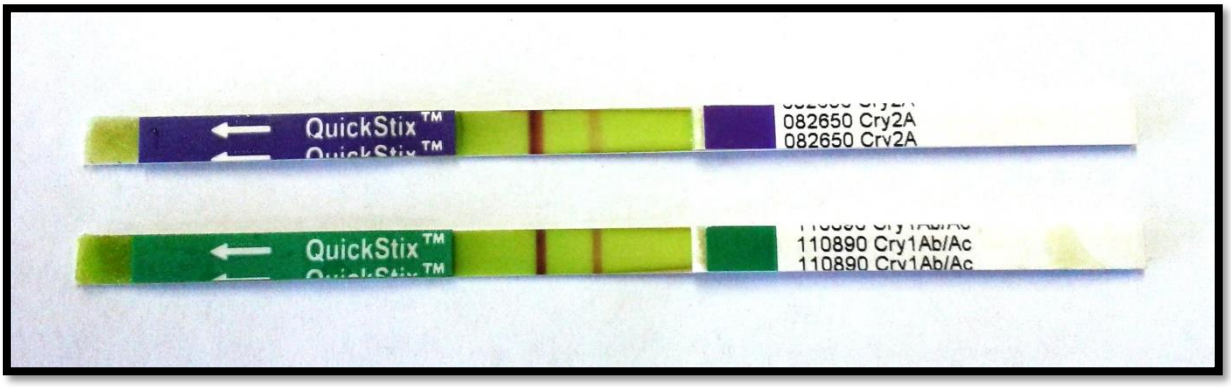

Figure 2.3: Envirologix QuickStix strip tests were used to detect Cry1Ab/Ac and Cry2A proteins to ensure that the correct maize plants were used in feeding bioassays.

2.2.3 Data analysis

The life history parameter data for larval survival and mass were analyzed by means of repeated measures ANOVA and one way ANOVA (Genstat 17th addition). Means were separated by using the Tukey test to correct for multiple comparisons. The number of days until 50% larval mortality (lethal time, LT50) on each of the three hybrids was determined by means of logistic regressions of larval survival over time. Chi-square analysis was used to determine if there were statistically differences in the LT50 and sex ratio between treatments of different populations. Corrected percentage mortality (mortality corrected according to survival on control treatment), larval duration, pupation percentage, male and female pupal mass, pupal period and moth longevity was compared between treatments by means of student t-tests.

2.3 Results

2.3.1 Evaluation of larval survival and growth

Results are provided below in graphical and table format. While observations over time present a comparative picture of mortality, the data at the end of all experiments (26 days) are considered to be more important and to be a more accurate representation of the comparative levels of susceptibility of different stem borer populations.

A significant difference was observed in larval survival between treatments in the Bethlehem population (Fig. 2.4). The larvae feeding on MON810 treatment showed a higher survival compared to the control treatment between day 1 and 15. On day 26 a higher survival percentage was observed on the control treatment compared to survival on MON810. No survival was recorded on MON89034 after day 8. A significant difference in

mean larval mass was observed between treatments, with the larvae feeding on non-Bt maize being nearly three-fold heavier than those feeding on MON810 after 26 days. No larvae survived for longer than 8-12 days on the MON89034 treatment for the Bethlehem or any of the other populations.

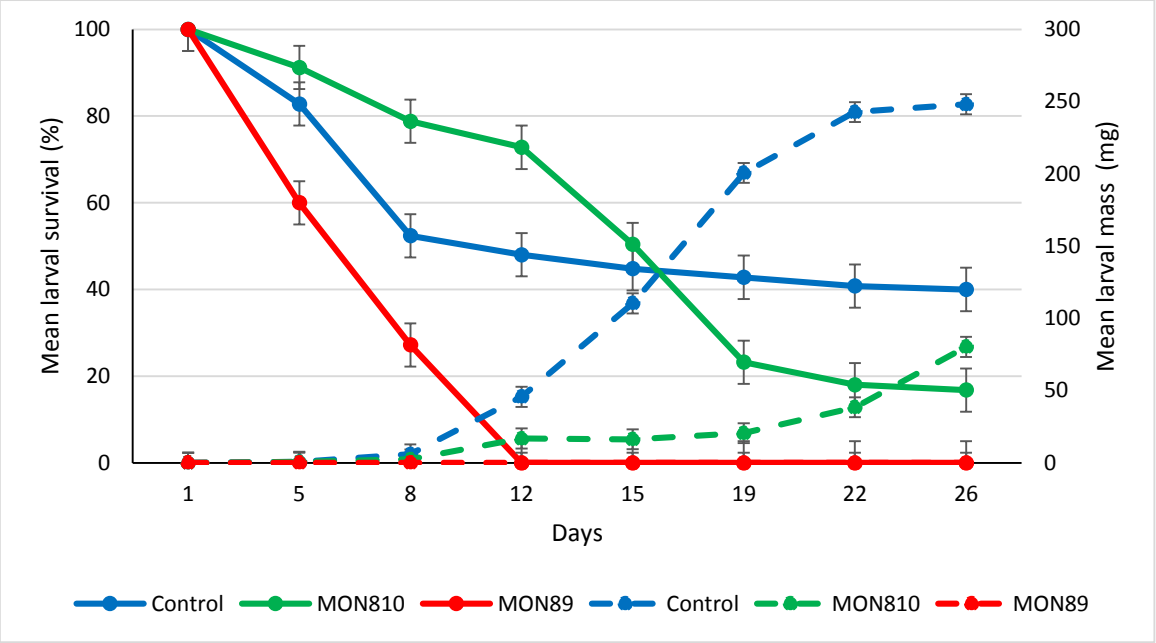

Figure 2.4: Mean larval survival and mass (Bethlehem population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=16.15; P<.001) (Mass: $F_{(14;96)}$=141.26; P<.001) (Bars = Least Significant Difference (LSD-value). Dotted lines indicate larval mass while solid lines indicate larval survival.

Survival of larvae from the Bothaville population differed significantly between the three treatments (Fig. 2.5). There was significantly higher survival on the control treatment compared to MON810 and MON89034. Although the larval survival decreased on MON810 from day 12 onwards there was still a significantly higher percentage larval survival on MON810 compared to MON89034. No survival was recorded on MON89034 after day 15 (0.4%). There was a statistical significant difference observed in mean larval mass between treatments with larvae feeding on non-Bt maize being significantly heavier than those feeding on MON810 after 26 days.

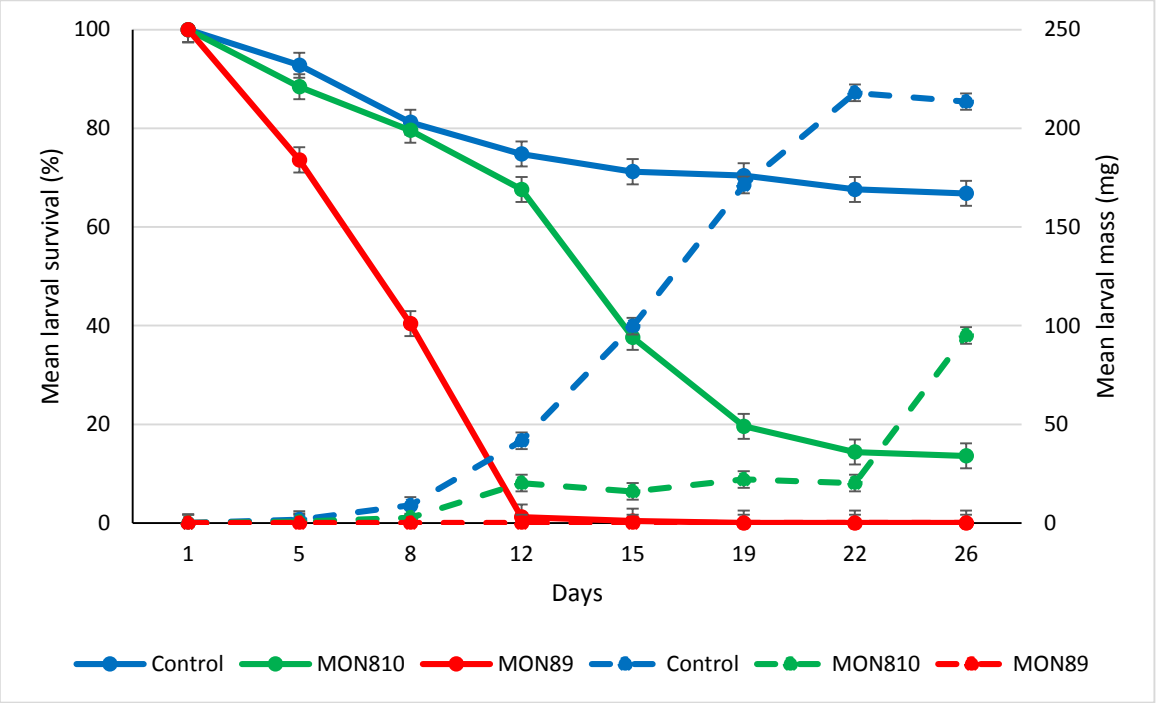

Figure 2.5: Mean larval survival and mass (Bothaville population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=93.76; P<.001) (Mass: $F_{(14;96)}$=279.79; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

