Susceptibility of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to Bt maize in South Africa

AS Botha

orcid.org 0000-0002-2701-9882

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Supervisor:	Prof J van den Berg
Co-supervisor:	Prof MJ du Plessis
Assistant Supervisor:	Dr A Erasmus

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Abstract

Spodoptera frugiperda, the fall armyworm (FAW), invaded Africa during 2016 and is now considered the number one maize pest in Africa. The destructive feeding habits of FAW larvae threaten maize production in Africa. Bt maize is effective against African stem borer species and is expected to be approved for control of these pests in several African countries. Bt maize that express Cry proteins have been used effectively for control of the FAW in the United States, Canada, and several countries in South America. Although most Cry proteins provide effective control of the FAW, this pest evolved resistance to Cry1F Bt maize in Puerto Rico, Brazil and United States, and Cry1Ab Bt maize in Brazil. Proactive management of resistance evolution requires continued monitoring studies. The aim of this study was to provide baseline data on the control efficacy of Bt maize and the frequency of resistance alleles in field populations of *S. frugiperda* to single- and pyramid-gene Bt maize in South Africa. In order to determine the efficacy of Bt maize for the control of FAW a phenotypic screen was conducted and nine populations of *S. frugiperda* were evaluated, including a laboratory reared reference population. Larval feeding bioassays were conducted in which plant tissue of maize expressing Cry1Ab (single-toxin event) or Cry1A.105 + Cry2Ab2 (pyramid-toxin event), were fed to larvae. Results indicated moderate levels of survival (4-35%) on Cry1Ab maize, which supports field observations of commercial level control provided by this event. Considering Cry1A.105 + Cry2Ab2 maize, very high levels of mortality occurred with only one larvae being able to complete its life cycle. Although survival is low and effective control will definitely be achieved, resistance alleles seemed to be present and a genotypic evaluation was therefore done during 2019. During the second part of this study, a F_2 screen was conducted to estimate frequency of resistant alleles and 117 families were established of two different field collected populations. Three of the 117 established families carried major resistance alleles against Cry1A.105 + Cry2Ab2 maize, with a low overall estimated frequency of 0.0084 (95% credibility interval of 0.0023 - 0.0181). The frequency of Cry1Ab resistance alleles was 0.0819 (95% credibility interval of 0.0617 - 0.1036). The high frequency of resistance alleles and moderate susceptibility of *S. frugiperda* to Cry1Ab could be ascribed to the latter being a low-dose event for this pest, as well as the fact that the individuals which initially arrived on the continent may have carried resistance alleles. This study provides base-line data regarding resistance of FAW in South Africa to single- and pyramid-gene Bt maize. Results include at what frequency resistance alleles occur naturally, how effective single- and pyramid-gene Bt maize are in controlling this pest and what effect Bt maize has on the life history parameters of the resistant individuals. These results predict that single-gene Bt maize will only provide short term control of this pest in Africa, and that pyramid-gene Bt maize will be more effective and sustainable within the parameters of IRM strategies to control this pest. We therefore advise that pyramid-gene Bt maize should be commercialized and that single-gene Bt maize should be retracted due to possible enhanced resistance development if these two events are cultivated simultaneously.

Key words: Cry protein, fall armyworm, insect resistance management, monitoring, resistance

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1 Chapter 1: Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a polyphagous insect (Sparks, 1979) with major economic impacts (Cruz & Turpin, 1983; Stokstad, 2017) in the western hemisphere, that invaded Africa from the Americas early in 2016 (Goergen *et al*., 2016). According to Hulme *et al*. (2008) six possible pathways of entry exist, of which only three are applicable to the fall armyworm's introduction into Africa, namely unaided dispersal, contaminated commodities and stowaway individuals on a vector. Cock *et al*. (2017) considered the most likely transfer to be by means of stowaways on a direct flight.

The FAW is regarded the new primary pest of maize in African countries because of its destructive feeding habits on foliage and on the ears of maize during the reproduction stage (Day *et al*., 2017), causing both quantitative and qualitative losses (Cruz & Turpin, 1983; Lima *et al*., 2010; Day *et al*., 2017; Silva *et al*., 2018). This attack subsequently allows secondary pests and pathogens to cause indirect damage to the grains. The production of maize and food security in Africa, is threatened if appropriate control measures for this pest is not applied.

The two preferred control tactics are application of insecticides, or the planting of genetically modified Bt maize that expresses insecticidal proteins derived from a soil living bacterium, *Bacillus thuringiensis* (Bt). The presence of FAW infestation of plants is usually observed when damage is already severe. Larvae feed deep in the whorl region of maize plants, making it difficult for contact insecticide sprays to reach the larvae. The spraying of insecticides usually leads to inadequate control of this pest. Chemical control strategies are only effective when larvae are small which require timely or regular applications that are harmful to non-target organisms (Yu, 1991; Romeis *et al*., 2018).

Planting of Bt maize to control FAW results in reduced insecticide application which limits negative environmental effects that are caused by insecticide applications. According to Brookes and Barfoot (2018), the aggregate income benefit of GM maize in South Africa alone between 1996 and 2016 was $ 2 238.4 million. Bt crops prevents direct and indirect damage by pests which could otherwise be responsible for severe yield losses, especially in developing countries (Qaim & Zilberman, 2003).

Ismael *et al*. (2002) reported that Bt crop adopters on the east coast of South Africa gained economically due to higher yields, and by reducing insecticidal expenses through the elimination pest spraying. Transgenic Bt maize is considered one of the most environmentally friendly methods (Romeis *et al*., 2006; Romeis *et al*., 2018; Koch *et al*., 2015) for the control of FAW in North (Buntin *et al*., 2004; Storer *et al*., 2012; Reay-Jones *et al*., 2016) and South American countries (Storer *et al*., 2012; Buntin *et al*., 2008; Bernardi *et al*., 2016).

Although both the above mentioned control methods are used successfully to control the FAW, field-evolved resistance to insecticides (Gutiérrez-Moreno *et al*., 2018) and Bt maize (Storer *et al*., 2010,) have been reported in Puerto Rico and several other countries (Young & McMillian, 1979; Yu, 1991; Huang *et al*., 2014; Farias *et al*., 2014; Omoto *et al*., 2016; Chandrasena *et al*., 2018). In order to preserve control methods, especially those related to biotechnology, management practices need to be implemented to comply with biosafety legislation (Head & Greenplate 2012, Johnston *et al*., 2004).

According to Johnston *et al*. (2004) management strategies such as refuge plantings have been developed for commercial large-scale cultivation systems and result in challenges for subsistence farmers in Africa. The challenges faced by smallholder farming practices include small fields in close vicinity of other farmers fields, recycling and sharing of maize seeds amongst farmers to use for the next cropping season and the planting of different varieties together in a single field (Aheto *et al*., 2013; Johnston *et al*., 2004; Van den Berg, 2013). These challenges along with the lack of understanding the importance of good management practices among small scale farmers are most likely to result in poor stewardship compliance that might increase resistance development and thereby threaten the long term effectiveness of Bt maize (Kotey *et al*., 2017).

The aim of the study is to assess the susceptibility of *S. frugiperda* to Bt maize in South Africa. This will be done through development of a base-line data set of different populations to the two Bt maize events that are currently approved for cultivation in South Africa , i.e. MON810 and MON89034.

Two different approaches will be followed. Firstly, the effect of Bt maize on survivorship and life history parameters of the FAW will be determined and secondly, the frequency of resistant alleles present in FAW populations in South Africa will be determined. This base-line data is essential to detect future changes in FAW response to Bt proteins and will facilitate detection of shifts in susceptibility. In order to prolong the longevity of Bt maize in Africa, IRM strategies need to be implemented. The results of this study will provide valuable data that can be used in the future to aid in resistance monitoring and the development of effective IRM strategies.

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2 Chapter 2: Literature study

2.1 Fall armyworm

2.1.1 Fall armyworm history, identification and arrival in South Africa

The 1st reports of fall armyworm (FAW) as a pest date back to 1797, when this species was first described by Smith and Abbot as *Phalaena frugiperda* (Lepidoptera: Noctuidae). The scientific name of this pest changed several times (Luginbill, 1928) before it became known as the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). The FAW originates from the central and northern parts of South America and the southern parts of North America and migrates on an annual basis to the central parts of USA and southern parts of Canada (Luginbill, 1928; Nagoshi & Meagher, 2008).

The most common method used to distinguish lepidopteran species is based on visible distinctive phenotypic markings of either the larva or the moth (Figure 2:1). The distinctive identification markings on the larva of *S. frugiperda*, are the four larger dark spots in the form of a square on the last body segment and the white inverted “Y” on the forehead. To further ensure that the identification is correct, larvae should be reared until adults to confirm whether male moths have white markings on their wing tips and golden copper patterns on the upper surface of the forewing.

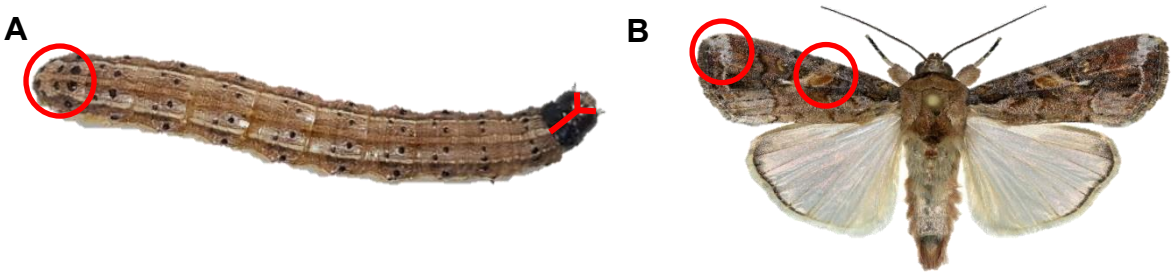

Figure 2:1 Fall armyworm larva (A) and moth (B)

Photos (A) Botha, (B) Goergen *et al.,* (2016)

Early *et al*. (2018) indicated that there is considerable potential for a near global invasion of the fall armyworm. Since 2016 the fall armyworm invaded Africa (Goergen *et al.,* 2016) and Asia (Sharanabasappa *et al*., 2018). Although Early *et al*. (2018) indicated that the probability of colonisation in North Africa, along the Nile valley, or seasonal invasion into Europe due to migration, is hard to predict, FAW was reported in Egypt during May 2019 (Anonymous, 2019). High possibilities of invasion and establishment is predicted for Australia (Early *et al*., 2018), due to transportation of agricultural commodities.

Spodoptera frugiperda was recorded for the first time early in 2016 as an invasive species in West and Central Africa (Goergen *et al.,* 2016). There are six possible types of introduction pathways as set forward by Hulme *et al*. (2008), according to Cock *et al.* (2017) only three of the possible six pathways are relevant regarding this case. The three considered introduction pathways are unaided dispersal, contaminant of a commodity and stowaway on a vector. There are multiple speculations regarding the different pathways of entry into Africa. The most likely speculation suggests egg batches that arrived in or on parts of an aircraft (Cock *et al*., 2017). Instead of already laid egg batches, gravid female moths could have been present in some parts of an aircraft such as the cargo holds or wheel bays, therefor no wind is required to disperse newly hatched larvae. Other speculations suggest that the pest arrived via the shipment of maize, e.g. maize ears with the sheath in place, into Africa (Cock *et al*., 2017). Regardless of the introduction and vagility of *S. frugiperda*, this pest species will establish as an endemic, multigenerational pest species in Sub-Sahara Africa because of suitable agroecological conditions and the presence of host pants (Goergen *et al*., 2016; Prasanna *et al*., 2018).

2.1.2 Host strains and host plant preference

Two different *Spodoptera frugiperda* strains can be identified by means of molecular analyses (Nagoshi *et al*., 2007), although these strains are morphologically identical. Moths of these different strains prefer different host plant species and larval performance is influenced by different host plants (Nagoshi *et al*., 2007; Pashley *et al*., 1985; Pashley, 1986; Prowell *et al*., 2004). The sympatric speciation of this species leads to the development of two different strains, i.e. the corn-strain (C

strain) and rice-strain (R strain). Behavioural differences occur among moths of the different strains with moths of the former preferring to lay eggs on maize, cotton and sorghum, while moths of the latter prefer to lay their eggs on rice and various pasture grasses, thereby compelling larvae to feed on plants on which the eggs are laid. Montezano *et al*. (2018) reported 353 larval host plants species of *S. frugiperda*. *Spodoptera frugiperda* is currently regarded a serious pest of maize in South Africa (DAFF, 2018) with confirmed presence of the maize strain (Jacobs *et al*., 2018). This is worrying, since the maize strain is considered less susceptible to Bt toxins (Ingber *et al*., 2017). Fortunately laboratory and field studies found similar levels of susceptibility to insecticides regardless of the resistance status to Bt maize (Muraro *et al*., 2019).