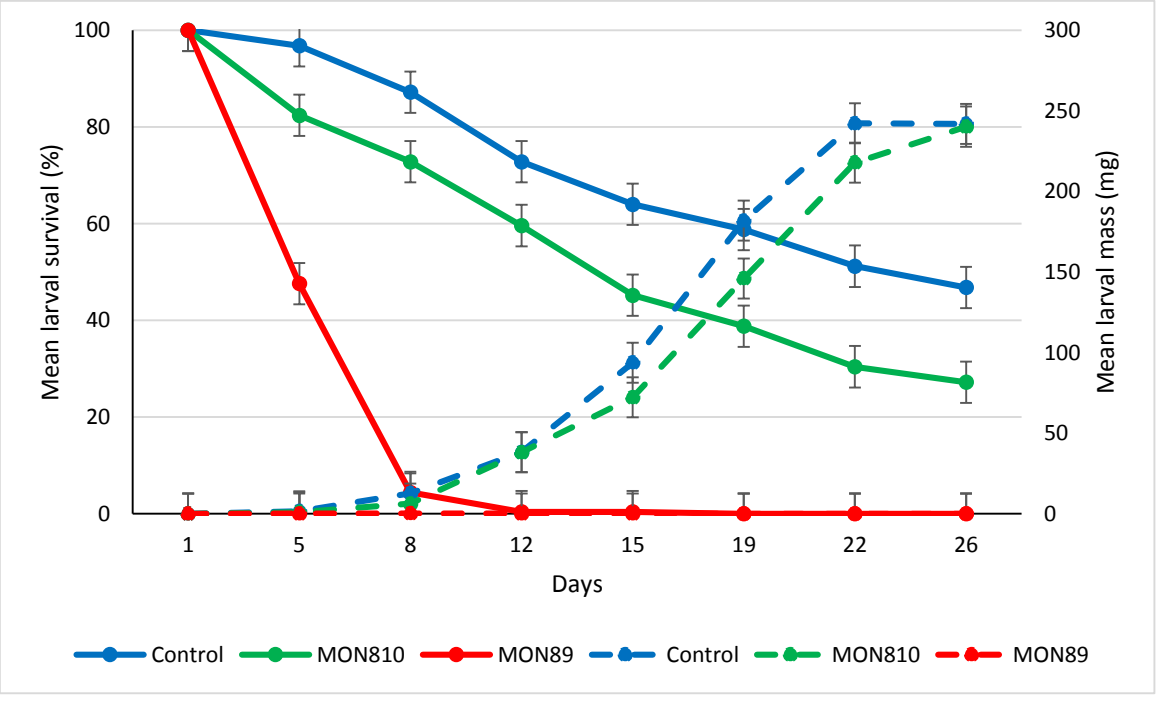

Figure 2.6: Mean larval survival and mass (Douglas population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=19.59; P<.001) (Mass: $F_{(14;96)}$=44.90; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

A statistical significant difference in larval survival between treatments was observed for the Douglas population (Fig. 2.6). Larval survival on the control treatment was significantly higher than on MON810 after 26 days. No larval survival was recorded after day 15 (0.4%) on the MON89034 treatment. No significant difference was observed in mean larval mass between the control and MON810 treatments on day 26, indicating no difference in fitness between these two treatments.

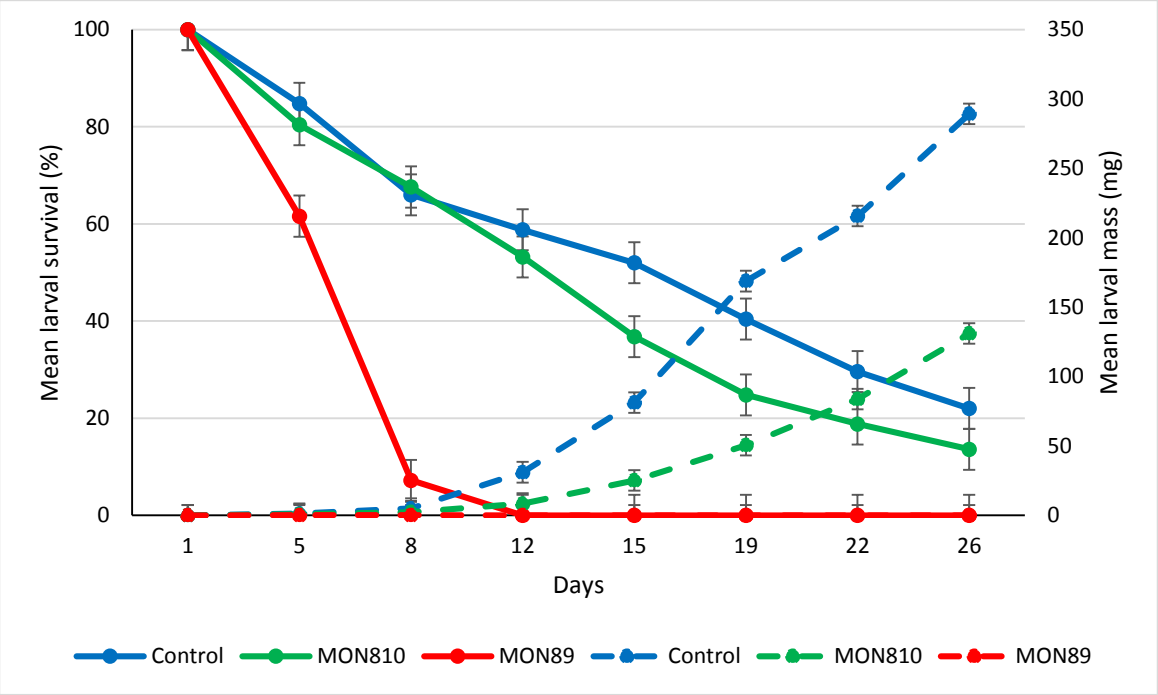

Figure 2.7: Mean larval survival and mass (Ficksburg population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=16.38; P<.001) (Mass: $F_{(14;96)}$=114.88; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

Survival of larvae of the Ficksburg population did not differ significantly between those that fed on non-Bt and MON810 plants (Fig. 2.7). On day eight 7.2% surviving larvae were recorded on the MON89034 treatment but no larvae survived after 8 days. The mean larval mass recorded on the control treatment was significantly higher compared to the MON810 treatment on day 26.

A significant difference was observed in survival of larvae of the Grootpan population with the control treatment having a slightly higher survival compared to MON810 (Fig. 2.8). Similar to other populations, larval survival decreased rapidly on the MON89034 treatment. However, a few surviving larvae were recorded on day 15 (0.4%). Mean larval mass on the control treatment was higher compared to the MON810 treatment.

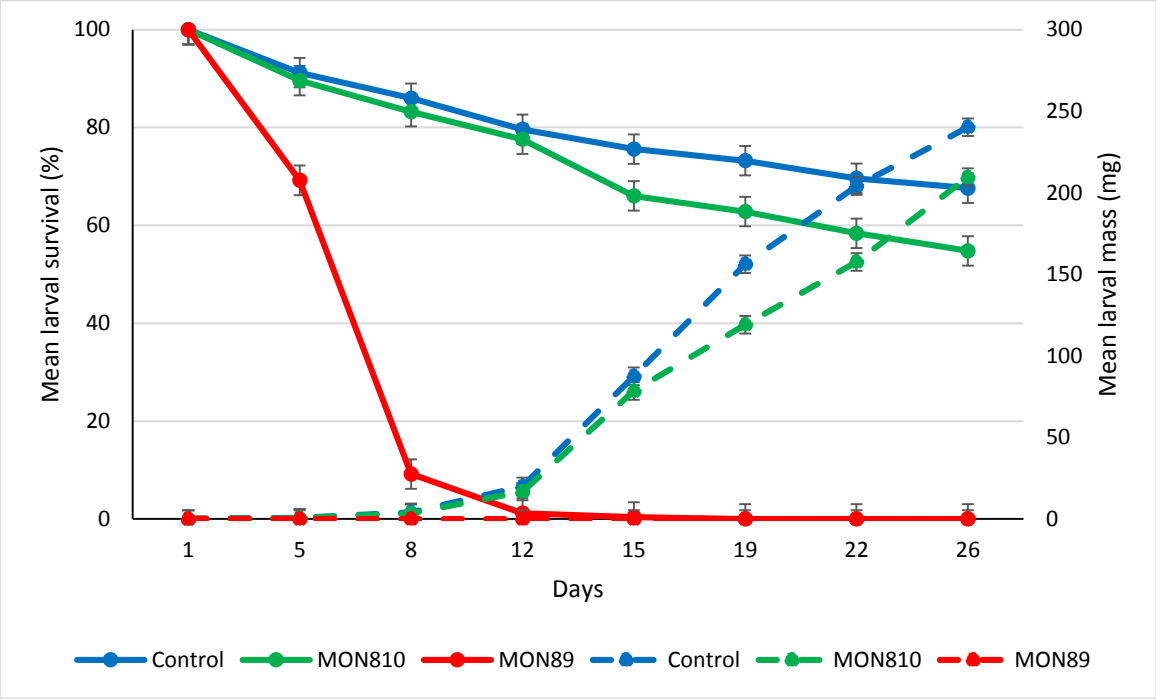

Figure 2.8: Mean larval survival and mass (Grootpan population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=59.52; P<.001) (Mass: $F_{(14;96)}$=190.90; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

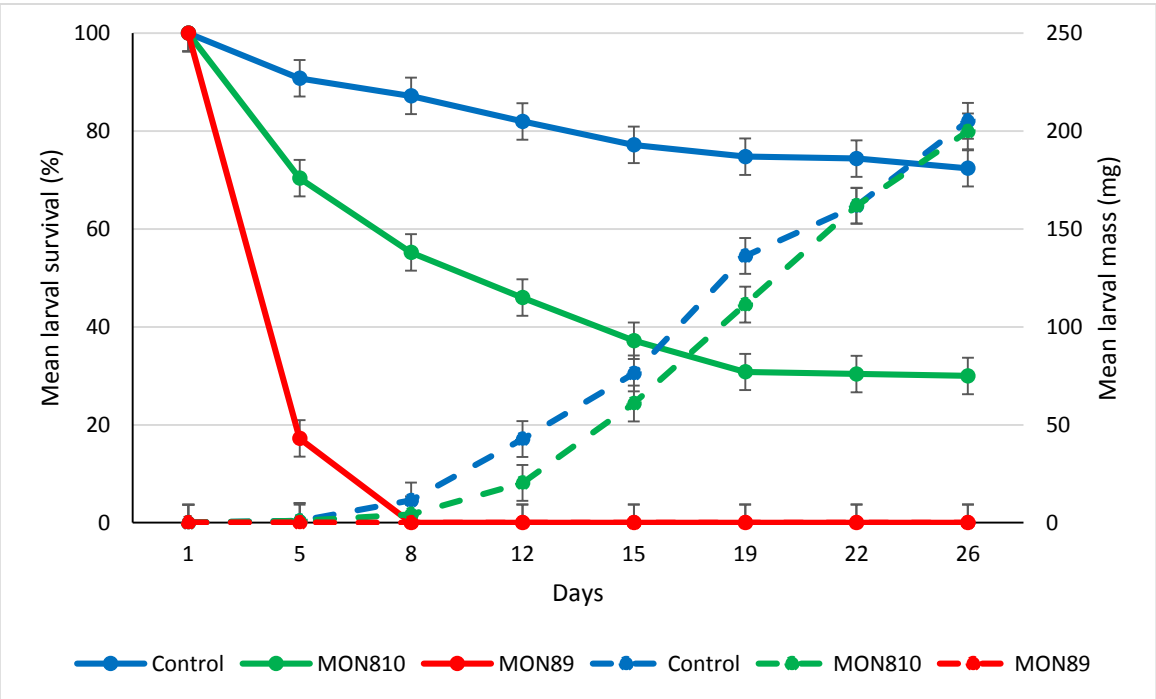

Figure 2.9: Mean larval survival and mass (Lichtenburg population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=31.11; P<.001) (Mass: $F_{(14;96)}$=49.66; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

Statistical significant differences in survival of larvae of the Lichtenburg population were observed between treatments (Fig. 2.9). Significantly higher numbers of larvae survived on non-Bt maize than on MON810 maize after 26 days. While 17.2% survival was recorded on MON89034 on day 5, no survival was observed from 8 days onwards. No significant difference was observed between mean larval mass on the non-Bt and MON810 treatment.