Studies conducted by Adamczyk et al. (1997) as well as Ríos-Díez and Saldamando-Benjumea (2011) found differential responses to several chemicals of the nerve and muscle target sites, specifically to the pyrethroid, organophosphate and carbamate families. However, there will always be variability associated with past selection pressures, considering the lack of knowledge regarding previous exposure patterns of the tested strains to insecticides or toxins. A behavioural difference between the two strains have also been reported by Meagher and Nagoshi (2013), after they observed that that attraction of males to corresponding-strain females did not appear to be a premating mechanism that results in assortative mating between corn and rice host strains. Clearly other premating or perhaps even post-mating mechanisms are important for the maintenance of host strains in *S. frugiperda*. No studies have been conducted, in which the timing of infestation between the two strains were monitored.

2.1.3 Biology

The life stages of *S. frugiperda* are illustrated in Figure 2:2. None of the life stages have adapted to survive low winter temperatures (Luginbill, 1928). The lack of a diapause mechanism assures that overwintering only takes place in mild climates with temperatures above 10℃, for continuous reproduction to occur (Sparks, 1979). The two climatic limits which influence the year-round distribution of FAW, are the minimum annual temperature and the amount of rainfall during the rainy season (Early *et al*., 2018). The life cycle of this pest can vary considerably, but the average

duration of the life cycle is 24-30 days under optimal conditions (Sparks, 1979). Climatic conditions such as mean temperature and the amount of rain during the rainy season is the strongest natural factors influencing the biology of the FAW.

2.1.3.1 Eggs

Nocturnal behaviour of the adults causes oviposition to occur only at night time, mainly on the underside of the leaves of maize plants and other host species (Luginbill, 1928). Eggs hatch within three days if the mean temperature is 26.6° C, or it will extend to four days when temperature decreases to below 20.5° C (Luginbill, 1928). Several egg batches are laid in clusters and eggs are protected by a dense covering of scales (Vickery, 1929; Sparks, 1979).

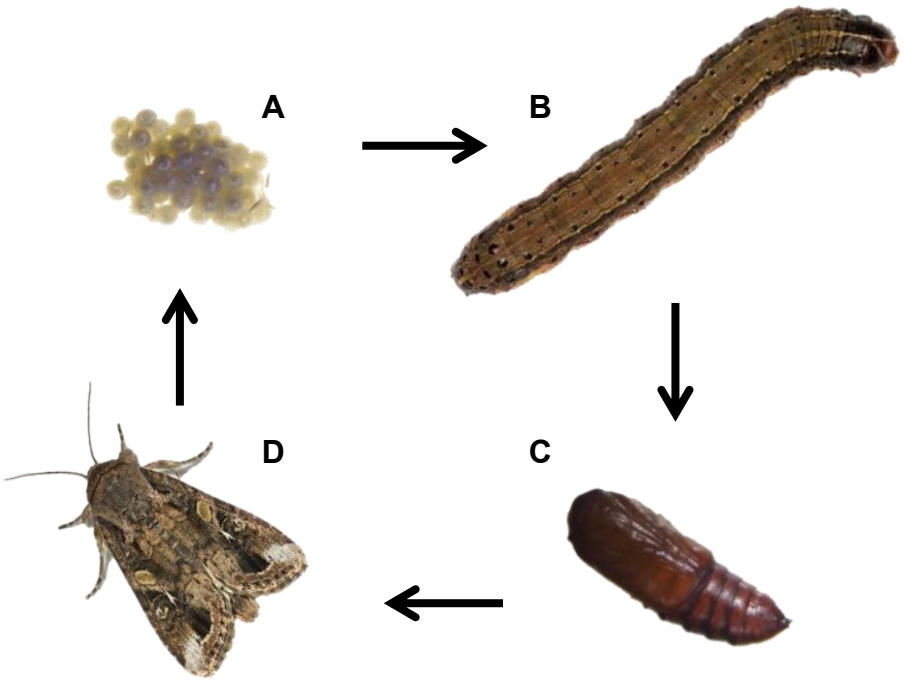

Figure 2:2 Different stages of the life cycle of *Spodoptera frugiperda*
A) An individual egg measures around 0.3 mm in height and 0.4 mm in diameter. B) Larvae consist of several different colours, mainly dependent on the instar stage, ranging from light brownish to dark greenish, and attain lengths of about 1 mm (instar 1) to 45 mm (instar 6). C) Both sexes have a reddish brown pupal colour, and measures around 14 to 18 mm in length and 4.5 mm in width. D) Moths have a wingspan of 32 to 40 mm, the forewing of the female moth is pale in colour where the male moths is more colourful.

2.1.3.2 Larvae

The larval stage has six different instars (Sparks, 1979). The first three instars are the smaller and less cannibalistic stages, were in the fourth to sixth instar it is not uncommon to find one larva feeding on another of the same species (Luginbill, 1928). Newly hatched larvae may live for more than a day without food, other than the egg shells, while being active most of the time in search of food (Luginbill, 1928). Mean larval duration was determined to be 3.3, 1.7, 1.5, 1.5, 2.0, and 3.7 days for instars one to six, respectively, when larvae were reared at 25° C (Pitre & Hogg, 1983). During the warmer summer months larvae are very active, feed voraciously, grow rapidly, and consequently have shorter instars (Luginbill, 1928).

2.1.3.3 Pupa

Pupation usually occurs within the soil (Luginbill, 1928) and seldom inside stalks of the host plant. In loose soil the larva burrows to a depth of 2.5 to 7.5 cm and spin together soil particles with silk to form a loose cocoon (Luginbill, 1928). The duration of the pupal stage is also highly dependent on soil temperature. The duration of this stage can range from seven days under ideal conditions, to 37 days under harsh conditions (Vickery, 1929), with a mean pupal duration of eight to nine days under favourable conditions.

2.1.3.4 Moth

Food and temperature are the factors that largely influence the longevity and fertility of the moths (Luginbill, 1928). Under optimal temperature conditions, moths will emerge, irrespective of the season, and live for four to six days in natural environments (Sparks, 1979). The average number of eggs laid by female moths is 1,024 (Vickery, 1929). The variation in number of eggs laid by a female is ascribed to the quantities of food ingested during the larval stage, or it is possible that some moths are naturally more fertile (Luginbill, 1928).

2.1.4 Crop injury and economic importance

Spodoptera frugiperda larvae feed inside the whorls of maize plants, causing distinctive holes that are visible in the leaves, which increase drastically in size as the larvae ages (Figure 2:3) (Sena, 2003). Damage to maize during vegetative stages is visible on young leaves and the soft nutritious parts inside the whorl

(Goergen *et al.,* 2016). During the reproductive stages of older maize plants, older larvae can bore into the developing reproductive structures such as maize ears, reducing yield quantity and quality (Cruz & Turpin, 1983; Lima *et al*., 2010; Day *et al*., 2017; Silva *et al*., 2018).

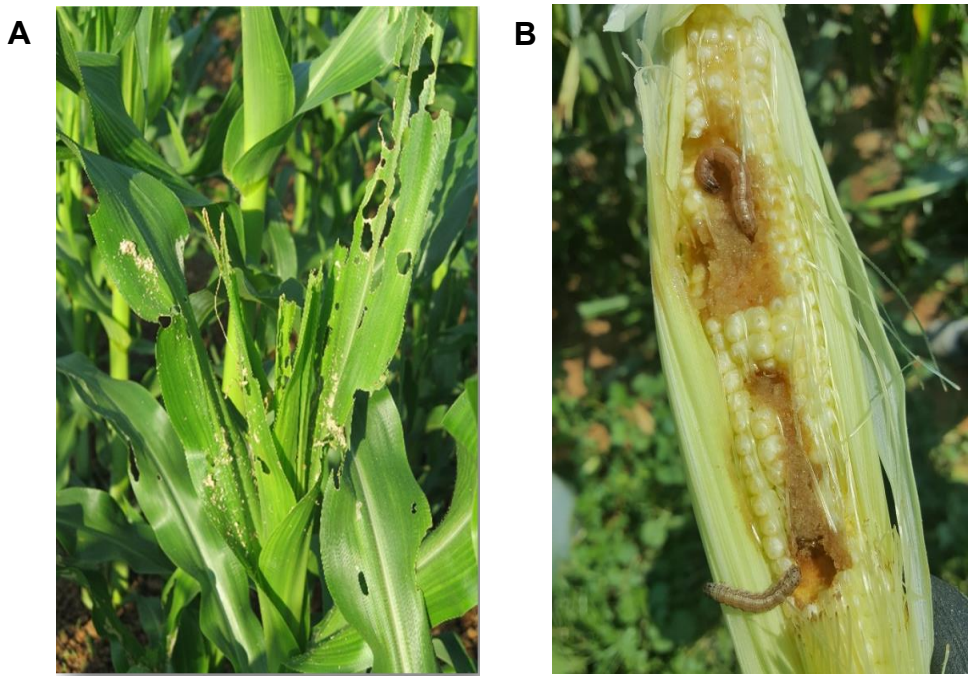

Figure 2:3 A) Foliar and B) ear damage

Conspicuous damage caused by fourth/fifth instar larvae of *Spodoptera frugiperda* to the whorl leaves of a maize plant (Du Plessis *et al*., 2018).

Young larvae (first to third instar) skeletonize the leaves of plants upon which they feed, while older larvae (fourth to sixth instar) cause conspicuous damage to plants. First instar larvae usually feed on the yellow/green leaf tissue low inside the base of the plant whorl, and rarely eat entirely through the leaf (Cruz *et al*., 1999). This colourless membranous epidermis is prominently visible against the dark-green back ground of the remaining leaves. Second and third instar larvae eat small pinholes through the leaves, otherwise they eat from the edges of the leaves inward. Fourth to sixth instar larvae often completely destroy small plants and strip larger ones of their leaves (Cruz, 1995). Chapman *et al.* (1999) calculated the average maize leaf area consumed by a single *S. frugiperda* larva, from hatching until pupa formation to be 302.5 cm^2. Of this total consumed leaf area, 78.3% is ingested by the 6^{th} instar larva (Day *et al*., 2017), while a total of 95.2% is consumed by the last three instars (Figure 2:4).

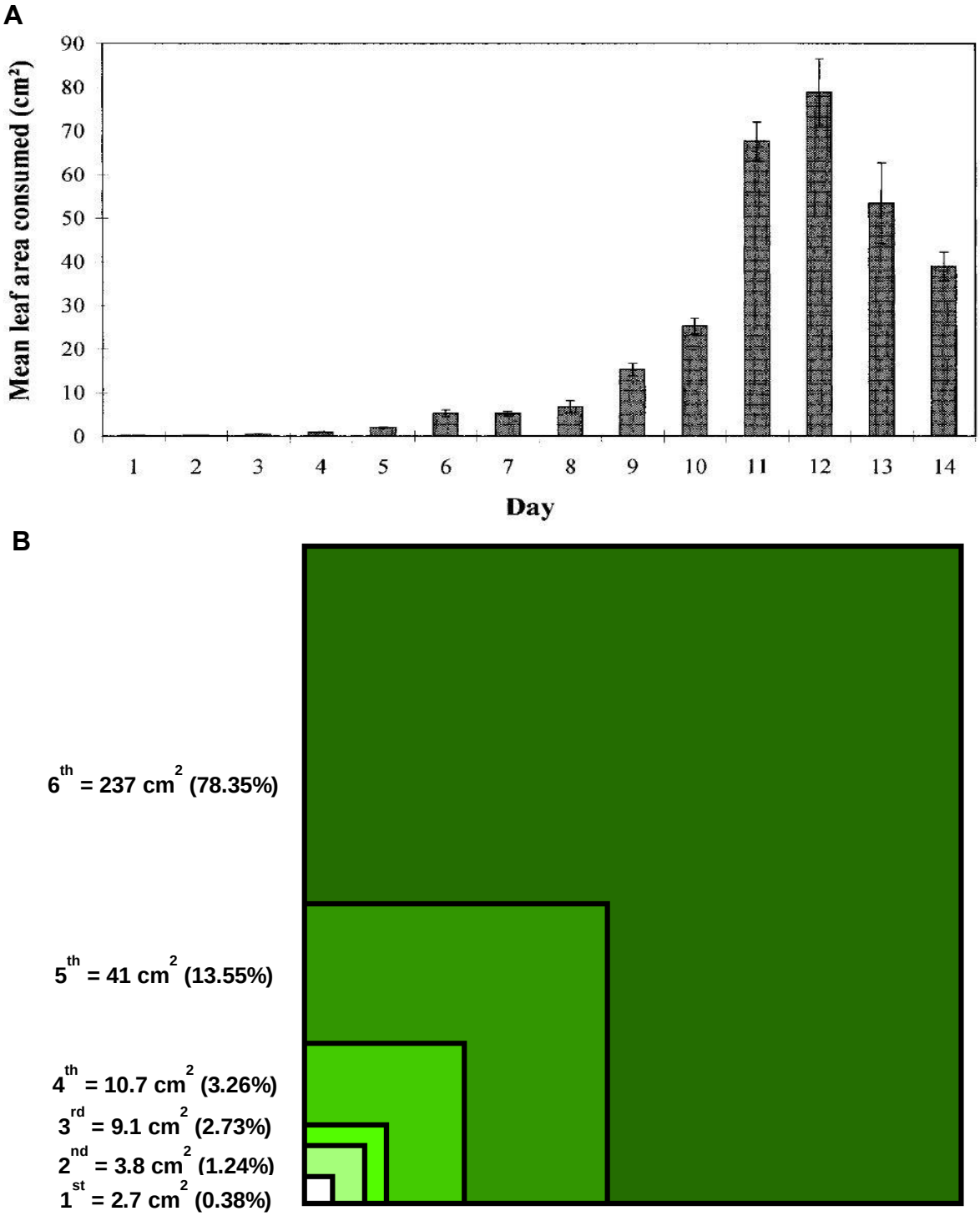

Figure 2:4 A) Daily consumption, B) Respective instar consumption

A) Mean area (± 1 S.E.) of maize leaves consumed daily (Chapman *et al.,* 1999), B) mean area of maize leaves consumed during respective instars by a single larva, with an abundant food supply every 24 hours throughout larval development (*n* = 30).

In order to limit yield loss caused by FAW, control methods needs to be implemented. The two commercial successful approaches for the control of FAW are insecticide applications, or planting Bt maize that expresses insecticidal proteins. Not only does insecticide applications have negative environmental effects, it also requires timely or regular applications that are often ineffective due to factors such as unreachable feeding sites, weather conditions and applications being done when larvae are already too large. By planting Bt maize, none of the above factors have an impact on the efficacy of controlling *S. frugiperda*. Bt maize is therefor considered an environmentally friendly and cost effective way of controlling this pest on commercial scale.

The economic importance of *S. frugiperda* is determined by the severity of the outbreaks (Luginbill, 1928). In North America two types of outbreaks occur, namely local and general outbreaks. Both types of outbreaks originate from migrating FAW populations from south Florida and Texas where there is year-round survival due to advantageous weather conditions and abundant host plants (Luginbill, 1928). Local outbreaks, a consequence of cold winters, occur when only the southern parts of USA are invaded. General outbreaks refer to a near complete invasion of North America. Migrating adults depend on prevailing winds to migrate as far as 1600 km northward (Rose *et al*., 1975), where they infest maize and other crops in the northern regions of the United States. Severe outbreaks usually coincide with the onset of the wet season, especially when the new cropping season follows a long period of drought (Goergen *et al.*, 2016).

Assessing yield losses caused by FAW remains difficult as yield can be decreased through foliar- (quantitative) and grain (qualitative) damage. Attacks at different developmental stages complicate the assessment of yield loss even further as certain plant growth stages are more vulnerable to injury. Furthermore, calculation of economic losses proves to be complex due to varying prices and value changes over time (Cruz *et al*., 1999; Day *et al*., 2017).

Spodoptera frugiperda is considered a major insect pest of maize in Latin American countries (Andrews, 1988), causing yield losses of 17% in Mexico (Galt & Stanton, 1979), 34% in Brazil (Lima *et al*., 2010), up to 40% in Honduras (Wyckhuys & O'Neil, 2006), 45-60% in Nicaragua (van Huis, 1981; Hruska & Gladstone, 1988) and 72%

in northern Argentina (Perdiguero *et al*., 1967). Cruz and Turpin (1983) reported yield losses of 17% when 20-100% of plants were inoculated with FAW egg masses. These losses were mainly due to grain damage as there were no correlations between foliar damage and yield loss. Williams and Davis (1990) recorded a reduction of 13% in yield, due to foliar damage and Buntin *et al*. (2001) a yield loss of between 28-71% due to grain damage by the FAW in the USA. According to the above mentioned studies it seems that qualitative damage may affect yield loss more severely than quantitative damage. This is ascribed to the direct influence of larval feeding damage on grain quality and a reduction grain mass. Accurate calculation of economic losses due to FAW damage is complex as several factors influence these estimations (Oliveira *et al*., 2014). Foliar and ear damage caused by FAW result in annual economic losses estimated between $300 to $500 million in the United States (Mitchell, 1979), and US$400 million in Brazil (Figueiredo *et al*., 2005).

An evidence note published by the Centre for Agriculture and Bioscience International (CABI) (Day *et al.*, 2017), estimated yield losses caused by FAW in ten major maize producing countries in Africa (excluding South Africa and Kenya), to be between 8.3 and 20.6 million tons per year. This represents a range of 21-53% of the annual production of maize averaged over a three-year period in these countries, with an estimate economic loss of between US$2.48 billion and US$6.19 billion, in the absence of any appropriate control measures. These economic implications of the establishment of *S. frugiperda* on the African continent may not be limited to its direct effects on agricultural production but also has the potential to adversely affect access to foreign markets (Goergen *et al.*, 2016; Day *et al*., 2017).

2.2 *Bacillus thuringiensis* (Bt)

Bacillus thuringiensis (Bt) is a gram-positive spore forming bacterium, typically found in soil (Höfte & Whiteley, 1989). *Bacillus thuringiensis* produces four types of insecticidal proteins, namely crystal proteins (Cry), cytolytic proteins (Cyt), vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip) by some strains. All these insecticidal proteins have virulent effects on: lepidopteran, coleopteran and dipteran insect orders (Höfte & Whiteley, 1989). While Cry and Cyt toxins are synthesized during sporulation (Hannay & Fitz-James, 1955) and the late exponential growth phase (Salamitou *et al*., 1996), Vip and Sip proteins are

produced during the vegetative growth phase (Estruch *et al*., 1996). *Bacillus thuringiensis* can be distinguished from closely related species *B. cereus* and *B. anthracis* by the production of large crystalline parasporal inclusions during sporulation, these inclusions contain crystal proteins that exhibit highly specific insecticidal activity (Aronson *et al*., 1986; Whiteley & Schnepf, 1986; Höfte & Whiteley, 1989; Schnepf *et al*., 1998).

The nomenclature regarding Cry and Vip proteins are based on their primary sequence identity, comparing the degree amino acid identity of new proteins to previously named proteins (Pardo-Lopez *et al*., 2013; Palma *et al*., 2014) (Figure 2:5). However, this does not imply similar protein structures, target pests, or even mode of action for all Cry or Vip proteins (Palma *et al*., 2014). The first section of the identification code used in the nomenclature of these proteins refers to the protein type (Cry or Vip), followed by a rank number assigned according to the similarity in amino acid identity (Figure 2:5). Bt proteins with an amino acid identity similarity of less than 45% are assigned a different primary rank indicated by an Arabic number, e.g., Cry2 and Vip3. Proteins sharing less than 78% pairwise identity similarity are differentiated by the secondary rank indicated with a capital letter, e.g., Cry2A and Vip3B. Proteins sharing less than 95% amino acid sequence similarity are assigned a different tertiary rank, a lowercase letter (e.g., Cry2Ab and Vip3Aa), while proteins with an amino acid identity similarity of more than 95% are indicated at quaternary rank with an Arabic number (e.g., Cry2Ab2 and Vip3Aa1) (Palma *et al*., 2014).

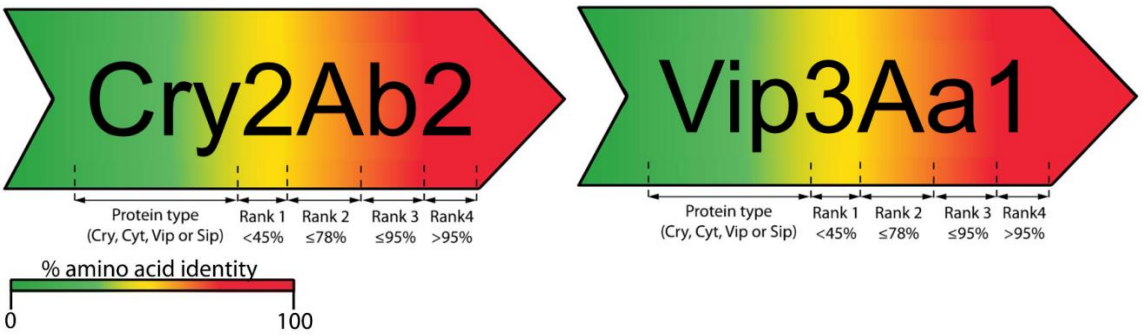

Figure 2:5 A graphical presentation of Cry protein nomenclature (Palma *et al*., 2014)

Although the largest group of Cry proteins, 53 of the 73 subgroups, belongs to the three-domain Cry-toxin family (Crickmore *et al*., 1998), other Cry protein families, such as the Mtx-like Cry toxins and Bin-like Cry toxins, also exist. Toxins of the three-domain Cry toxin family are globular shaped molecules which contain three distinct domains attached by single linkers (Pardo-Lopez *et al*., 2013) (Figure 2:6).

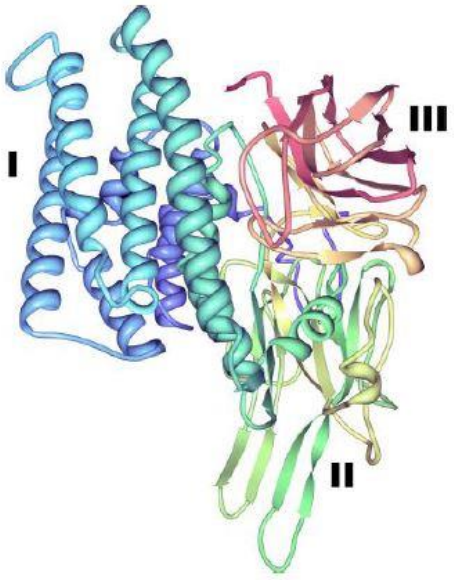

Figure 2:6 Three dimensional structure of a three-domain Cry toxin

Different domains are indicated by Roman numbers: (I) perforating domain, (II) central domain, and (III) galactose-binding domain (Palma *et al*., 2014).

All three-domain Cry toxins share roughly the same structure and core mode of action steps but display differences among their amino acid sequences and exhibit different specificities (De Maagd *et al*., 2001; 2003; Bravo *et al*., 2007). Once the protein is ingested and solubilized the respective domains exhibit different functions such as, receptor identification and binding, oligomerization and pore formation, and membrane insertion (Bravo *et al*., 2007; Vachon *et al*., 2012; Pardo-Lopez *et al*., 2013). Domain I is revered to as the perforating domain, and is most probably responsible for toxin insertion into the membrane and pore formation (Schnepf *et al*., 1998; Xu *et al*., 2014); domain II or the central domain is involved in toxin-receptor interactions (Xu *et al*., 2014); domain III or the galactose-binding domain, is also involved in receptor binding and pore formation (Xu *et al*., 2014).

2.2.1 Bt and its Mode of Action

Three different models that describe the modes of action of three-domain Cry toxins have been proposed, mainly with regard to their action in lepidopteran insects (Bravo *et al*., 2007; Vachon *et al*., 2012) (Figure 2:7). The first and most agreed upon model is referred to as the classical model, the second is the sequential binding model while the third is known as the signaling pathway model (Palma *et al*., 2014).

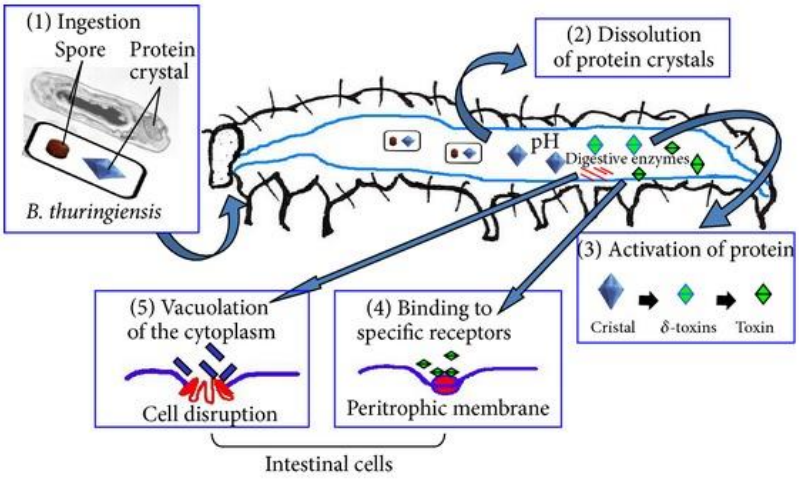

Figure 2:7 *Bacillus thuringiensis* mode of action

The mode of action of *Bacillus thuringiensis* in Lepidoptera involves the consecutive completion of several steps, hours after ingestion in order to result in insect mortality. These steps are: (1) ingestion of protein, (2) solubilization of the toxins, (3) activation toxins, (4) binding of toxins to midgut receptors, (5) membrane pore formation and cell lysis (Schünemann *et al*., 2014).