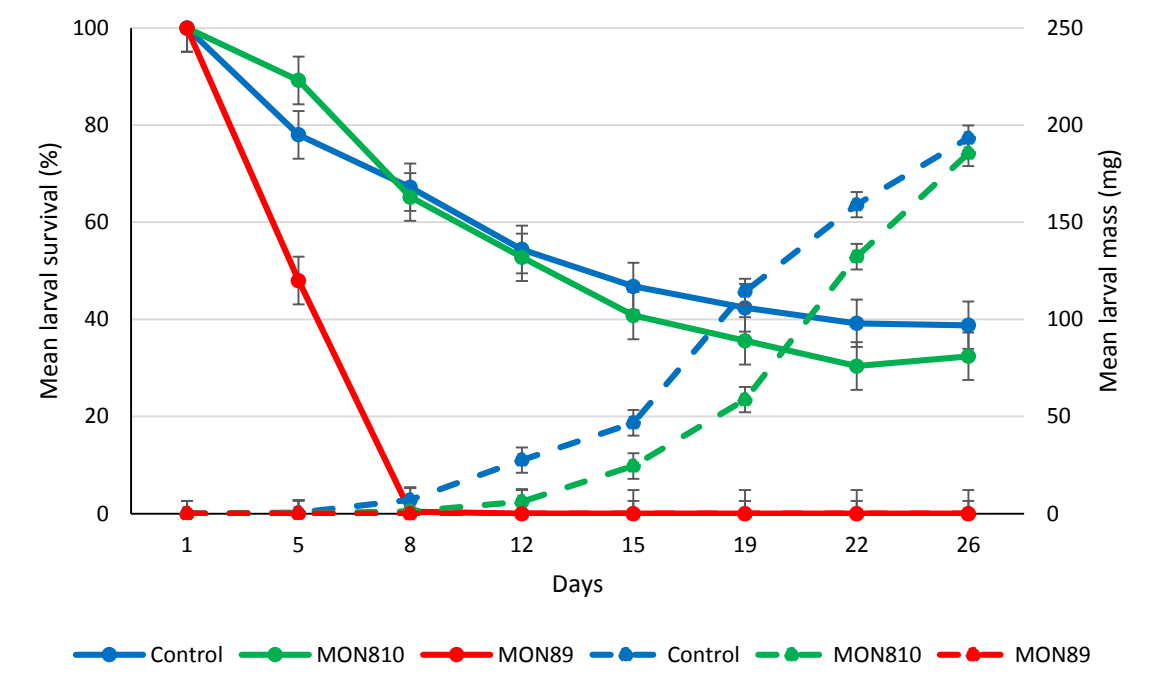

Figure 2.10: Mean larval survival and mass (Petrusburg population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=10.71; P<.001) (Mass: $F_{(14;96)}$=86.54=; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

There was no significant difference in survival of the Petrusburg population between the control and MON810 on day 26 (Fig. 2.10). There was also no difference in mean larval mass between the control and MON810 treatment. Some larvae (0.4%) survived for 8 days on the MON89034 treatment.

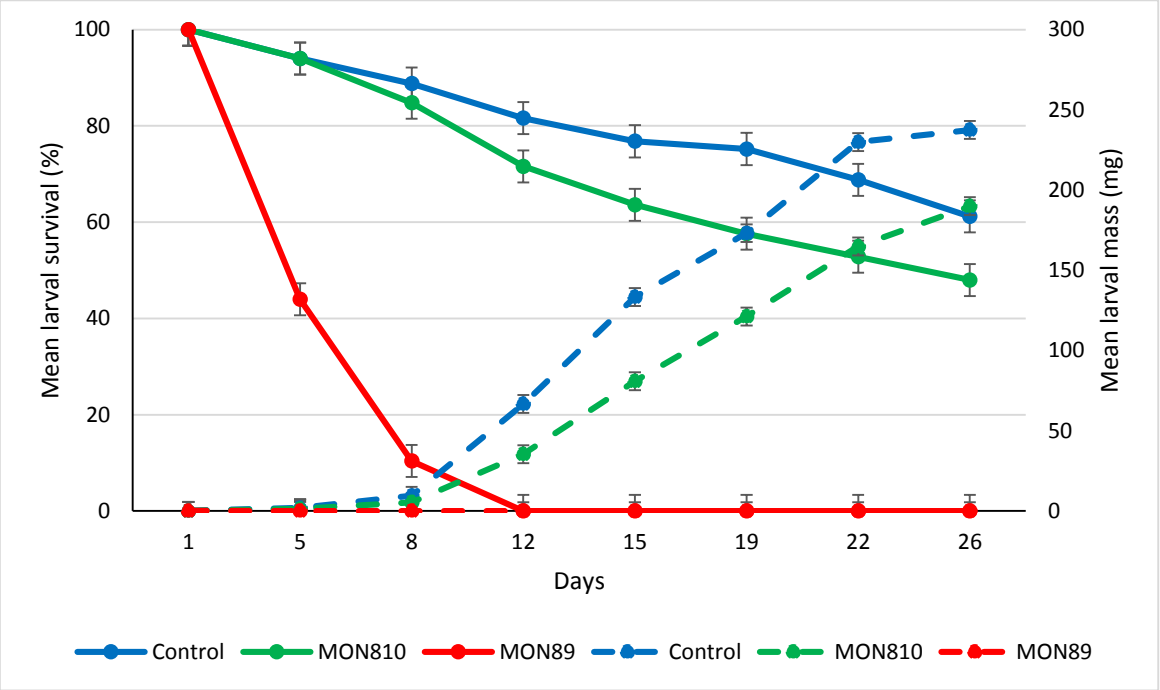

Figure 2.11: Mean larval survival and mass (Potchefstroom population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=38.98; P<.001) (Mass: $F_{(14;96)}$=175.75; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

A statistical significant difference in survival of larvae of the Potchefstroom population was recorded between the control and MON810 treatments (Fig. 2.11). On day 26, larvae feeding in non-Bt maize had a higher survival compared to the MON810 treatment. Larval survival (10.4%) on MON89034 was recorded until day 8. A significantly higher larval mass was recorded for larvae feeding on non-Bt maize than MON810 maize at the end of the experiment.

Survival of larvae of the Vaalharts 2013 population did not differ significantly between the control and MON810 treatments (Fig. 2.12). Although no larvae survived until the end of the experiment, survival was recorded up to day 15 (0.8%) on the MON89034 treatment. Throughout the trial period there was no significant difference in larval mass between the control and MON810 treatment, indicating that the larvae feeding on MON810 was just as fit as larvae in the control treatment.

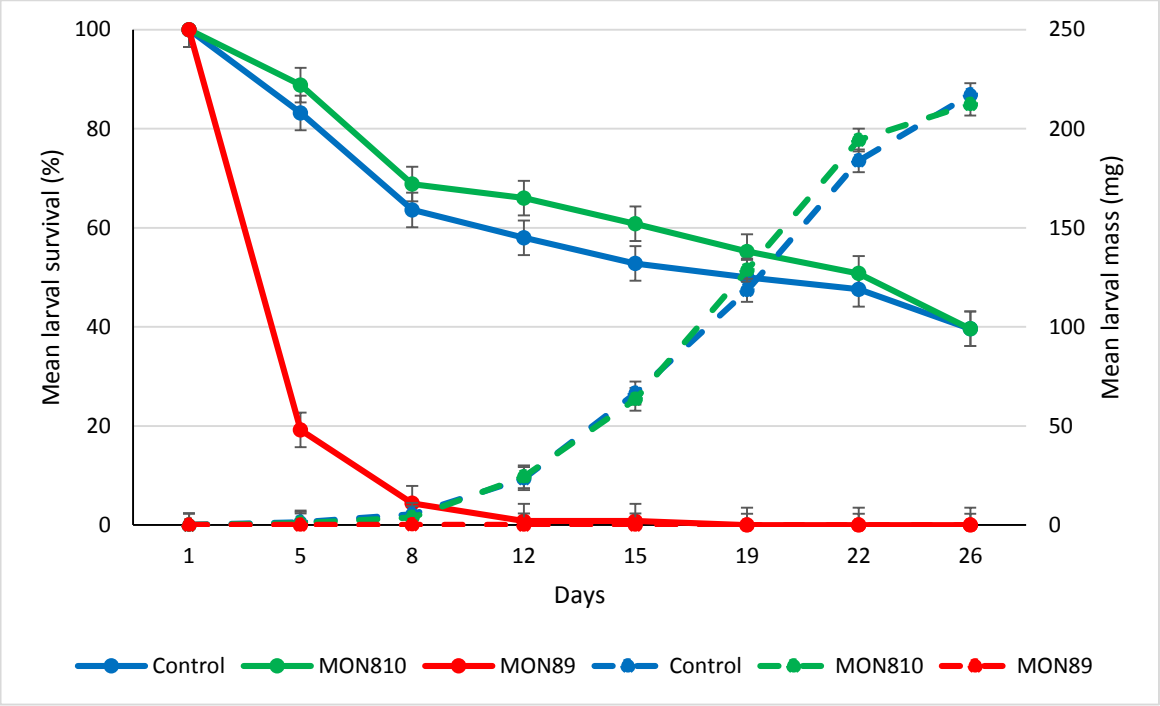

Figure 2.12: Mean larval survival and mass (Vaalharts 2013 population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=24.87; P<.001) (Mass: $F_{(14;96)}$=152.05; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