According to the classical model δ-endotoxin crystals must be ingested by susceptible larvae to have an effect. When ingested, the alkaline conditions (pH 9 to 12) in the insect midgut are responsible for solubilization of the crystals (Bravo *et al*., 2007). Subsequently the crystals are broken down into smaller polypeptides or amino acids, considered as a toxic core fragment (De Maagd *et al.,* 2003). Affinity of activated toxins ensures the binding of toxins to specific receptors located on the apical microvillus membranes of epithelial midgut cells (Pigott & Ellar, 2007). After the binding of activated toxins, the formation of a cation-selective channel happens when the toxin is inserted into the cell membrane, after which it is believed that oligomerization follows. Oligomers form a pore or ion channel, induced by an

increase in cationic permeability within the functional receptors contained on the brush border membrane vesicles (BBMV) (Bravo *et al*., 2004). Once a sufficient number of these channels have formed, extra cations enter the cell. Osmotic imbalance occurs within the cell, and the cell compensates by absorbing water. This process, referred to as colloid-osmotic induced lysis, continues until the cell ruptures and exfoliates from the midgut microvillar membrane. After a sufficient number of cells have been destroyed, the midgut epithelium loses its integrity. This allows the bacteria and alkaline gut juices to enter the haemolymph, causing septicemia within the larval body and finally resulting in death. Alteration in any of the above steps (solubilization, proteolytic activation, receptor binding, membrane insertion, pore formation, and osmotic lysis of midgut cells) could result in resistance development, although resistance usually develops through alteration of receptor binding on the BBMV in the midgut (Ferré & Van Rie, 2002). The mode of action of certain vegetative insecticidal proteins seems to be similar to those of Cry proteins, regarding the activation, binding and cell lysis caused by of Vip3 toxins (Yu *et al*., 1997), although the binding sites and the ionic channels are different than those of Cry1A toxins (Lee *et al.,* 2003).

2.2.2 Commercial use and naming of Bt cultivars in South Africa

Parasporal inclusions of Bt exhibit highly specific toxicity to larvae of lepidopteran, dipteran and coleopteran species and is therefore used to control pests of these groups, whether by the use of Bt spray applications or transgenic plants. Transgenic plants are genetically engineered to possess desired genes derived from other species. In the case of transgenic Bt plants, *Bacillus thuringiensis* serves as the donor organism for the genes that confer insect resistant properties of these plants. These genes are known as Cry, Cyt or Vip genes and within the transgenic plants they encode for proteins that are responsible for the insecticidal activity against larvae of different insect orders. Endotoxins exhibit highly specific insecticidal activity with Cry1 being exclusively active against lepidopterans. Cry2 exhibits dual toxicity against lepidopteran and dipteran pests, Cry3 is active against coleopteran pests and Cry4 exclusively controls dipterans. Regarding the insecticidal activity of Vip toxins, Vip1 and Vip2 toxins are toxic against certain coleopteran species (Warren *et al*., 1998), whereas Vip3 toxins control lepidopteran pests (Estruch *et al.,* 1996). Species controlled by Vip4 toxins have not been identified yet (Palma *et al.,* 2014).

Table 2:1 List of Bt maize events approved for, or in the approval phase in South Africa (ISAAA, 2018)

Event	Bt gene	Gene source	Approved for cultivation
MON810	Cry1Ab	*Bacillus thuringiensis* subsp. *kurstaki*	1997
Bt11	Cry1Ab	*Bacillus thuringiensis* subsp. *kurstaki*	2003
4114	Cry1F	*Bacillus thuringiensis* var. *aizawai*	*
TC1507	Cry1Fa2	synthetic form of Cry1F gene derived from *Bacillus thuringiensis* var. *aizawai*	2012
MON89034	Cry1A.105 + Cry2Ab2	*Bacillus thuringiensis* subsp. *kumamotoensis* *Bacillus thuringiensis* subsp. *kumamotoensis*	2010
MIR162	Vip3Aa20	*Bacillus thuringiensis* strain AB88	*

Data obtained from ISAAA, 2018 and were last updated on October 23, 2017. *not yet approved for cultivation.

The successful transfer of a desired gene from one species to another is referred to as a transformation event. The event into which the successful incorporation of the desired gene occurred is named after the specific DNA recombination experiment. Events approved in South Africa consist of single- and pyramided toxins. According to (ISAAA, 2018) six insect-resistant maize events have been approved in South Africa (Table 2:1). All these Bt maize events confer resistance to lepidopteran insects by selectively damaging their midgut lining (ISAAA, 2018).

2.2.3 Cry protein expression in plants

Expression levels of Cry proteins do not only differ between events but also among plant structures (Mendelsohn *et al.*, 2003) (Table 2:2). To ensure that a sufficient amount of Cry proteins is produced during the vegetative growth phases of plants, the United States Environmental Protection Agency (USEPA) has stipulated that transgenic Bt plants should meet high-dose expression levels. A high-dose expression is defined as the level that is 25 times higher than that required to kill

99% of heterozygous insects (USEPA, 1998a). However, a high dose against one pest species cannot be considered a high dose against another species since some pests tend to be inherently less susceptible to certain Bt proteins (Storer *et al*., 2012b). According to USEPA (1998b, 2001), Cry1Ab maize does not meet the high-dose criteria for *S. frugiperda*. Sousa *et al*. (2016) confirmed that Cry1Ab expressed by maize plants of the single-gene event MON810, is regarded a low-dose expression for FAW, with >5% of the heterozygous insects being able to survive on Bt maize. Low-dose Bt maize events increases the risk of resistance evolution, since most homozygous susceptible insects are killed but heterozygous insects (carrying a single resistance allele, see 2.4 Resistance evolution) survive, ensuring that heritable resistance alleles are present in subsequent generations. This increases the number of resistant alleles in the population over time and subsequently the rate of resistance evolution (Gould, 1998).

Table 2:2 Quantity of Cry protein expression amongst different structures of maize plants (USEPA, 2010)

All values reflect fresh tissue weight (ng/mg) unless otherwise noted.

Event	Cry protein	Leaf	Root	Pollen	Seed	Whole plant
Bt11	Cry1Ab	3.3	2.2-37.0	<90 ng/g	1.4	-
MON810	Cry1Ab	10.34	-	<90 ng/g	0.19-0.39	4.65
TC1507	Cry1F	56.6-148.9	-	113.4-168.2	71.2-114.8	830.2-1572.7
MON89034	Cry1A.105	14	-	-	5.1	-
	Cry2Ab2	12	-	-	1.1	-

2.3 Commercialized Bt maize and target pests in South Africa

In 2017/18, 1.62 million hectares of Bt maize was planted in South Africa (71% of the total maize area) (ISAAA, 2017). Brookes and Barfoot (2016) estimated the economic gains from biotech crops in South Africa during the period 1998-2015 as US$2.1 billion and US$237 million for 2015 alone. Transgenic Bt maize (MON810) has been planted in South Africa since 1998 (Gouse *et al*., 2005) and successfully

controls the target pests, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Van Rensburg, 1999; Van Wyk *et al*., 2009). The efficacy of Cry1Ab was threatened by *B. fusca* which developed resistance to this protein after nine years of successful control (Van Rensburg, 2007). This occurrence of resistance resulted in the deployment of a pyramid event (MON89034) during the 2011/12 growing season in South Africa. Plants of event MON89034 express two Cry proteins (Cry1A.105 and Cry2Ab2), and therefore provides a more effective insect resistant management tool (Van den Berg *et al*., 2013).

As from November 2018 the FAW has been included as a target pest of MON89034 (Botha *et al*., 2019). Although Cry1Ab maize is not registered for control of FAW due to the lack high-dose expression, field observations during 2017 and 2018 indicated that Bt maize provided protection against FAW in South Africa (Prasanna *et al*. 2018). Due to significantly reduced injury levels and complete mortality of FAW larvae feeding on foliar tissue of Cry1A.105 + Cry2Ab2 maize (Bernardi *et al*., 2016; Siebert *et al*., 2012), along with no reports of field-evolved resistance, MON89034 is currently considered capable of providing effective control under field conditions. Bt maize events that express Cry1F, Cry1Ab and Cry1A.105 + Cry2Ab2 proteins, have been used effectively for the control of FAW in the USA and Canada (Buntin *et al*., 2004; Storer *et al*., 2012a; Reay-Jones *et al*., 2016) and several countries in South America (Storer *et al*., 2012a; Buntin *et al*., 2008; Bernardi *et al*., 2016). The presence of the FAW maize strain in South Africa (Jacobs *et al*., 2018), raises concerns since this strain is considered less susceptible to Bt toxins (Ingber *et al*., 2017).

2.4 Resistance evolution

Resistance is defined by Tabashnik (1994) as a genetically heritable decrease in susceptibility to a pesticide in a population. In practice the term field-evolved resistance is preferred as this refers to the genetically-based decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field (Tabashnik, 1994). Resistance development is a lengthy process when selection factors are absent. The presence of a selection factor favouring a certain genotype will increase the development rate of resistance exponentially. The

development of resistance to transgenic Bt crops relies on individual variation within a population and inheritance of resistant alleles due to selection pressure.

Resistance within insects is conferred by a recessive allele and these alleles are found at very low frequencies in populations (Gould, 1998). There are two possible forms of the resistance-gene, namely r (the mutant allele conferring for resistance) and S (the normal allele conferring for susceptibility), encoding for three possible genotypes. Each insect has two copies of the allele within the gene, thus three possible genotypes (SS, Sr and rr) of insects exist (Cohen *et al*., 2000). Gould (1998) stated that for an insect to be resistant (able to survive toxins above the high dose rate) both recessive resistance alleles should occur at the same locus, and therefor only homozygous resistant insects (rr) are assumed to be resistant and heterozygous insects (Sr) as susceptible. Homozygous resistant individuals capable of surviving the selection pressure determine the alleles that are transferred to their offspring.

At first, resistance evolution, where low frequencies of resistant genotypes occur, is slow, until the number of individuals with resistance proliferates within a population. Resistance evolution of insects to Bt maize, threatens the durability and longevity of this technology (Tabashnik *et al*., 1994; Gould, 1998; Carrière *et al*., 2010; Huang *et al*., 2014), which emphasizes the importance of insect resistance management (IRM) strategies to delay or even prevent resistance development. In order to design appropriate IRM strategies it is essential to understand the biochemical mechanisms and genetic basis of resistance to Bt proteins (Ferré & Van Rie, 2002).

2.4.1 Mechanisms of resistance

A mechanism of resistance is defined by Tabashnik *et al*. (2014) as ‘‘a genetically based change in a particular phenotypic trait that decreases susceptibility to a toxin, such as a change in physiology, morphology or behavior’’. Viable mutations in certain receptor genes within individual insects of a population, responsible for low frequencies of variation, could result in resistance evolution to Cry toxins when alteration at any step of the sequential procession of intoxication occur (Ferré & Van Rie, 2002; Tabashnik *et al*., 2003; Wu, 2014). Peterson *et al*. (2017) reviewed 123 papers regarding resistance mechanisms of lepidopteran pests and reported all to be highly complex. Ferré and Van Rie (2002) categorized insect resistance mechanisms

to Cry toxins into three groups of which the first group is considered the most common mechanism of resistance. This group refers to alteration in receptor binding of Cry toxin to BBMV in the midgut, because of the reduction in binding sites or decreased binding affinity. The second type of resistance mechanisms alter the proteolytic activation of the Cry toxins causing a decrease in protoxin solubilization, decreased rates of activation or increased rates of toxin degradation. The third category of resistance mechanisms ensures efficient repair of damaged midgut epithelium cells to avoid septicemia.

Several studies regarding the biochemical mechanisms of resistance within *S. frugiperda* indicates that two of the three groups of resistance mechanisms occur. Aranda *et al*. (1996) found Cry1Ab toxins to have a low affinity for midgut tissue sections and the isolated BBMV of the FAW. Subsequent studies by Jurat-Fuentes *et al*. (2011) and Jakka *et al*. (2015) indicated low binding affinity of Cry1Fa on BBMV midgut tissue occur, because of reduced levels of membrane-bound alkaline phosphatase (ALP) that serves as a receptor for Cry1Fa. Another study done by Miranda *et al*. (2001) indicated that faster degradation of the Cry proteins occur within the midgut of *S. frugiperda* compared to more susceptible insects. The above protein binding assays explained why there was reduced susceptibility of FAW to Cry1 proteins. The main mechanism of resistance reported in field resistant *S. frugiperda* populations is the alteration of binding sites (Herrero *et al*., 2016; Peterson *et al*., 2017). Improvement of resistance management strategies is difficult due to the limited amount of information regarding the resistance mechanisms that are present in *S. frugiperda* and which enables their survival when exposed to Cry toxins.

2.4.2 Field-evolved resistance

Field-evolved resistance occurs when exposure of a field population to a toxin leads to increases in the frequency of recessive resistance alleles in the subsequent progeny (Tabashnik *et al*., 2009). The key concept of field-evolved resistance is the decrease in susceptibility to toxins due to previous exposure of the target insect to the toxin in the field (Sumerford *et al*., 2012). In order to show that field-evolved resistance was responsible for failure of a Bt crop to control a target species, four requirements need to be met (Farias *et al*., 2014). The first requirement is that the Bt

crop must have previously provided economic control of the target pest population, and secondly, excessive damage to the Bt crop occurred later on. A third aspects that needs to be considered is the presence of a resistant target pest phenotype in the population, and lastly, that the resistance is genetically inherited.

The term “field-evolved resistance” does not necessarily imply product failure in the field but rather indicates the necessity of management strategies to prevent field control failure (Tabashnik *et al*., 2009; 2013, Sumerford *et al*., 2012). To avoid this confusion, four levels of field-evolved resistance to Bt crops were defined by Tabashnik *et al*. (2013, 2014). These levels range from “incipient resistance”, with less than one percent of individuals considered resistant, to severe cases of resistance “practical resistance”, were significantly reduced efficacy of a product to control a pest is observed. Since the commercialization of Bt maize only three cases of “practical resistance” (>50% resistant individuals and reduced efficacy reported) have been reported on Bt maize. Two of these reports are lepidopteran pests and the other a coleopteran pest (Sumerford *et al*., 2012; Wu, 2014).

The lepidopteran pest for which practical resistance has been observed most commonly and over the largest geographical areas is *S. frugiperda,* with resistance to more than one Bt event reported from the USA as well as from several countries in South America. According to Storer *et al*. (2010) maize expressing Cry1F proteins has been grown in field trails since 1996 and has been planted on a much bigger scale since 2003 on commercial maize silage farms in Puerto Rico. *Spodoptera frugiperda* was found to be resistant to Bt maize of the event TC 1507 which expresses Cry1F proteins, in Puerto Rico (Storer *et al*. 2010; Matten *et al*., 2008). Two more recent reports of field-evolved resistance to the same Bt event by *S. frugiperda* was made by Farias *et al*. (2014), only three years after commercialization in Brazil, and Huang *et al*. (2014), 13 years after introduction of Cry1F maize into the Southern USA. Omoto *et al*. (2016) reported field-evolved resistance of FAW against an event of Bt maize expressing Cry1Ab proteins, six years after commercialization (Sousa *et al*., 2016). Monnerat *et al*. (2006) reported that *S. frugiperda* is known for variable responses against Cry1Ab across geographies, due to differences in selection pressure over long periods of time. The most recent report of field-evolved resistance to Cry1F was made by Chandrasena *et al*. (2018) in Argentina, eight

years after commercialization. Regarding these cases of resistance, it seems to take around five years for FAW to develop resistance, depending on environmental conditions (Storer *et al*., 2010) and geographical factors (Monnerat *et al*., 2006; Farias *et al*., 2014) (Table 2:3).

Table 2:3 A list of cases of field-evolved resistance of *Spodoptera frugiperda*.

Country	Cry protein	Year of commercial release	Year of 1st resistance report	Years of effective control	Reference
Puerto Rico	Cry1F	2003	2006	3	Storer *et al*., 2010
Brazil	Cry1F	2009	2011	3	Farias *et al*., 2014
USA	Cry1F	2001	2012	11	Huang *et al*., 2014
Brazil	Cry1Ab	2007	2013	6	Omoto *et al*., 2016
Argentina	Cry1F	2005	2013	8	Chandrasena *et al*., 2018

2.4.3 Cross-resistance

Resistance to a certain toxin that subsequently results in resistance to other toxins is defined as cross-resistance (Tabashnik *et al*., 2014). Cross-resistance to Bt toxins generally occur among insecticidal crystal proteins (ICPs) with specific similarities in their mode of action. Therefore, when a resistance mechanism such as altered binding sites is responsible for resistance to one toxin it will lead to resistance to another toxin, if these toxins are highly similar (Wu *et al*., 2014). Cross-resistance also commonly results in multiple resistance. Additionally, multiple resistance could develop by independent resistance evolution to two or more toxins. Multiple resistance refers to resistance of a single organism to a range of toxins due to the exposure of a population to different toxins (Tabashnik *et al*., 2014).

Cross-resistance relies on similar toxin properties, these similarities of toxins is ascribed to protein structure and receptor binding sites, which contribute to the ability of existing resistance mechanisms to result in resistance to a different toxin. This explains why related toxins, with shared binding sites for instance, could more easily result in cross-resistance. Receptor binding studies helped determine which Cry toxins share binding sites within the midgut of *S. frugiperda*, subsequently followed by cross-resistance studies. Commercialized Cry proteins such as Cry1Ab,

Cry1A.105 and Cry1Fa share midgut binding sites, whereas Cry2Ab2 and Vip3A have independent binding sites within the midgut of *S. frugiperda* (Luo *et al*., 1999; Sena *et al*., 2009; Hernández-Rodríguez *et al*., 2013; Monnerat *et al*., 2015). Cry1F-resistant *S. frugiperda* showed none to low indications of cross-resistance to Cry1Ab and Cry1Ac (Storer *et al*., 2010; Vélez *et al*., 2013; Monnerat *et al*., 2015) and low levels of cross-resistance to Cry1A.105 (Huang *et al*., 2014). In contrast, significant levels of cross-resistance to Cry1Ab and Cry1A.105 were assumed (Niu *et al*., 2013) and confirmed (Bernardi *et al*., 2015). Cross-resistance to Cry1A.105 is intelligible considering the association among the gene structure and amino acid sequence of Cry1A.105, Cry1Ab and Cry1F. Cry1A.105 is a chimeric gene, comprised of domains I and III of Cry1Ab and Cry1F respectively, with an overall amino acid sequence identity of 90.0% to Cry1Ab, and 76.7% to Cry1F (BCH, 2018).

Although some level of cross-resistance to Cry2Ab2 was assumed previously, since Cry1F resistant *S. frugiperda* larvae survived on three pyramid Bt events containing Cry2Ab2 (Niu *et al*., 2013), this was later rejected (Niu *et al*., 2014). Hernández-Rodríguez *et al*. (2013) and Monnerat *et al*. (2015) reported that Cry2Ab2 showed low toxicity to susceptible and Cry1Fa-resistant *S. frugiperda* larvae but reported no cross-resistance, as was probably the case with Niu *et al*. (2104). Furthermore, no significant levels of cross-resistance to Cry2A and Vip3A proteins have been observed (Vélez *et al*., 2013; Huang *et al*., 2014; Niu *et al*., 2014; Bernardi *et al*., 2015; Monnerat *et al*., 2015, Li *et al*., 2016). Low levels of cross-resistance between Cry1F, Cry2Ab2 and Vip3 can be attributed to the difference within insecticidal protein structure, hence different modes of action and separate binding receptors (Sena *et al*., 2009; Storer *et al*., 2012b; Hernández-Rodríguez *et al*., 2013).

2.5 Resistance management strategies

Since the commercialization of Bt maize, there have been concerns about resistance development in target pests (Tabashnik *et al*., 1994; Gould, 1998). Resistance evolution is considered to be the single most important threat to the long-term efficacy of this technology (Tabashnik *et al*., 2011; Carrière *et al*., 2016). Biotechnology is highly beneficial to producers and the environment (Romeis *et al*., 2006), but along with the benefits comes the responsibility to ensure the sustainable use of this technology (Head & Greenplate, 2012). The constant monitoring of target

pests to detect shifts in susceptibility combined with the implementation of resistance management strategies is necessary for a successful resistance management program (Bates *et al*., 2005).

Insect resistance management is considered as all the practices aimed at preventing insect pests from evolving resistance to an insecticidal toxin (Glaser & Matten, 2003). The main goal of resistance monitoring is to detect field-evolved resistance early enough to ensure that proactive management is enabled before control failures occur (Tabashnik *et al*., 2004; 2009). In this way, mitigation strategies can be deployed if needed to delay resistance before it becomes widespread (Bates *et al*., 2005). The aim of IRM is not only to monitor and apply strategies on commercialized transgenic crops but to constantly develop and improve the biotechnology of insect resistant transgenic crops (Glaser & Matten, 2003). Numerous cases of field-evolved resistance have been reported (Van Rensburg, 2007; Storer *et al*., 2010; Omoto *et al*., 2016), this indicates that IRM strategies are far from ideal (Bates *et al*., 2005). In several of these cases poor refuge compliance (Kruger *et al*., 2009; Farias *et al*., 2014) and low dose Bt expression seems to be the cause (Omoto *et al*., 2016). The main IRM strategies are, high-dose/refuge and gene-pyramiding (Carrière *et al*., 2010), since these appear to be the most effective in delaying resistance evolution (Cohen *et al*., 2000; Gould, 2000; Huang *et al*., 2011; Storer *et al*., 2012b; Tabashnik *et al*., 2013).

2.5.1 High-dose/refuge strategy

The high-dose/refuge strategy depends on several assumptions and consists of two concepts (Tabashnik *et al*. 2004). Firstly, the high-dose expression of Bt toxins within transgenic plants are compulsory, and secondly, the planting of non-Bt plants (refuge), is necessary in order to delay resistance evolution. This strategy relies on the following assumptions: (1) only homozygous resistant (rr) insects can survive high dose concentrations, (2) these individuals are rare within a population (Gould, 1998), thus refuge plants will (3) produce susceptible insects in abundance to ensure (4) random mating between resistant and susceptible insects occur (Bates *et al*., 2005). Therefore, the main objective of the high-dose/refuge strategy is to keep resistance traits functionally recessive.

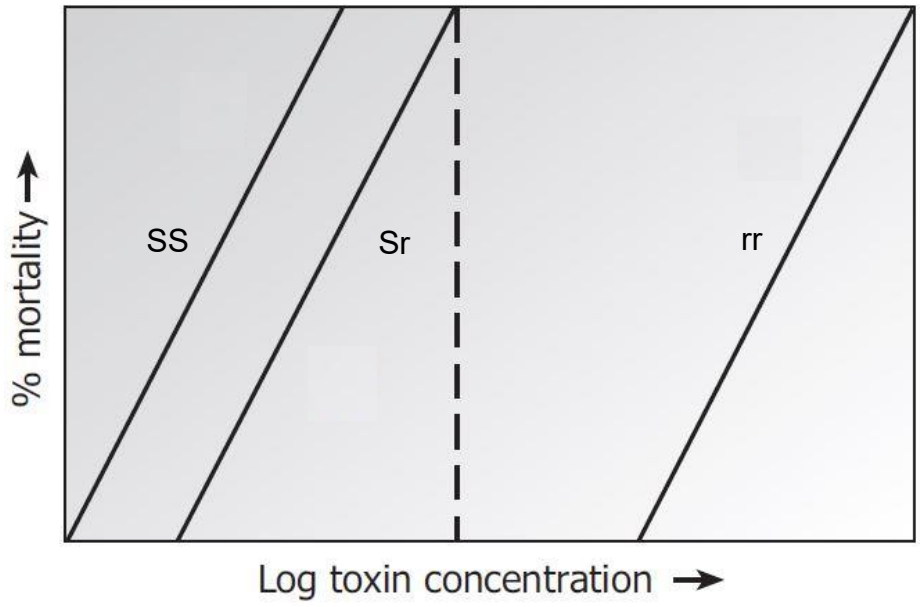

Figure 2:8 Dose-response lines indicating the mortality of three insect genotypes at increasing concentrations of an insecticidal toxin.

The dotted line indicates the concentration required for a “high dose.” S, allele conferring susceptibility; r, allele conferring resistance (Cohen *et al*., 2000).

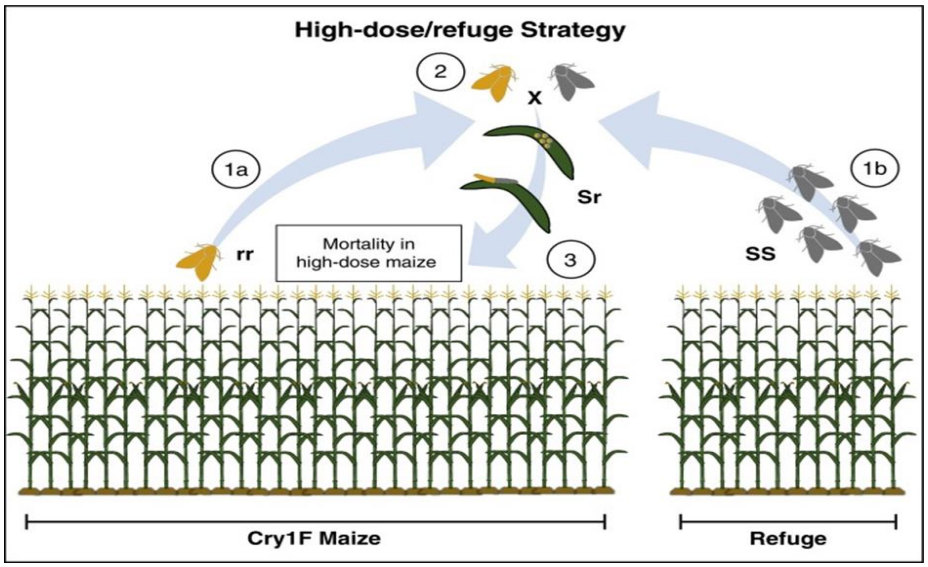

Figure 2:9 Illustration of the mechanisms through which the high-dose/refuge strategy functions.