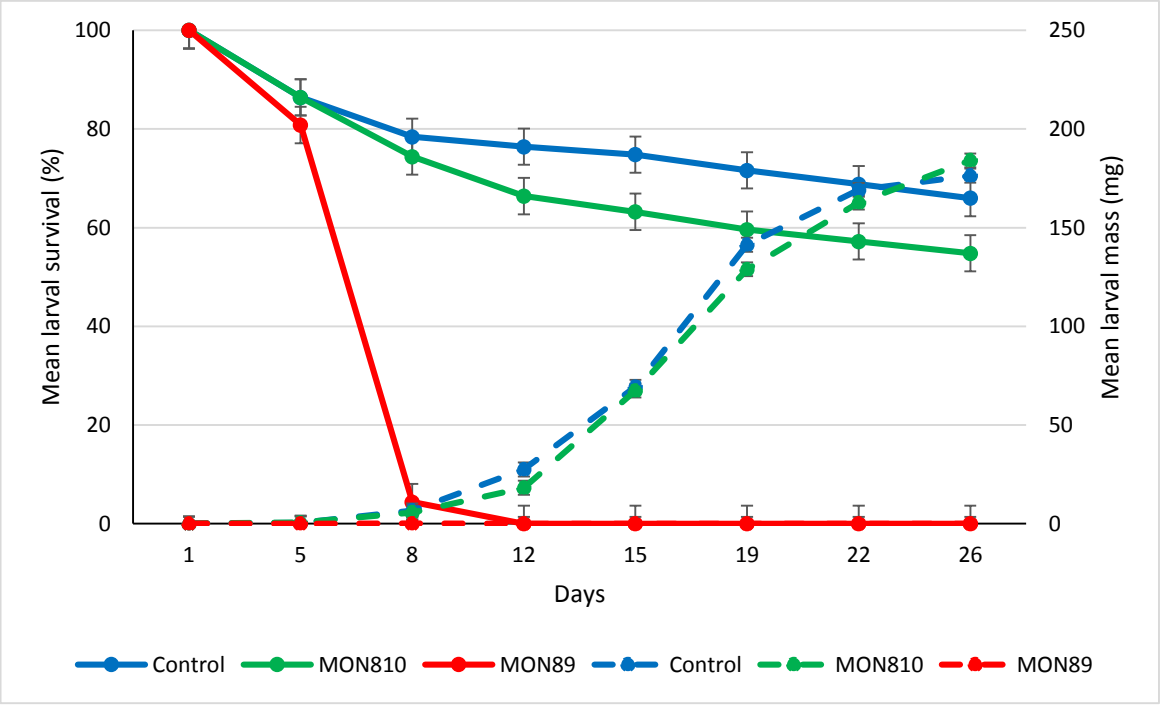

Figure 2.13: Mean larval survival and mass (Vaalharts 2014 population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=44.62; P<.001) (Mass: $F_{(14;96)}$=313.31; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

A significant difference was observed in larval survival between the control and Bt treatments in the case of the Vaalharts 2014 population (Fig. 2.13). On day 26 the larval survival in the control treatment was somewhat higher compared to MON810. No larvae survived for longer than 8 days on the MON89034 treatment. No significant difference was observed in larval mass between the control and MON810 treatments on day 26.

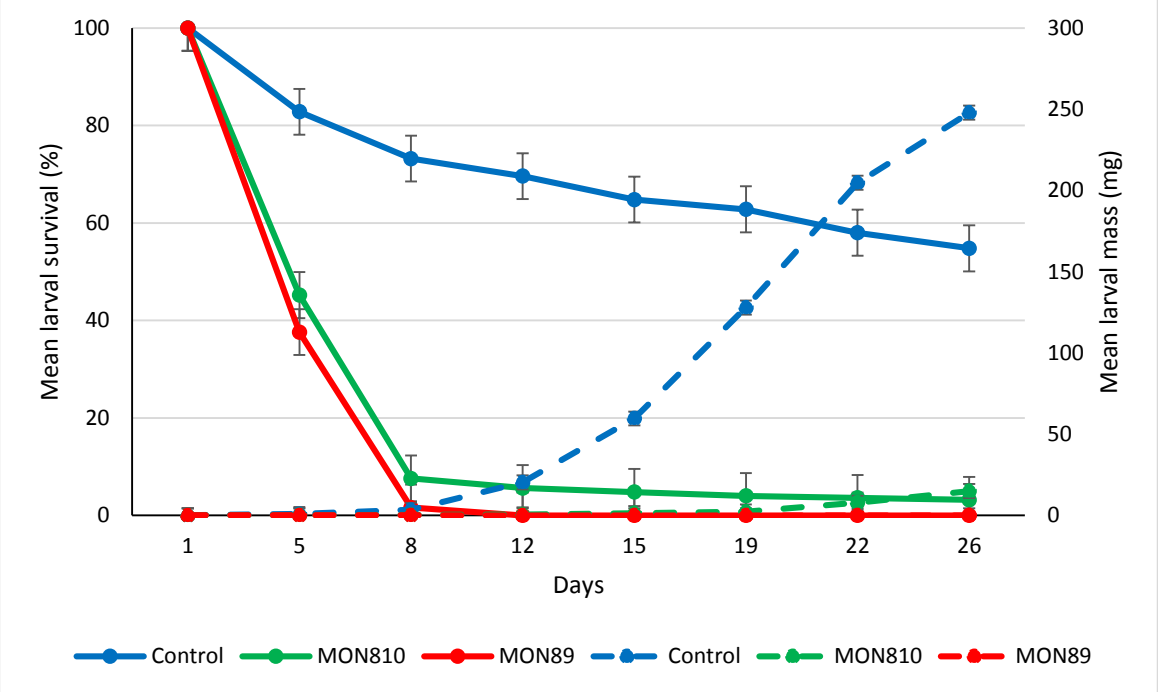

Figure 2.14: Mean larval survival and mass (Venda population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=14.97; P<.001) (Mass: $F_{(14;96)}$=330.26; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

There was a high statistical significant difference in survival between the control and Bt treatments for the Venda population (Fig. 2.14) but survival did not differ between the two Bt treatments. Larval survival at the end of the experiment on non-Bt maize was 55%. While some larvae (<8%) survived on MON810 maize after 8 days, no survival was observed on the MON89034 treatment. No significant difference in mass was observed between the two Bt maize treatments.

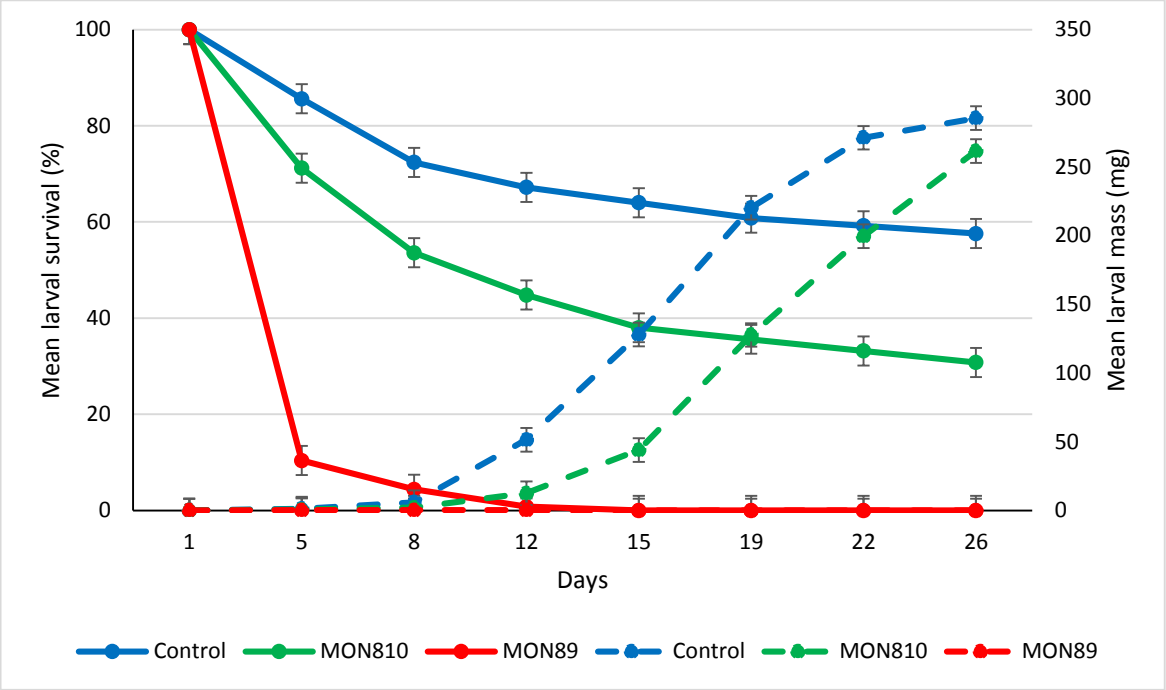

Figure 2.15: Mean larval survival and mass (Ventersdorp population) on Bt and non-Bt maize varieties over time. (Survival: $F_{(14;96)}$=33.98; P<.001) (Mass: $F_{(14;96)}$=122.03; P<.001) (Bars = LSD). Dotted lines indicate larval mass while solid lines indicate larval survival.

Statistically significant differences in survival of larvae of the Ventersdorp population were observed between the different treatments (Fig. 2.15). Larval survival on the MON89034 treatment was recorded only until day 12 (0.8%). A significant difference was observed in larval mass between the different treatments, with larvae feeding on the non-Bt treatment weighing more than those that fed on MON810.