(1a) Few homozygous resistant (rr) insects will emerge from Bt maize; (1b) multiple homozygous susceptible (SS) insects will emerge from the non-Bt maize refuge; (2) homozygous susceptible (SS) insects will mate with homozygous resistant (rr) insects; (3) functionally recessive resistance will generate heterozygous (Sr) offspring that will eventually die with the high-dose expressed in Bt maize seed mixture (Vélez *et al*., 2016).

Within the high dose/refuge strategy, high dose is defined as a dose that kills almost all the heterozygous (Sr) insects. High dose plants theoretically express toxins at a level high enough to kill almost all the heterozygous (Sr) insects (Figure 2:8). This high dose has been defined by the USEPA (1998a) as a level that is 25 times higher than that required to kill 99% of heterozygous (Sr) insects. In order to determine the high dose for a target pest, it is necessary to use heterozygous insects (Ranjekar *et al*., 2003). The only possible way in which resistance can develop within a population is through resistant genotypes (rr), as these are the only insects capable of surviving the high dose toxin concentration expressed by transgenic plants. It may appear that high dose plants, by killing all the susceptible (SS) and heterozygous (Sr) insects, can lead to rapid resistance development, but this will only occur if refuge planting compliance is poor. The second component of this strategy is the refugia, which consist of non-Bt host plants, which is essential for sustaining a population of susceptible insects (Tabashnik *et al*., 2003). These homozygous-susceptible insects (SS) will mate with homozygous-resistant (rr) insects ensuring the persistence of heterozygous (Sr) insects which are susceptible to high dose rates (Cohen *et al*., 2000; Glaser & Matten, 2003). It is necessary for refuge plantings to be located in close proximity to Bt fields to ensure optimal random mating between susceptible moths from the refuge and any resistant survivors from the Bt maize fields (Glaser & Matten, 2003) (Figure 2:9).

2.5.2 Gene-pyramiding

First-generation Bt maize refers to insect resistant maize expressing a single insecticidal protein with only one mode of action, active against only a few pest species, and which mainly relied on the high-dose/refuge strategy to delay insect resistance evolution (Storer *et al*., 2012b). Second-generation Bt maize refer to Bt crops that produce two or more distinct Bt toxins expressed at a high dose, equally active against the same target pests, resulting in complete ‘redundant killing’ of susceptible insects (Comins, 1986; Gould, 1986, Carrière *et al*., 2016). Therefore, second-generation Bt maize in itself serves as an IRM strategy and is considered to be more effective in delaying resistance evolution as resistance to two modes of actions is extremely rare (Gould, 1998). Although two or more proteins are combined within a pyramided event, the need for the distinct insecticidal proteins to be present

in all tissues expressed throughout the larval feeding period and to provide high dose expression is essential (Storer *et al*., 2012b).

Gould *et al*. (2006) reported that when two distinct toxins are produced at high dose concentrations, resistance evolution is expected to increase slowly even if refuge plantings are small and initial resistance frequencies are high. According to Roush (1998), pyramided Bt plants with a 5% refuge can delay resistance up to eight times longer than a single-gene Bt plants with a 20% refuge (Figure 2:10). Ives *et al*. (2011) confirmed that pyramided traits more effectively delay resistance evolution with only a small refuge (2-5% of the crops acreage) by providing hundreds of generations of durable use. Even though resistance is expected to be more recessive in pyramided events (Gould, 1998; Carrière *et al*., 2010), refuge plantings are compulsory as they increase the durability of this technology (Gould, 1986).

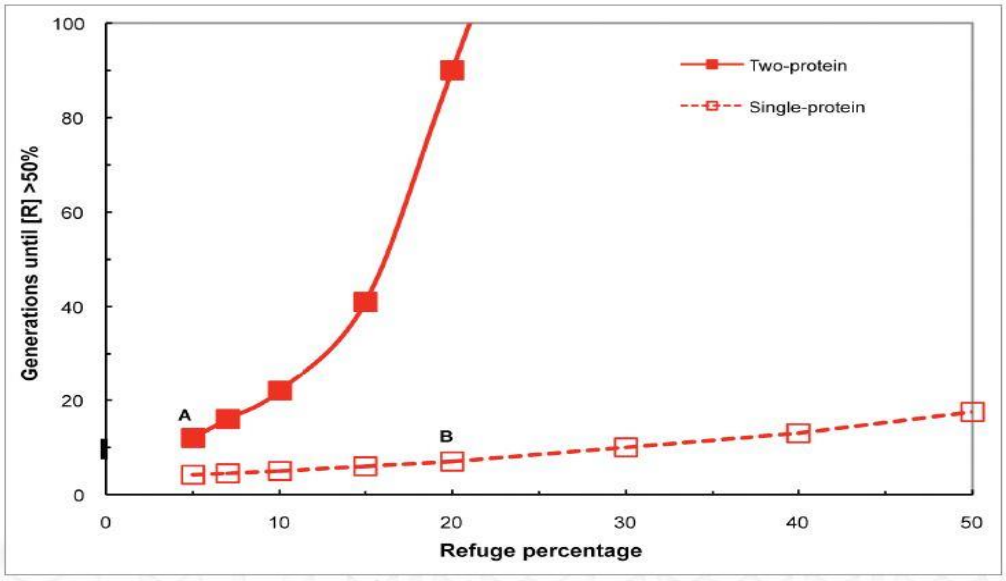

Figure 2:10 The effect of refuge size on rate of resistance evolution to pyramided Bt proteins compared to single Bt proteins (Roush, 1998).

The concept behind gene pyramiding is based on the requirements identified by Tabashnik (1989) in managing resistance by means of multiple pesticides. The criteria required for durable gene pyramiding are: (1) each Cry protein within the cultivar ensures high mortality among homozygous susceptible insects individually (complete redundant killing), (2) low probability of cross-resistance between Cry proteins, (3) refugia prevent selection pressure amongst the whole population of target insects, and (4) both proteins express equal persistence (Storer *et al*. 2012b;

Gressel *et al*., 2017). Compared to first-generation Bt crops that only produce a single Bt toxin, pyramid traits improved cross-resistance management and are assumed to cover a broader spectrum of target pests, due to the presence of multiple Bt toxins. Insecticidal toxins are highly specific with regards to binding sites, and since toxins are pyramided in second generation hybrids, the variety of binding sites within the midgut of the target pest requires an individual to possess two mutations in order to become resistant to both toxins (Hernández-Rodríguez *et al*., 2013).

Therefore, when Cry proteins in pyramid Bt crops share binding sites, a single mutation conferring for alteration with regards to the binding affinity or target site, the likelihood of resistance development to both proteins are expected (Tabashnik *et al*., 2009). Therefor when a high possibility of cross-resistance is present among Cry proteins, these proteins should not be pyramided as it will make this resistance strategy useless. Niu *et al*. (2013) and Bernardi *et al*. (2015) emphasized the fact that Bt proteins should be carefully selected for pyramid events, as the authors found that Cry1F-resistant FAW larvae showed significant levels of cross-resistance to proteins expressed by three other pyramid events. Since Cry and Vip proteins bind to different receptors in the midgut of susceptible insects, these proteins are recommended for pyramiding in transgenic crops to improve resistance management (Roush, 1998; Zenas & Crickmore, 2012). According to Sena *et al*. (2009), Vélez *et al*. (2013), Yang *et al*. (2013), Niu *et al*. (2014) and Santos-Amaya *et al*. (2015) Vip3A is highly effective against susceptible and Cry1 resistant *S. frugiperda* larvae, and should therefore be combined with Cry1 proteins to counter and delay resistance evolution.

The pyramid strategy is expected to be effective if recessive resistance to each Bt toxin occurs, refugia is planted, and cross-resistance among Bt toxins are absent (Roush 1998; Zhao *et al*., 2005; Gould *et al*., 2006). Head and Greenplate (2012) reported that second generation Bt technologies, responsible for multiple modes of action within the Bt crop, will most probably increase the durability of Bt crops as this strategy is mainly dependent on manufacturers and less dependent on the behavior of the producer. The validation of gene-pyramiding is based on the presence of distinct binding sites for different proteins that are pyramided, so that multiple

mutations, which seem to be recessive, are needed for the target pest to become resistant (Roush, 1998; Zenas & Crickmore, 2012; Hernández-Rodríguez *et al*., 2013).

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3 Chapter 3: Efficacy of Bt Maize for Control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in South Africa

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Field and Forage Crops

Efficacy of Bt Maize for Control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in South Africa

A. S. Botha,[1] Annemie Erasmus,[2] Hannalene du Plessis,[1] and Johnnie Van den Berg[1,3]

[1]Unit for Environmental Sciences and Management, IPM Program, North-West University, Potchefstroom 2520, South Africa, [2]Agricultural Research Council, Grain Crops, Private Bag X1251, Potchefstroom 2520, South Africa, and [3]Corresponding author, e-mail: johnnie.vandenberg@nwu.ac.za

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Abstract

Spodoptera frugiperda (J.E. Smith) is an invasive pest species that threatens maize production by small holder farmers in Africa. Bt maize that express Cry proteins have been used effectively for control of this pest in the United States, Canada, and several countries in South America. *Spodoptera frugiperda* has evolved resistance to Cry1F Bt maize in Puerto Rico, Brazil and United States, and Cry1Ab Bt maize in Brazil. This study provides the first data on the efficacy of Bt maize for the control of *S. frugiperda* in Africa. Susceptibility levels of nine *S. frugiperda* populations were evaluated between January 2018 and May 2018, including a laboratory reared reference population. Larval feeding bioassays were conducted in which plant tissue of maize expressing Cry1Ab (single-toxin event - designated Bt1) or Cry1A.105 + Cry2Ab2 (pyramid-toxin event - designated Bt2), were fed to larvae. Survival and different life history parameters were recorded. Results indicate moderate survival on Bt1 maize, which supports field observations of commercial level control provided by this event. Very high levels of *S. frugiperda* larval mortality occurred on Bt2 maize (<1% survival). The moderate susceptibility of *S. frugiperda* to Cry1Ab could be ascribed to the latter being a low-dose event for this pest, as well as the fact that the individuals which initially arrived on the continent may have carried alleles with resistance to this protein. Moderate overall survivorship (4–35%) of *S. frugiperda* on Cry1Ab maize in South Africa reflects the possible presence of alleles resistant to this toxin, indicating the importance of future resistance monitoring.

Key words: Cry protein, fall armyworm, insect resistance management, monitoring, resistance

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), invaded Africa from the Americas early in 2016 (Goergen et al. 2016). *Spodoptera frugiperda* is highly polyphagous (>300 host plants) but moths prefer to lay eggs on plants of the Poaceae family (Montezano et al. 2018). Bt maize events that express Cry1F, Cry1Ab, and Cry1A.105 + Cry2Ab2 proteins, have been used effectively for control of *S. frugiperda* in the United States and Canada (Buntin et al. 2004, Siebert et al. 2012, Storer et al. 2012, Reay-Jones et al. 2016) and several countries in South America (Buntin et al. 2008, Storer et al. 2012, Bernardi et al. 2016). In South Africa MON810 maize (designated Bt1), expressing Cry1Ab protein, and MON89034 (Bt2), which expresses Cry1A.105 + Cry2Ab2 proteins, are cultivated for control of maize stem borers. *Spodoptera frugiperda* has been included as a target pest of MON89034 from November 2018.

Bt maize has been planted in South Africa since 1998 (Gouse et al. 2005) for control of target pests, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae). Although *B. fusca* evolved resistance to Cry1Ab Bt maize (Kruger et al. 2014), this problem was successfully addressed after the release of Bt2 maize during 2011 (Van den Berg et al. 2013). During 2017/2018 cropping season 1.62 million hectares of Bt maize were planted in South Africa (71% of the total maize area), which is also the only African country where cultivation of Bt maize has been approved (ISAAA 2017). Along with the benefits of biotechnology comes the responsibility to ensure sustainable use of this technology (Head and Greenplate 2012).