Table 2.1: Comparison of larval survival and mean larval mass of different *Busseola fusca* populations after a feeding period of 26 days on Bt and non-Bt maize.

Population	Larval survival (%)								Mean larval mass (mg)							
	Control		MON810		MON89034		F-value	P-value	Control		MON810		MON89034		F-value	P-value
Bethlehem	40.0	a	16.8	b	0	c	38.99	<.001	248.17	a	80.26	b	0	c	170.86	<.001
Bothaville	66.8	a	13.6	b	0	c	424.85	<.001	213.51	a	95.07	b	0	c	253.84	<.001
Douglas	46.8	a	27.2	b	0	c	158.12	<.001	241.95	a	240.19	a	0	b	108.98	<.001
Ficksburg	22.0	a	13.6	a	0	b	22.33	<.001	289.26	a	131.01	b	0	c	148.44	<.001
Grootpan	67.6	a	54.8	b	0	c	345.39	<.001	240.24	a	209.53	b	0	c	367.21	<.001
Lichtenburg	72.4	a	30.0	b	0	c	117.59	<.001	205.25	a	199.94	a	0	b	224.07	<.001
Petrusburg	38.8	a	32.4	a	0	b	32.78	<.001	193.31	a	185.50	a	0	b	285.85	<.001
Potchefstroom	61.2	a	48.0	b	0	c	150.77	<.001	237.40	a	189.96	b	0	c	775.22	<.001
Vaalharts 2013	39.6	a	39.6	a	0	b	65.56	<.001	217.14	a	212.35	a	0	b	218.08	<.001
Vaalharts 2014	66.0	a	54.8	a	0	b	109.29	<.001	176.33	a	184.08	a	0	b	970.59	<.001
Venda	54.8	a	3.2	b	0	b	164.23	<.001	247.90	a	14.97	b	0	b	436.37	<.001
Ventersdorp	57.6	a	30.8	b	0	c	346.16	<.001	285.56	a	261.61	a	0	b	184.97	<.001

Means within rows followed by different letters differ significantly at P=0.05.

Larval survival and mean mass was compared between treatments within each population after 26 days of feeding (Table 2.1). For 4 of the 12 populations (Ficksburg, Petrusburg, Vaalharts 2013 and Vaalharts 2014) no significant differences in larval survival were observed between the non-Bt and MON810 treatments. However, for 6 of the 12 populations (Douglas, Lichtenburg, Petrusburg, Vaalharts 2013, Vaalharts 2014 and Ventersdorp) no differences in mean larval mass were observed between the non-Bt and MON810 treatments after 26 days. Only in the case of the Venda population did survival and mean larval mass not differ significantly between the two Bt treatments.

Larval survival and mean mass was also compared between populations (Table 2.2). Larval survival in the control treatment ranged between 22% (Ficksburg population) to 72.4 % (Lichtenburg population) on day 26. The highest larval survival after 26 days on MON810 was recorded from the Grootpan and Vaalharts 2014 populations with 54.8% compared to Venda with a larval survival percentage of 3.2%. In many cases larval survival recorded on the MON810 treatment for the Grootpan and Vaalharts 2014 populations was much higher than survival of some populations on non-Bt maize (Bethlehem, Douglas, Ficksburg, and Petrusburg).

Larval mass is an important parameter to determine whether a population can be identified as resistant and a good indicator of fitness of a population (Kruger *et al.*, 2014). Larger larvae are more fit and will develop into large reproducing adults that can give rise to a greater number of offspring. The mean larval mass recorded on non-Bt maize ranged between176.33 mg for the Vaalharts 2013 population to 289.26 mg for the Ficksburg population. On the MON810 treatment larval mass ranged from 14.97 mg for the Venda population to 261.61 mg for the Ventersdorp population. Mean larval mass recorded from some of the MON810 treatments were higher than that recorded on some of the non-Bt control treatments.

Table 2.2: Comparison of larval survival and mean larval mass between different *Busseola fusca* populations after 26 days of feeding on Bt and non-Bt maize.

Population	Larval survival (%)				Mean larval mass (mg)			
	Control		MON810		Control		MON810	
Bethlehem	40.0	bc	16.8	abc	248.17	cde	80.26	b
Bothaville	66.8	e	13.6	ab	213.51	abc	95.07	b
Douglas	46.8	bcd	27.2	bcd	241.95	cde	240.19	ef
Ficksburg	22.0	a	13.6	ab	289.26	e	131.01	bc
Grootpan	67.6	e	54.8	f	240.24	bcd	209.53	def
Lichtenburg	72.4	e	30	cd	205.25	abc	199.94	de
Petrusburg	38.8	ab	32.4	d	193.31	ab	185.50	d
Potchefstroom	61.2	de	48	ef	237.40	bc	189.96	de
Vaalharts 2013	39.6	ab	39.6	de	217.14	abc	212.35	def
Vaalharts 2014	66.0	e	54.8	f	176.33	a	184.08	cd
Venda	54.8	bcde	3.2	a	247.90	cde	14.97	a
Ventersdorp	57.6	cde	30.8	d	285.56	de	261.61	f
	F	P	F	P	F	P	F	P
	17.76	<.001	35.22	<.001	11.95	<.001	41.64	<.001

Means within columns with different letters differ significantly at P=0.05

The LT50 values, which indicate the number of days until 50% mortality of larvae in each of the populations, are provided in Table 2.3. The LT50 values on non-Bt maize in this study ranged between 16 days for the Ficksburg population and 35 days for the Lichtenburg population. In the MON810 treatment the LT50 ranged between 6 days for the Venda population to 25 days for the Vaalharts 2014 and Grootpan populations. In the MON89034 treatments the LT50 was between 4 days (Venda) and 8 days (Bothaville).

Table 2.3: LT50 values of the different populations of *Busseola fusca* feeding on Bt and non-Bt under laboratory conditions.

Population	LT50 (days)								
	Control (95% fiducial limits)	**Chi-square**	**P - value**	**MON810 (95% fiducial limits)**	**Chi-square**	**P-value**	**MON89034 (95% fiducial limits)**	**Chi-square**	**P-value**
Bethlehem	16.52 (14.24 – 19.25)	406.14	<0.0001	15.47 (14.62 – 16.35)	190.03	<0.0001	6.07 (5.68 – 6.45)	143.23	<0.0001
Bothaville	30.23 (26.81 – 35.78)	165.53	<0.0001	14.29 (13.60 – 14.98)	131.39	<0.0001	7.01 (6.67 – 7.26)	59.08	.016
Douglas	22.28 (20.79 – 24.16)	197.93	<0.0001	16.08 (14.87 – 17.37)	212.74	<0.0001	5.00 (3.64 – 6.06)	1285.9	<0.0001
Ficksburg	15.91 (14.60 – 17.32)	264.47	<0.0001	13.31 (12.47 – 14.14)	143.97	<0.0001	5.50 (5.35 – 5.65)	24.34	.958
Grootpan	31.32 (28.00 – 36.51)	139.77	<0.0001	24.94 (23.25 – 27.13)	110.89	<0.0001	5.85 (5.33 – 6.37)	346.60	<0.0001
Lichtenburg	34.68 (29.48 – 44.87)	218.46	<0.0001	13.50 (11.89 – 15.11)	261.94	<0.0001	3.85 (3.67 – 4.02)	14.42	1.00
Petrusburg	17.17 (15.27 – 19.42)	324.10	<0.0001	15.67 (14.29 – 17.14)	261.61	<0.0001	4.94 (4.79 – 5.07)	56.58	0.27
Potchefstroom	28.84 (26.99 – 31.26)	75.16	<0.0001	22.48 (20.88 – 24.53)	190.95	<0.0001	5.02 (4.76 – 5.27)	77.83	<0.0001
Vaalharts 2013	19.01 (17.24 – 21.22)	225.90	<0.0001	20.69 (19.03 – 22.80)	201.62	<0.0001	4.02 (-6.65 – 6.73)	3545.3	<0.0001
Vaalharts 2014	32.23 (28.41 – 38.43)	125.84	<0.0001	24.30 (21.68 – 28.30)	235.74	<0.0001	6.03 (5.89 – 6.17)	25.44	.941
Venda	25.22 (22.20 – 30.12)	254.33	<0.0001	5.15 (2.49 – 7.12)	1422.7	<0.0001	4.67 (4.47 – 4.83)	61.71	.009
Ventersdorp	25.49 (22.76 – 29.65)	184.75	<0.0001	13.89 (12.18 – 15.62)	267.73	<0.0001	3.72 (-0.058 – 5.38)	2447.3	<0.0001

The corrected percentage mortality (Table 2.4) calculated for all the populations provided a good indication of the resistance levels for both the single and stacked gene Bt maize events. No larvae survived on MON89034 for longer than 15 days. The Petrusburg and two Vaalharts populations, on MON810 maize had the lowest corrected percentage mortality while the populations from Bothaville and Venda had the highest mortality. This shows that the Petrusburg and Vaalharts populations had high levels of resistance to MON810 maize and that large numbers of larvae were able to complete their lifecycles on maize plants that express Cry1Ab protein. Larval survival of populations such as those from Grootpan and Potchefstroom, for which low corrected percentage mortalities were observed, indicated that problems with field resistance may also be experienced at these localities in future. The highest corrected percentage mortality was recorded for the Venda population. This population is considered to still be a susceptible population because of this high level of larval mortality. The Bothaville population also had a high mortality percentage, indicating susceptibility.

Table 2.4: The corrected percentage mortality of *Busseola fusca* larvae calculated for each population in the different Bt maize treatments.