Since the commercialization of Bt maize, there have been concerns about the evolution of resistance in target pests (Tabashnik 1994, Gould 1998). Monnerat et al. (2006) reported that *S. frugiperda* populations from different geographical regions in South America showed variable responses to Cry proteins and that responses correlated with genetic background of populations. In Puerto Rico *S. frugiperda* was found to be resistant against the Bt maize event TC 1507, which expresses Cry1F proteins (Matten et al. 2008, Storer et al. 2010). Two recent reports of field-evolved resistance to Cry1F maize by *S. frugiperda* are that by Farias et al. (2014) 5 yr after commercialization in Brazil and by Huang et al. (2014), 13 yr after introduction of Bt maize into the southern states of the

United States. Omoto et al. (2016) reported field-evolved resistance of *S. frugiperda* against Cry1Ab Bt maize in Brazil, 8 yr after its introduction in 2007 (ISAAA 2017). The above-mentioned examples indicate that *S. frugiperda* can evolve resistance to Cry proteins over a relatively short period of time, depending on environmental conditions (Storer et al. 2010) and genetic background (Monnerat et al. 2006, Farias et al. 2014). *Cry*1A.105 is a chimeric gene, comprising of domains I and II of Cry1Ab and Cry1Ac, respectively, and domain III of Cry1F, with an overall amino acid sequence identity of 90.0% to Cry1Ab, 93% to Cry1Ac, and 76.7% to Cry1F (BCH 2018). Niu et al. (2013), Bernardi et al. (2015), and Li et al. (2016) reported that Cry1F resistant *S. frugiperda* larvae had significant levels of cross-resistance to Cry1A.105 protein. However, cross-resistance to Cry1A.105 is not expected in this study since a recent study by Nagoshi et al. (2017) showed that the genetic marker for Cry1F resistance was not detected in the West African population of *S. frugiperda*.

Fall armyworm in Africa probably originated from the Americas (Goergen et al. 2016, Nagoshi et al. 2018). This means they probably were exposed to Bt maize and could have resistance to one or more Cry proteins. Nagoshi et al. (2018), however, reported a haplotype in the African *S. frugiperda* population which they did not find in American populations they tested. Thus, the introduction history of *S. frugiperda* in West Africa still has some uncertainty. The aim of this study was not to evaluate resistance and compare between different populations but to evaluate the efficacy of Bt1 and Bt2 maize against *S. frugiperda* in South Africa.

Material and Methods

Collection and Rearing of Different *S. frugiperda* Populations

Larvae of *S. frugiperda* were sampled in non-Bt maize fields from six of South Africa's nine provinces, between January 2017 and March 2018. Attempts were made to collect at least 300 larvae at each of the nine different localities. All populations were kept separate during rearing and trials. These localities were in the districts of Groblersdal (designated GLD18), Settlers (SET17-F15), Dundee (DNE18), Koster (KOS18), Stofberg (SFB18), Vaalharts (VAA18), Venda (VEN18), Nelspruit (NSP18), and East London (ELD18) (Table 1). The SET17-F15 population used in this study was the F15 generation of the first *S. frugiperda* population collected in South Africa during January 2017. This population was maintained on artificial diet until these experiments were conducted. Although the numbers of larvae that were collected in the Vaalharts region were very low (n = 34) this population (VAA18) was used since this area was the first where *B. fusca*, evolved resistance to Bt maize and where Bt cotton is also planted. The northern most and most southern localities where *S. frugiperda* was sampled were 1,200 km apart, with a distance of 680 km between the populations that were collected east and west.

Collected larvae were reared individually in plastic aerated containers (50 ml) until pupation. Non-Bt maize whorl leaves collected from field grown maize were provided as food. Containers were kept in insect rearing chambers at 28 ± 2°C, 60 ± 10% relative humidity (RH), and a photoperiod of 14:10 (L:D) h until pupation. Male and female moths were then paired and put into plastic containers to mate and lay eggs. A minimum of 15 moth pairs from each population was used to ensure that a genetic diverse population of the F1-larvae was used in the study and not only the offspring of a limited number of females. The F1 instars derived from different egg masses were used in bioassays.

Larval Feeding Bioassays

Bioassays were conducted with each population. Each bioassay had three treatments and 10 replicates. The treatments were a non-Bt near iso-hybrid (DKC 80-10), maize that expresses Cry1Ab protein (DKC 80-12B) and maize that expresses both Cry1A.105 + Cry2Ab2 proteins (DKC 80-12BGEN). Each replicate consisted of 10 plastic aerated containers (50 ml) with one larva per container (10 replicates per treatment × 10 containers per replicate = 100 larvae per treatment). Envirologix QuickStix strip tests were used to confirm the Bt status of the maize fed to the larvae.

All maize plants used in these bioassays were grown under field conditions. Maize plant tissue fed to the larvae was derived from the whorl of maize plants during the V6-V8 stages. Larvae were provided with fresh leaf tissue and larval mass was determined 7, 10, and 14 d after inoculation. One 5-cm-long cutting of the inner furl role was provided on each of these days. Pupal mass and duration of the pupal period until the moths emerged were also determined. The total developmental period was calculated by adding the duration of larval period to that of pupal period.

Inhibition of larval growth on Bt maize leaf tissue was calculated, relative to the control larvae, using the method described by Storer et al. (2010). Mean mass of larvae that fed on Bt plant tissue was divided by the mean mass of larvae that fed on non-Bt tissue and expressed as a percentage value, providing an indication of what larvae should have weighed at that time, compared to non-Bt feeding larvae. The following equation was used: 1-(a/b) = c (%), where a = mass of larvae on Bt, and b = mass of larva on non-Bt).

Data Analysis

The percentage larval survival, pupation, and moth emergence were compared by means of binomial distribution tests. For the populations where survival on both the Bt1 and Bt2 maize occurred, pairwise comparisons to the control treatment were made,

Table 1. Information regarding populations of *Spodoptera frugiperda* collected at different localities in South Africa

Population code	Collection area	Date of collection	Number of larvae collected	Latitude	Longitude
GLD18	Groblersdal	22 Jan. 2018	400	S 25° 16′ 28.21″	E 29° 25′ 23.74″
SET17-F15	Settlers	20 Jan. 2017	300	S 24° 54′ 43.46″	E 28° 32′ 24.89″
DNE18	Dundee	29 Jan. 2018	300	S 28° 02′ 28.01″	E 30° 22′ 45.90″
KOS18	Koster	08 Feb. 2018	300	S 25° 49′ 07.31″	E 26° 58′ 32.00″
SFB18	Stofberg	07 Feb. 2018	500	S 25° 25′ 31.61″	E 29° 50′ 30.30″
VAA18	Vaalharts	05 Mar. 2018	34	S 27° 53′ 16.60″	E 24° 49′ 14.10″
VEN18	Venda	12 Mar. 2018	350	S 22° 28′ 31.66″	E 30° 19′ 21.22″
NSP18	Nelspruit	20 Mar. 2018	800	S 25° 26′ 14.20″	E 30° 59′ 34.60″
ELD18	East London	30 Mar. 2018	180	S 33° 03′ 14.00″	E 27° 35′ 50.20″

using data collected after 10 d. Larval and pupal mass as well as developmental time was analyzed by means of *t* tests. For the populations from which larval mass were recorded on both Bt1 and Bt2 maize, data were subjected to a one-way analysis of variance (ANOVA). Significant differences between the treatments were determined using the Tukey HSD test ($P < 0.05$). The duration of developmental period on Bt and non-Bt maize was compared by means of *t* tests. The data were checked for normal distribution before analyses and since data were not normally distributed, larval masses were log-transformed before *t* tests were conducted. Untransformed means are provided in tables. All statistical analyses were done using STATISTICA (version 13.3; StatSoft, Inc. 2018).

Results

Larval Survival and Pupation

Larval survival after 10 d of feeding on non-Bt maize leaf tissue ranged between 80% (SET17-F15) and 96% (DNE18) for the respective populations (Table 2). Survival of larvae that fed on Bt1 maize tissue ranged between 20 (VAA18) and 86% (VEN18) for the different populations, but the growth of these surviving larvae was drastically inhibited (>81.7%) (Table 3). While 86% of larvae of the VEN18 population survived on the Bt1 maize after 10 d, only 3% of the larvae of this population survived up to 10 d on the Bt2 maize with a growth inhibition of 95%.

The percentage pupation of larvae that fed on non-Bt maize ranged between 71 and 94%, with moth emergence of 50 (SFB18) to 92% (VAA18), for the respective populations (Table 4). The percentage pupation on Bt1 maize ranged between 7 and 54% and moth emergence of between 4 (KOS18) and 35% (VEN18) (Table 4). Only a single larva from all the populations that fed on leaf tissue of Bt2 maize pupated.

Larval and Pupal Mass

Larval mass of the respective non-Bt treatments ranged between 0.404 g (VAA18) and 0.305 g (SET17-F15), and 0.009 g (DNE18) and 0.061 g (VEN18) for the respective Bt treatments after 10 d of feeding (Table 2). Larval mass was significantly lower on Bt1 maize, on days 7 and 10, compared to non-Bt maize and larval growth was highly inhibited (between 81.7 and 97.6%) (Table 3), which was also evident in reduced pupal mass (Table 3). Pupal mass of the larvae that fed on the non-Bt maize tissue ranged between 0.180 g (NSP18) and 0.237 g (GLD18) (Table 4), while the larvae that fed on Bt maize had a 9.1–33.5% reduced pupal mass compared to those from non-Bt maize (Table 3).

Developmental Period

Comparative developmental periods of larvae that fed on Bt or non-Bt maize for the different populations are presented in Fig. 1. The developmental periods were significantly longer (5 to 10 d) on Bt maize for all populations. The mean duration of the larval period was significantly longer on Bt1 maize than on non-Bt maize for all the populations (Table 2). The duration of the larval period ranged between 11.9 and 15.2 d on non-Bt maize and 18.3 and 23.6 d on the Bt1 event. The larval duration of the single larva that survived on the Bt2 event was 29 d. Duration of the pupal period ranged between 6.5 and 8.8 d for the different populations on non-Bt maize and between 7.9 and 9.6 d on Bt1 maize (Table 4). Duration of the pupal period differed significantly between non-Bt and Bt maize for five of the populations (Table 4).

Discussion

Survival of larvae from each population was significantly lower on Bt1 maize than non-Bt maize. Based on the low survival of larvae, very strong growth suppression, a long extension of larval periods and the

Table 2. Percentage larval survival, larval mass, larval duration, and growth inhibition after 10 d of feeding on non-Bt- Bt1 and Bt2 maize, of different *Spodoptera frugiperda* populations

	Populations								
Treatment	GLD18	SET17-F15	DNE18	KOS18	SFB18	VAA18	VEN18	NSP18	ELD18
% Larval survival (%± SE)									
Non-Bt	93 (0.2)	80 (0.3)	96 (0.2)	95 (0.2)	82 (0.5)	94 (0.2)	93 (0.3)	92 (0.3)	93 (0.2)
Bt1	56 (0.4)	57 (0.7)	37 (0.6)	21 (0.4)	32 (0.4)	20 (0.1)	86 (0.3)	56 (0.3)	70 (0.3)
Bt2	-	2[a]	-	-	-	-	3[a]	2[a]	7[a]
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.11	<0.001	<0.001
Larval mass (g ± SE)									
Non-Bt	0.373a (0.008)[b]	0.305a (0.070)	0.356a (0.014)	0.373a (0.010)	0.380a (0.018)	0.404a (0.006)	0.344a (0.017)	0.322a (0.011)	0.375a (0.012)
Bt1	0.052b (0.007)	0.023b (0.020)	0.009b (0.002)	0.010b (0.003)	0.014b (0.004)	0.011b (0.002)	0.061b (0.007)	0.018b (0.001)	0.027b (0.005)
Bt2	-	0.003b (0.014)	-	-	-	-	0.005c (0.005)	0.001b (0.001)	0.003b (0.003)
df	18	27	18	18	18	18	27	27	27
t value	91.77	-	23.97	34.89	19.81	17.71	-	-	-
F value	-	159.19	-	-	-	-	300.21	783.85	808.07
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Larval duration (days ± SE)									
Non-Bt	13.1 (0.03)	15.2 (0.25)	13.7 (0.15)	14.1 (0.03)	14.0 (0.02)	12.8 (0.07)	11.9 (0.11)	12.5 (0.13)	12.5 (0.08)
Bt1	18.3 (0.30)	22.7 (0.66)	20.7 (0.75)	21.6 (1.91)	23.6 (1.86)	21.1 (0.94)	19.6 (0.34)	21.5 (0.55)	21.3 (0.88)
Bt2	-	-	-	-	-	-	-	-	29 (0)[b]
df	124	104	95	91	77	103	145	110	105
t value	−24.21	−12.81	−13.33	−14.28	−16.07	−22.14	−25.67	−23.12	−13.76
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a]Not included in analysis.

[b]Means within columns followed by the same lower case letter do not differ significantly.