Population	**Corrected % mortality**		**t-value df (4)**	**P - value**
	MON810	**MON89034**		
Bethlehem	58	100	-6.33	<0.0001
Bothaville	79.64	100	-7.90	<0.0001
Douglas	41.88	100	-13.88	<0.0001
Ficksburg	38.18	100	-7.01	<0.0001
Grootpan	18.93	100	-22.68	<0.0001
Lichtenburg	58.56	100	-14.30	<0.0001
Petrusburg	16.49	100	-13.99	<0.0001
Potchefstroom	21.57	100	-12.48	<0.0001
Vaalharts 2013	0	100	-10.52	<0.0001
Vaalharts 2014	16.97	100	-10.31	<0.0001
Venda	94.16	100	-2.36	<0.0001
Ventersdorp	46.53	100	-16.61	<0.0001

The different levels of resistance between the different *B. fusca* populations are indicated in Figure 2.16. Venda and Bothaville were the most susceptible populations and had the highest mortality percentages compared to the Vaalharts 2013 population with no mortality recorded on the MON810 treatment.

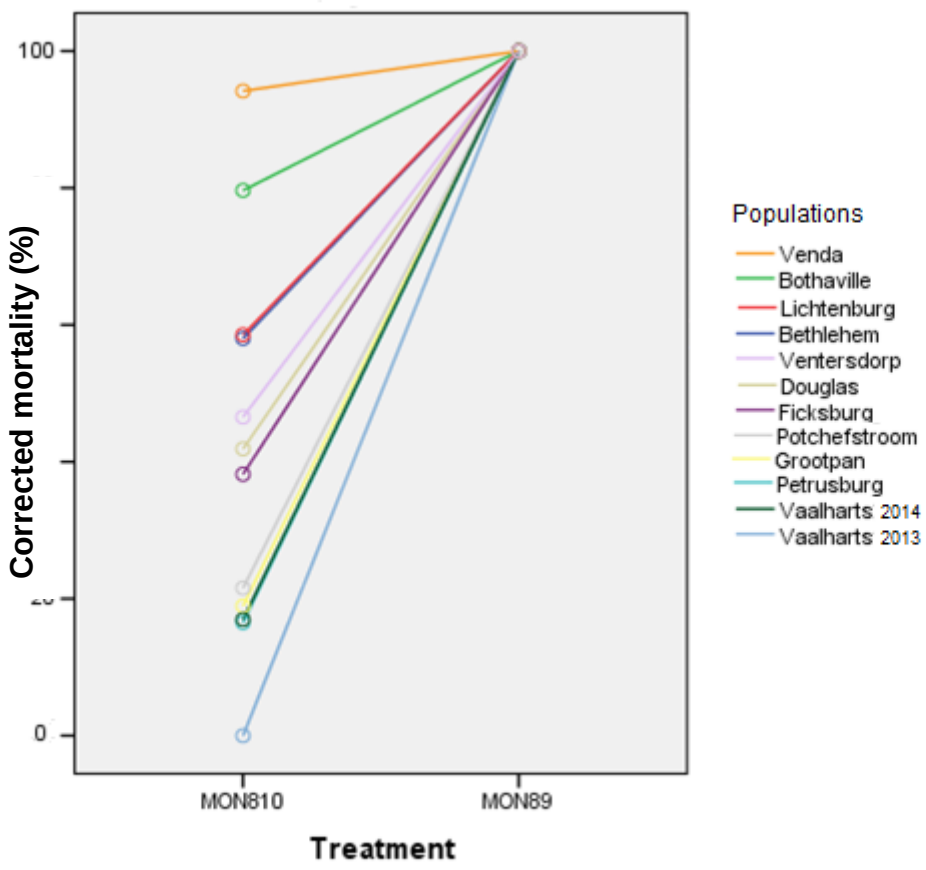

Figure 2.16: The corrected percentage larval mortality calculated for different populations of *Busseola fusca* collected at different localities in South Africa.

Duration of the larval period was shorter on the non-Bt treatment compared to the MON810 treatment (Table 2.5). On non-Bt maize larval duration ranged between 34 and 47 days compared to 33 to 55 days on the MON810 treatment. Larval duration period until pupation could not be determined for the Venda and Bothaville populations on MON810 treatment since no larvae pupated. On the MON810 treatment it was noteworthy to note that both Vaalharts populations had the shortest larval development periods compared to other populations on Bt maize as well as the non-Bt treatments.

2.3.2 Evaluation of larval development on Bt and non-Bt maize

Table 2.5: Mean duration of the larval period (days) of *Busseola fusca* populations on non-Bt and Bt treatments.

Population	Larval duration (number of days) (SE)		t-value (df)	P-value
	Control	MON810		
Bethlehem	44.22 (1.24)	49.33 (1.22)	-1.980 (59)	0.05
Bothaville	36.82 (1.38)	*	17.771 (30)	1.79
Douglas	33.43 (0.88)	46.78 (2.73)	-5.910 (70)	1.13
Ficksburg	43.33 (1.33)	54.7 (4.36)	-1.380 (11)	0.20
Grootpan	43.91 (1.08)	51.85 (1.88)	-3.924 (97)	0.0001
Lichtenburg	40.95 (0.86)	39.62 (0.96)	1.010 (65)	0.32
Petrusburg	35.30 (1.25)	41.70 (1.27)	-3.176 (38)	0.003
Potchefstroom	40.68 (1.12)	45.84 (1.69)	-2.639 (113)	0.01
Vaalharts 2013	33.08 (0.77)	32.92 (1.28)	0.112 (36)	0.91
Vaalharts 2014	46.29 (2.66)	32.25 (5.66)	2.572 (9)	0.03
Venda	39.31 (0.79)	*	18.15 (83)	1.25
Ventersdorp	35.52 (0.63)	36.11 (0.95)	-0.500 (132)	0.62

* SE = Standard error

2.3.3 Pupal development

Only populations of which significant numbers of larvae pupated were used in assessment of the effect of Bt maize on this life history parameter. Percentage pupation was compared between treatments for each population and significant differences were observed (Table 2.6). The highest incidence of pupation on the control treatment was observed for the Ventersdorp population with 39.2% pupation. On the MON810 treatment the Potchefstroom population had the highest pupation with 19.6%. No pupation was recorded on the MON89034 treatment. In all of the populations, significant differences in percentage pupation were observed between the control and MON810 treatments, except for the Potchefstroom population.

Pupation percentage of larvae that fed on Bt and non-Bt maize

Table 2.6: Pupation (%) of different *Busseola fusca* populations after larval feeding on non-Bt and Bt maize.

Population	Pupation (%)							
	Control		MON810		MON89034		t-value	P-value
	%	Pupae	%	Pupae	%	Pupae		
Bethlehem	19.6	49	4.8	12	0	0	25.56	<0.001
Douglas	19.6	49	9.2	23	0	0	10.97	<0.001
Grootpan	26	65	13.6	34	0	0	12.10	<0.001
Lichtenburg	16.4	41	10.4	26	0	0	3.88	0.049
Petrusburg	10.8	27	5.2	13	0	0	5.33	0.021
Potchefstroom	26.4	66	19.6	49	0	0	3.26	0.071
Vaalharts 2013	10.4	26	4.8	12	0	0	5.58	0.018
Venda	30	75	0	0	0	0	88.24	<0.001
Ventersdorp	39.2	98	14.4	36	0	0	39.19	<0.001

* Analysis done on the actual number of pupae from each treatment.

Pupal mass of larvae that fed on Bt and non-Bt maize

Results on pupal mass are provided in Table 2.7. Male and female pupal mass differed statistically significant between populations but not between treatments. The duration of the pupal period of male and female pupae of the different populations is provided in Table 2.8. Male pupal duration on the control treatment ranged between 12 and 15 days compared to 13 and 15 days for the MON810 treatment. Female pupal duration on both the control and MON810 treatments were 13 to 14 days.

Table 2.7: Mean mass of male and female pupae of different *Busseola fusca* populations of which larvae fed on non-Bt and Bt maize.

Population	Male pupal mass (mg)				t-value (n)	P-value	Female pupal mass (mg)				t-value (n)	P-value
	Control		MON810				Control		MON810			
	Mass	(SE)*	Mass	(SE)			Mass	(SE)	Mass	(SE)		
Bethlehem	0.2011	0.0062	0.2083	0.0155	-0.474 (26)	0.64	0.2685	0.0087	0.2565	0.0188	0.619 (31)	0.54
Douglas	0.1586	0.0051	0.1512	0.0061	0.907 (45)	0.37	0.2518	0.0092	0.2293	0.0357	0.900 (23)	0.38
Grootpan	0.1599	0.0065	0.1528	0.0063	0.702 (53)	0.49	0.2039	0.0076	0.1823	0.0105	1.667 (42)	0.10
Lichtenburg	0.1861	0.0064	0.1905	00102	-0.380 (43)	0.71	0.2298	0.0084	0.2598	0.0127	-1.969 (20)	0.06
Petrusburg	0.1898	0.0099	0.1786	0.0121	0.670 (17)	0.51	0.2413	0.0372	0.2705	0.0195	-0.755 (10)	0.47
Potchefstroom	0.1579	0.0070	0.1442	0.0068	1.402 (53)	0.17	0.1955	0.0059	0.1785	0.0067	1.819 (58)	0.07
Vaalharts 2013	0.1762	0.0065	0.1932	0.0158	-1.025 (27)	0.31	0.2245	0.0171	0.2010	0.0125	0.7430 (6)	0.49
Ventersdorp	0.1966	0.0045	0.1959	0.0059	0.078 (68)	0.94	0.2706	0.0082	0.2799	0.0148	-0.569 (62)	0.57

* SE = Standard error

Pupal duration on Bt and non-Bt maize

Table 2.8: Mean duration of the pupal period of male and female pupae of *Busseola fusca* of which larvae developed on non-Bt and Bt maize.

Population	Male pupae				t-value (df)	P-value	Female pupae				t-value (df)	P-value
	Control		MON810				Control		MON810			
	Days	(SE)	Days	(SE)			Days	(SE)	Days	(SE)		
Bethlehem	13.50	0.54	15	1.0	-1.308 (16)	0.21	14.00	0.33	13.80	0.91	0.213 (26)	0.83
Douglas	13.29	0.23	12.17	0.55	1.944 (32)	0.06	12.44	0.38	13.75	0.25	-1.691 (18)	0.11
Grootpan	12.88	0.27	13.57	48	-1.343 (21)	0.19	12.79	0.47	12.33	0.71	0.486 (23)	0.63
Lichtenburg	14.22	0.67	14.10	0.53	0.123 (26)	0.90	13.40	0.60	12.73	0.71	0.586 (14)	0.57
Petrusburg	12.76	0.45	13.33	0.67	-0.664 (21)	0.51	12.71	0.61	13.43	0.57	-0.857 (12)	0.41
Potchefstroom	12.53	0.49	13.31	0.33	-1.278 (26)	0.21	12.94	0.42	12.93	0.38	0.027 (30)	0.98
Vaalharts 2013	12.86	0.93	14.50	0.87	-0.898 (16)	0.38	12.60	1.12	14.00	0	-0.509 (4)	0.64
Ventersdorp	12.93	0.30	13.09	0.53	-0.282 (37)	0.78	13.28	0.54	12.38	0.78	0.944 (24)	0.35

* Initial value of larvae inoculated = 250

* SE = standard error

Sex ratio on Bt and non-Bt maize

No significant differences were observed in the sex ratio between the Bt and non-Bt treatments in the populations where pupae formed and data did not deviate from the expected sex ratio of 1:1 (Table 2.9). It seems that the Cry1Ab proteins in the MON810 treatment did not have an effect on the sex ratio.

Table 2.9: The sex ratio of different *Busseola fusca* populations on non-Bt and Bt maize treatments.

Population	**Sex ratio**				
	Sex ratio (M:F)		**Observations (*n*)**	**Chi-square**	***P*-value**
	Control	**MON810**			
Bethlehem	1: 1.13	1: 1.40	61	0.11	0.743
Douglas	1: 0.69	1: 0.28	72	2.51	0.113
Grootpan	1: 0.81	1: 0.79	99	0.00	0.962
Lichtenburg	1: 0.52	1: 0.73	67	0.45	0.501
Petrusburg	1: 0.35	1: 1.17	40	3.01	0.083
Potchefstroom	1: 1.36	1: 0.81	115	1.81	0.178
Vaalharts 2013	1: 0.30	1: 0.20	38	0.20	0.652
Venda	1: 1.08	*	75	*	*
Ventersdorp	1: 0.92	1: 0.89	134	0.01	0.940

Moth longevity was only determined for populations that had sufficient numbers of moths that emerged from pupae (Table 2.10). There was no statistical significant difference in moth longevity between the non-Bt and Bt treatments in any of the populations. Mean male moth longevity on the control and MON810 treatments ranged between 5 and 7 days. Mean female moth longevity on the control and MON810 treatments ranged between 6 and 8 days.

2.3.4 Moth longevity

Table 2.10: Mean longevity of *Busseola fusca* moths of different populations of which larvae were reared on non-Bt and Bt maize.

Population	Male moth longevity				t-value (df)	P-value	Female moth longevity				t-value (df)	P-value
	Control		MON810				Control		MON810			
	Days	(SE)*	Days	(SE)*			Days	(SE)*	Days	(SE)*		
Bethlehem	6.57	0.29	5	1.15	1.978 (16)	0.07	6.5	0.39	6.5	0.5	0 (26)	1.00
Douglas	5.43	0.49	6.31	0.31	-1.320 (32)	0.20	8	0.76	8	1	-0.040 (18)	0.97
Ficksburg	5	0	5	1.53	0 (2)	1	5.50	0.50	7	0	-1.732 (1)	0.33
Grootpan	5.38	0.40	5.86	0.59	-0.672 (21)	0.51	6.68	0.54	5.67	0.88	0.938 (23)	0.36
Lichtenburg	5.11	0.61	5.80	0.71	-0.705 (26)	0.49	5.60	0.87	6.64	0.36	-1.318 (14)	0.21
Petrusburg	5.63	0.47	5.83	0.75	-0.232 (20)	0.82	5.71	0.61	5.57	0.57	0.172 (12)	0.87
Potchefstroom	5.80	0.68	5.08	0.51	0.824 (26)	0.48	5.17	0.49	6.21	0.55	-1.430 (30)	0.16

* SE = standard error

2.4 Discussion

Different levels of resistance were evident between the 12 test populations. The Venda population was the only population that had a very low larval survival percentage and mean mass in the MON810 treatment. Since there was no significant difference in survival of larvae of the Venda population in the MON810 and MON89034 treatments, it can be concluded that this population is still highly susceptible to both the single and stacked gene events.

The populations from Bethlehem, Bothaville and Ficksburg had higher larval survival on the MON810 treatment and can be considered to be tolerant to Cry1Ab protein expressed by this event. Although the survival of the larvae form the Ficksburg population was low, significant numbers survived and this population can be considered to be on the range of low tolerance for the Cry1Ab protein. The Grootpan and Potchefstroom population had very high survival percentages recorded on MON810 but the larval mass recorded from these populations was lower than that of the non-Bt treatments. These populations can however be regarded as moderately resistant.

Populations that showed high levels of resistance were those from Douglas, Lichtenburg, Petrusburg and Ventersdorp. Larvae from these populations survived for the whole trial period of 26 days and little or no differences in larval mass between the control and MON810 treatment were observed. Larvae from Ventersdorp population used in this study, seven years after a similar study by Van Rensburg (2007), responded differently to MON810 maize and were able to grow and survive successfully. The concerning results from this study is that there were no significant differences in larval mass between the control and MON810 treatments for all populations, indicating no difference in fitness. Larvae of these four populations are able to complete their lifecycles on MON810 maize and produce fit resistant offspring.

The Vaalharts populations are already known to be resistant to Bt maize that express Cry1Ab proteins (Van Rensburg, 2007; Kruger *et al.* 2011, 2014). The Vaalharts 2014 population had very high larval survival on MON810 and, as expected, there was no significant difference in larval mass between the control and MON810 treatments. Larvae collected in Vaalharts in 2013 are also regarded as highly resistant since there were no significant differences between either larval survival or mass on the non-Bt and MON810 treatments. Larvae are able to survive and develop into fit reproductive adults that are capable of passing on the resistant genes to following generations. The corrected percentage mortality model indicated a clear picture of resistance levels. High levels of resistance was recorded in the Vaalharts populations compared to the highly susceptible

Venda population. A 100% corrected mortality was observed for all populations on the MON89034 treatment indicating high level of susceptibility to the stacked event.

No larvae survived on MON89034 maize. Larvae from Lichtenburg feeding on MON89034 only survived for 5 days. Larvae from the Bethlehem, Ficksburg, Petrusburg, Potchefstroom, Vaalharts 2014 and Venda populations survived on the MON89034 treatment for 8 days. Larvae from the Ventersdorp population fed on MON89034 for 12 days while larvae from Bothaville, Douglas, Grootpan and Vaalharts 2013 were able to survive for 15 days. Although no larvae survived on MON89034 maize for the whole trial period, the 5-8 day period until death of all larvae, compared to the short period of 3-4 days observed in pre-release studies (Van Rensburg, 2001) with MON810, raises concern about resistance evolution of *B. fusca* to this stacked gene.

The lethal time until 50% of a population was killed differed between populations as some populations were more susceptible than others. It can be assumed that in populations where there were no differences between the LT50 in the control and MON810 treatments the larvae of the different treatments were equally fit and that the moths were able to produce offspring successfully. A study done by Kruger *et al.* (2011) on larvae collected from Vaalharts (resistant) and Viljoenskroon (susceptible) showed that the susceptible population had a LT50 of four days on Bt maize compared to the resistant population that had a LT50 of 9 days in a laboratory experiment. In the current study, conducted two and three years later, the LT50 of the Vaalharts population (2013 and 2014) was between 21 and 25 days on the MON810 treatment and between 5 and 7 days on the stacked event.

2.5 Conclusions

While the level of resistance of the field collected *B. fusca* populations to MON810 maize differed largely, no population showed resistance against the stacked event expressing Cry1A.105+Cry2Ab2 proteins. For some populations there were no differences between either larval survival or mass on the non-Bt or MON810 treatments. Although no larvae survived on MON89034 maize, the period of survival, which is long compared to the short period observed in pre-release studies (Van Rensburg, 2007) with MON810, raises concern. This study confirms resistance of *B. fusca* to Bt maize that express Cry1Ab proteins and highlights the importance of continuous monitoring of the resistance status of this pest. This study can be used as a baseline against which pest resistance can be compared in future for both single gene and the stacked gene maize event.

2.6 References

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Chapter 3: Conclusion

Several studies on the levels of resistance of *Busseola fusca* to MON810 Bt maize have been done since Bt maize was introduced into South Africa in 1998. Results from the literature as well as from this study are summarized in Table 3.1. Results from the current study can be compared with studies done by Van Rensburg (1999, 2011) and Kruger *et al.* (2011). Van Rensburg (1999) first evaluated different Bt maize events for the control of *B. fusca* in the growing seasons of 1995/96 and 1996/97 in South Africa from larvae collected at Ventersdorp (J.B.J. van Rensburg 2015, pers. comm., 26 November). Baseline susceptibility of *B. fusca* on protein-incorporated diets have never been determined because of difficulties rearing this pest on artificial diets. Efficacy data were collected by means of field and greenhouse evaluations of Bt maize using artificial infestation with the target pest. These efficacy evaluations were conducted using plants under optimal growing conditions in greenhouses and field plots (Van Rensburg, 1999). According to Van Rensburg (J.B.J. van Rensburg 2015, pers. comm., 26 November) inbred lines are homozygous and Bt expression may be higher in these lines compared to heterozygous hybrids that may have fluctuations in Bt expression. Therefore expression levels should be evaluated before hybrids are commercialized. In greenhouse studies (season 1995/96) with inbred lines of MON810, larval survival of 31 and 25% was observed after eleven days of feeding (Van Rensburg, 1999). However, on hybrids of MON810 (season 1996/97), larval survival was significantly lower with 2% for *B. fusca* after ten days of feeding. Under field conditions (season 1996/97), the levels of survival of *B. fusca* on the same MON810 hybrids were between 1.2 and 1.9% after early infestations and between 0.4 and 0.8% after late infestations (Van Rensburg, 1999).

Van Rensburg (2007, 2011) provided data on the status of resistance of *B. fusca* collected at different localities to Bt maize that express Cry1Ab and Cry1A.105+Cry2Ab2 proteins. Van Rensburg (2007, 2011) conducted a bioassay with Bt-transgenic hybrids during the 2006/07 season in which a Ventersdorp population was used as a susceptible (control) population to compare its survival and life history parameters with that of a resistant population from Christiana. The Ventersdorp population was chosen as a susceptible standard because Bt maize had at that time not been widely cultivated in that area whereas Christiana had a history of Bt maize use and resistance development. Van Rensburg (2011) reported larval survival for 12 to 16 days on Bt maize that expresses Cry1Ab for the susceptible population (Ventersdorp) while larvae of the resistant population (Christiana) had survived for the trial period of 20 days. Mean larval mass of the Ventersdorp population was very low, between 3.95 mg and 4.4 mg in comparison with that of the Christiana population (between 27.2 mg and 66.2 mg). Another Bt-susceptible population (Ventersdorp) (J.B.J. van Rensburg 2015, pers. comm., 17 November) also tested by Van

Rensburg (2011) in the 2006/07 season showed that larvae feeding on MON810 only survived for 13 days and on MON89034 for 9 days. During season 2007/08 Van Rensburg (2011) screened a Bt-susceptible Ventersdorp population but recorded larval survival of approximately 20% on day 16 on MON810 with larvae also surviving for 9 to 13 days on MON89034.

In the current study, the Ventersdorp population survived for the trial period of 26 days (31%) on MON810 with an average mean larval mass of 261.61 mg. Larval survival on MON89034 was recorded until day 12 but no significant weight gain was observed. These results indicate increased levels of resistance in the Ventersdorp population over a time frame of 19 years since the first trial was conducted with larvae from a Ventersdorp population in 1995/96.

Kruger *et al*. (2011) monitored survival of susceptible Christiana and Bethal populations and observed 100% mortality within 12 days. A susceptible population from Viljoenskroon feeding on MON810 had 100% mortality after six days of feeding (Kruger *et al.*, 2011). The Venda population that was screened in the current study had similar results although single surviving larvae were recorded on the MON810 treatment for the duration of the trial period. These larvae had no mass increase which indicate this population's susceptibility. From day 12 no survival was recorded from Venda larvae feeding on the MON89034 treatment.

A population collected from Rysmierbult (Potchefstroom) (Van Rensburg, 2011) showed signs of susceptibility as larval survival decreased sharply on day four to two percent and continued as such onward for the duration of the trial (day 30). Larvae of the Rysmierbult population that fed on MON89034 maize were killed within 4 days (Van Rensburg, 2011). The Potchefstroom population in the current study feeding on MON810 had a larval survival of 48% on day 26 and successfully gained mass over the trial period. In the current study larvae collected at Potchefstroom during the 2013/14 season fed for a period of eight days on MON89034 plants before 100% mortality was observed. However, although this was not compared statistically, the time that larvae survived in the latter study was twice the time it took until 100% mortality was reached in the Rysmierbult populations in 2011.

The Douglas population collected by Van Rensburg (2011) was also highly susceptible to MON810 and MON89034 and did not survive for more than four and nine days, respectively. The Douglas population monitored in the current study showed some concerning results. The larvae feeding on MON810 survived for the duration of the trial period (27.2%) and there was no significant difference in larval mass between the control (non-Bt) and MON810 treatment, which is a clear indication of resistance. Larvae from this population also survived on MON89034 for 15 days with a survival of 0.4% which raises concern.

Van Rensburg (2011) collected a population from Vaalharts during the 2009/10 season. Larval survival and mass was only recorded for a trial period of 13 days. Larval survival was recorded at about 45% on day 13 with an average larval mass of 6.5 mg on the MON810 treatment. Larvae only survived on MON89034 for 9 days. In the current study with a Vaalharts population collected during both 2013 and 2014 larval survival was recorded at 39.6% (2013) and 54.8% (2014) on MON810. Larvae gained mass faster compared to the report by Van Rensburg (2011). Mean larval mass was recorded at 212.3 mg (2013) and 184.0 mg (2014). Vaalharts larvae (2013/2014) from this study survived on MON89034 for a period of 12 and 19 days respectively. Therefore, over a period of four years larvae were able to survive ten days longer on MON89034 when compared to the initial study conducted during the 2009/10 season.

Results from the current study with larvae that fed on MON89034 tended to be similar to that observed with susceptible larvae that fed on MON810 when the first feeding studies was conducted by Van Rensburg during the mid-1990's. The observed trend in evolution of resistance is that there is a shift from a population where susceptible (SS) individuals dominate, to a population where heterozygotes (RS) dominate and an increase in the incidence of homozygous resistant (RR) individuals occurs over a period of a few years. Some populations that was observed as susceptible are becoming more resistant. This study provided a data set to assess the status of resistance of different *B. fusca* populations as well as a base line data set against which the status of resistance to MON89034 can in future be evaluated.

In 1998 MON810 was fist commercialized in South Africa and in 2006/07 season the first field evolved resistance was reported (Van Rensburg, 2007) six years after the first planting of MON810. Subsequently the first signs of survival of *B.* fusca on Bt maize were recorded in the field (Van Wyk *et al.,* 2008). MON89034 has been cultivated in South Africa since 2011 and within three years of commercialization of this event, laboratory studies indicated larval survival on this event, this raises concern.

Several factors played a role in the evolution of resistance on MON810 but one of the main reasons was the lack of refuge compliance in the region where resistance was first reported in South Africa. The effectiveness of the high/dose refuge strategy can be questioned since Kruger *et al.* (2011) reported that resistant larvae were also present in the refuge areas where larvae were sampled. Increased selection pressure in the Vaalharts area may have contributed to the rapid evolution of resistance as optimum conditions in the irrigated systems aided the abundance of *B. fusca* present (Kruger *et al.*, 2011).

Certain questions come to mind regarding resistance evolution against MON89034. Does non-compliance have a role in development of resistance and for how long will MON89034 be effective against key pests? If producers comply with refuge requirements, will

MON89034 technology be preserved for longer? Still the question needs to be asked whether the MON89034 event meets the requirements of the high dose needed for the high dose/ refuge strategy to be effective. MON89034 is stacked with a Cry1 protein which is also present in MON810 which resistance has been developed against. Could the MON89034 event only be relying on the Cry2 protein at the moment to control *B. fusca,* or do the two proteins together have a high-dose effect?

The importance to monitor the status of resistance of *B. fusca* populations over time to extend the period that Bt technology can be used effectively in South Africa, is highlighted throughout this study. Conducting this type of monitoring through support of the private sector and also by getting producers involved, will help provide early warning of resistance development.

3.1 References

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Table 3.1: Summary of available data on development and survival of *Busseola fusca* on Bt maize in South Africa since the first evaluations were done during 1995.

Population	Trial duration (days)		Survival (%)*		Mean larval mass (mg)**		LT50 (days)		Source
	MON 810	MON 89034	MON 810	MON 89034	MON 810	MON 89034	MON 810	MON 89034	
Ventersdorp 1995/96	11	-	30	-	0.43	-	-	-	Van Rensburg, 1999
Ventersdorp 1996/97	10	-	3		0.1	-	-	-	Van Rensburg, 1999
Ventersdorp 1999/2000	3	-	20	-	-	-	<1	-	Van Rensburg, 2001
Ventersdorp 2006/07	12	-	1***	-	3.95	-	-	-	Van Rensburg, 2007
Ventersdorp 2006/07	16	-	1***	-	4.4	-	-	-	Van Rensburg, 2007
Ventersdorp 2006/07	13	9	0	0	-	-	1	1	Van Rensburg, 2011
Ventersdorp 2007/08	16	9	25	10	5	0.1	-	-	Van Rensburg, 2011
Ventersdorp 2013/14	26	12	31	1	261.61	0.26	13.89	3.72	Current study
Vaalharts 2007/08	35	-	16	-	186.5	-	6.84	-	Kruger *et al.*, 2011
Vaalharts 2009/10	13	5	40	45	6.8	0.75	-	-	Van Rensburg, 2011
Vaalharts 2010/11	66	-	39	-	25	-	35.64	-	Kruger *et al.*, 2014
Vaalharts 2013/14	26	15	40	1	212.35	0.1	20.69	4.02	Current study
Vaalharts 2014/15	26	8	55	4	184.08	0.10	24.30	6.03	Current study
Christiana 2006/07	18	-	-	-	27.2	-	-	-	Van Rensburg, 2007
Christiana 2006/07	20	-	-	-	66.2	-	-	-	Van Rensburg, 2007
Christiana 2007/08	12	-			-	-	2.41		Kruger *et al.*, 2011
Douglas 2007/08	2	6	30	5	0.2	0.4	-	-	Van Rensburg, 2011
Douglas 2014/15	26	15	27	0.4	240.19	0.06	16.08	5.00	Current study
Potchefstroom 2007/08	30	4	2	0	50	-	-	-	Van Rensburg, 2011
Potchefstroom 2013/14	26	8	48	10	189.96	0.10	22.48	5.02	Current study
Bethal 2007/08	12	-	0	-	-	-	2.22	-	Kruger *et al.*, 2011
Viljoenskroon 2007/08	6	-	0	-	-	-	3.02	-	Kruger *et al.*, 2011
Bronkhorstspruit 2010/11	66	-	9	-	17	-	0.55	-	Kruger *et al.*, 2014
Venda 2013/14	26	8	3.2	-	14.97	0.08	5.15	4.67	Current study
Bethlehem 2013/14	26	8	17	27	80.26	0.07	15.47	6.07	Current study
Bothaville 2013/14	26	15	14	0.4	95.07	0.008	14.29	7.01	Current study
Grootpan 2013/14	26	15	55	0.4	209.53	0.04	24.94	5.58	Current study
Lichtenburg 2013/14	26	5	30	17	199.94	0.08	13.50	3.85	Current study
Petrusburg 2013/14	26	8	32	0.4	185.50	0.06	15.67	4.94	Current study
Ficksburg 2014/15	26	8	14	7	131.01	0.10	13.31	5.50	Current study

*Percentage survival as recorded on the day mentioned in the Trial duration (day) column
** Mean larval mass as recorded on the day mentioned in the Trial duration (day) column
*** Single surviving larvae as noted in article