Table 3. Larval growth inhibition after 7 and 10 d and the mean percentage reduction in pupal mass

	Populations								
Treatment	GLD18	SET17-F15	DNE18	KOS18	SFB18	VAA18	VEN18	NSP18	ELD18
% Growth Inhibition on day 7 (±SE)									
Bt1	90.7 (1.5)	95.5 (0.4)	98.3 (0.4)	98.9 (0.2)	97.6 (0.5)	98.4 (0.4)	93.0 (0.9)	96.3 (0.5)	94.5 (0.8)
Bt2	-	94.4 (3.2)	-	-	-	-	98.6 (0.4)	99.8 (0.2)	97.2 (1.3)
P value	-	0.44	-	-	-	-	0.003	0.016	0.13
% Growth Inhibition on day 10 (±SE)									
Bt1	85.9 (1.8)	91.8 (2.3)	97.6 (0.5)	97.4 (0.7)	96.6 (0.8)	97.4 (0.5)	81.7 (2.0)	94.3 (0.4)	92.9 (1.2)
Bt2	-	90.2 (8.9)	-	-	-	-	94.5 (1.7)	98.4 (0.2)	97.0 (0.9)
P value	-	0.78	-	-	-	-	0.007	0.002	0.098
% Reduction in pupal mass (±SE)									
Bt1	27.90 (2.19)	18.67 (3.40)	26.92 (4.80)	13.21 (4.28)	15.13 (9.43)	32.57 (6.20)	33.53 (2.65)	9.11 (3.92)	24.10 (4.36)
Bt2	-	-	-	-	-	-	-	-	30.2[a]

[a]Only one individual.

Table 4. Percentage pupation, pupal mass the day of pupation, pupal duration and moth emergence after feeding on non-Bt-, Bt1 and Bt2 maize, of different *Spodoptera frugiperda* populations

	Populations								
Treatment	GLD18	SET17-F15	DNE18	KOS18	SFB18	VAA18	VEN18	NSP18	ELD18
% Pupation (% ± SE)									
Non-Bt	86 (0.3)	75 (0.3)	88 (0.2)	86 (0.4)	71 (0.6)	94 (0.2)	93 (0.2)	84 (0.6)	91 (0.3)
Bt1	41 (0.3)	31 (0.5)	9 (0.4)	7 (0.2)	8 (0.2)	11 (0.2)	54 (0.3)	28 (0.2)	45 (0.3)
Bt2	-	-	-	-	-	-	-	-	1[a]
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Pupal mass (g ± SE)									
Non-Bt	0.237 (0.003)	0.196 (0.004)	0.203 (0.003)	0.219 (0.003)	0.214 (0.003)	0.208 (0.003)	0.205 (0.003)	0.180 (0.004)	0.219 (0.003)
Bt1	0.168 (0.007)	0.157 (0.006)	0.144 (0.013)	0.183 (0.010)	0.179 (0.016)	0.138 (0.012)	0.136 (0.005)	0.163 (0.008)	0.170 (0.006)
Bt2	-	-	-	-	-	-	-	-	0.155[a]
df	125	104	95	91	77	103	145	110	107
t value	10.41	5.58	5.37	3.07	3.67	7.52	12.78	2.26	8.37
P value	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	0.026	<0.001
Pupal duration (days ± SE)									
Non-Bt	7.4 (0.11)	7.8 (0.13)	8.1 (0.12)	6.5 (0.15)	6.5 (0.16)	7.7 (0.17)	8.6 (0.15)	8.2 (0.13)	8.8 (0.12)
Bt1	8.0 (0.19)	7.9 (0.26)	8.6 (0.30)	8.0 (0.41)	8.4 (0.75)	8.0 (0.37)	8.4 (0.25)	9.5 (0.28)	9.6 (0.37)
Bt2	-	-	-	-	-	-	-	-	9 (0)[a]
df	104	72	81	71	53	96	116	89	91
t value	−2.52	−0.14	−1.28	−2.36	−3.28	−0.41	0.81	−4.60	−2.66
P value	0.013	0.88	0.21	0.02	0.001	0.57	0.41	<0.001	0.009
% Moth emergence (% ± SE)									
Non-Bt	74 (0.3)	52 (0.5)	76 (0.4)	69 (0.7)	50 (0.7)	92 (0.2)	85 (0.4)	67 (0.4)	83 (0.4)
Bt1	33 (0.4)	22 (0.6)	7 (0.3)	4 (0.2)	5 (0.2)	6 (0.2)	35 (0.4)	24 (0.2)	33 (0.4)
Bt2	-	-	-	-	-	-	-	-	1[a]
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a]Actual number, not included in analysis.

lower incidence of pupae formation, all the populations were moderately susceptible to Cry1Ab maize. This supports field observations of commercial level control provided by this event (Prasanna et al. 2018). Although 86% (VEN18) of the larvae feeding on Bt1 maize tissue survived until day 10 the percentage that pupated, approximately 4 d later, was only 54%, significantly lower than the 93% of larvae feeding on non-Bt. Three larvae of the VEN18 population survived until day 10 on Bt2 maize, but none of these larvae pupated.

Despite the moderate larval survivorship on Bt1 maize, these larvae weighed significantly less than control larvae (>82%). Weight differences for the pupal stage, however, were not as pronounced. This measure was biased because only a few larvae pupated and those that survived were only slightly affected.

Similar results were reported for *S. frugiperda* by Omoto et al. (2016). The significantly lower mean mass of larvae that fed on Bt1 maize can be ascribed to growth inhibition, which indicates that *S. frugiperda* is still largely susceptible to Cry1Ab. Sousa et al. (2016) reported *S. frugiperda* survival on Cry1Ab maize of between 22 and 73%, depending on the collection site and reported growth inhibition of 81% on Cry1Ab maize. Similarly, Hardke et al. (2011) reported 25–76% pupation, and significantly lower *S. frugiperda* pupal mass on Cry1Ab maize.

The low pupation rate could be due to the extended duration of the larval period. The developmental periods of all populations were significantly delayed. Prolonged development periods on Cry1Ab maize were also reported by Buntin et al. (2001) and Omoto et al.

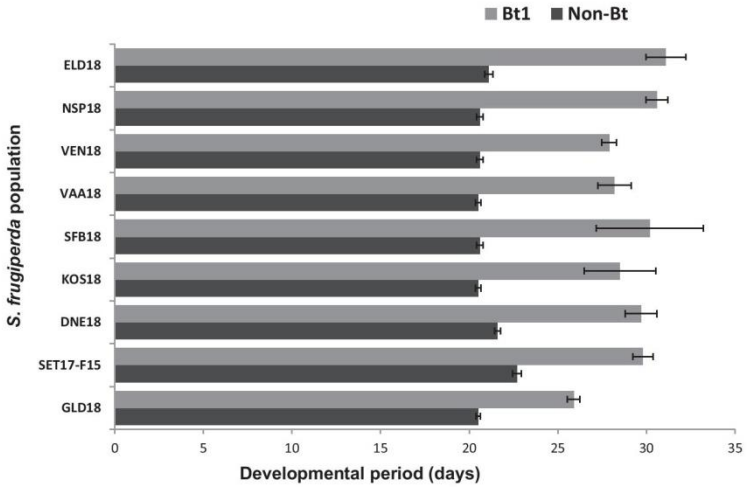

Fig. 1. Mean developmental period (days) of *Spodoptera frugiperda* on non-Bt and Bt1 maize (Bars represent standard error). Developmental period differed significantly ($P < 0.05$) between the Bt1 and non-Bt treatments for each population. No data provided for Bt2 since only one moth emerged.

(2016). This delay in development, under field conditions, would lengthen the exposure time of *S. frugiperda* larvae to harsh environmental conditions, natural enemies and pathogens (Mendes et al. 2012), and in this way contribute to delay resistance evolution.

Only one larva that fed on Cry1A.105 + Cry2Ab2 maize tissue survived until pupation. Bernardi et al. (2016) reported 100% mortality of *S. frugiperda* in leaf-disc bioassays with Cry1A.105 + Cry2Ab2 maize and concluded that it was completely susceptible to these proteins. Considering the efficacy of the Bt2 maize in this study, commercialized pyramid events seem to express Cry1A.105 + Cry2Ab2 toxins at a high enough level to provide highly effective control. Improvement of *S. frugiperda* control by pyramids was however not observed by Buntin et al. (2004) who reported no significant differences in survival between single toxin and pyramid Bt maize.

Bt maize hybrids expressing Cry1Ab and Cry1A.105 + Cry2Ab2 proteins are planted widely in South Africa for control of maize stem borers (Kruger et al. 2014) while while *S. frugiperda* has only recently been added to the list of target pests of Cry1A.105 + Cry2Ab2 maize. Field observations do however indicate indicate moderate to highly effective control of *S. frugiperda* in Bt maize fields. Cry1Ab maize will most likely be approved for cultivation in several other east and southern African countries within the next few years where field trials have occurred for a number of years (ISAAA 2017). Resistance to Bt proteins by *S. frugiperda* is a major threat as shown by the evolution of resistance to Cry1Ab in Brazil (Omoto et al. 2016). Thus, insect resistance management strategies should be established and baseline susceptibility data should be collected for *S. frugiperda* in Africa, where genetically engineered events in maize and other crops are introduced against this pest. This would allow future assessments of susceptibility shifts of *S. frugiperda* populations.

The aim of this study was not to screen for resistance alleles or compare the levels of susceptibility between populations, but to evaluate the efficacy of Bt maize against *S. frugiperda*. It is unlikely that any local differences in genetic background between populations already occurs since the founder populations of the current *S. frugiperda* mega-population in Africa only arrived in West Africa during January 2016 and in South Africa during December 2016. The time period for genetic differentiation between populations was therefore highly limited compared to that described by Monnerat et al. (2006) where differences in genetic backgrounds between populations sampled from different countries were associated with differential responses to Cry proteins.

Spodoptera frugiperda was first reported in West Africa during 2016 and in South Africa, which is the only country on the African continent that commercially grows Bt maize, in 2017. It is therefore unlikely that *S. frugiperda* has evolved resistance to Bt maize in South Africa because of limited time and opportunity for exposure. The levels of survival observed in this study could indicate the presence of Cry1Ab resistance alleles in the *S. frugiperda* population that invaded Africa, or that the Cry1Ab event does not meet the required high-dose criteria. USEPA (1998, 2001) reported that Cry1Ab maize did not meet the high-dose criteria for *S. frugiperda*. Gould (1998) defined a high-dose event, in the context of Bt crops, to be a dose that kills 99.99% of susceptible individuals in the field. It is reasonable to argue that the lack of meeting high-dose levels could be responsible for the moderate larval survival on Cry1Ab maize. Sousa et al. (2016) also concluded that Cry1Ab maize was a low-dose event for *S. frugiperda*. Bt maize was initially developed for the control of two Crambidae stem borers in North America (Armstrong et al. 1995, Ostlie et al. 1997, Archer et al. 2001) but was reported to have potential to control other Lepidoptera pests of maize (Koziel et al. 1993, Armstrong et al. 1995). Although Noctuidae pests were not initially considered the target pests of Cry1Ab maize, it was deployed from 1998 onwards against a noctuid stem borer in South Africa after successful control was observed in laboratory and field experiments (Van Rensburg 1999).

Moderate survival of *S. frugiperda* on Cry1Ab maize may lead to increased numbers of heterozygous individuals in the environment and will most likely contribute to resistance evolution (Gould 1998). The latter scenario will however only apply to South Africa and will not be relevant to other African countries until such time that Bt maize is cultivated in those countries.

Warm and humid conditions in many parts of Africa could lead to multiple generations of *S. frugiperda* that increase selection pressure for resistance where *S. frugiperda* populations occur with Bt

crops. Due to very low winter temperatures *S. frugiperda* does not overwinter in the main maize production regions of South Africa (du Plessis et al. 2018). Absence of a diapause phase in this pest species requires continues reproduction for them to survive (Sparks 1979). The permanent establishment of populations of *S. frugiperda* that will serve as source populations for annual southwards invasions into southern Africa is predicted to lay largely outside the borders of South Africa. No other country in Africa currently allows cultivation of Bt maize, thus selection for resistance currently is limited to areas in South Africa where permanent *S. frugiperda* populations might establish. If source populations establish permanently in environments with high Bt selection pressure in some climatically suitable areas of South Africa, it could be expected that the levels of susceptibility may change comparatively quicker.

Within the *S. frugiperda* species two genetically distinct but morphologically identical strains occur, namely the maize- and the rice strain (Pashley 1986). The maize- and rice strains tend to show differences in susceptibility to transgenic Bt plants and insecticides (Ríos-Díez and Saldamando-Benjumea 2011), respectively. Ingber et al. (2017) indicated that larvae of the maize strains are less susceptible to Bt toxins, especially to Cry1F, than larvae of the rice strain. Cock et al. (2017) found both strains to be present in Ghana and by means of DNA barcoding, both strains were also identified in South Africa (Jacobs et al. 2018). Reports that the maize strain is less susceptible to Bt toxins than the rice strain (Ingber et al. 2017), is concerning since the majority of South Africa's *S. frugiperda* populations seem to consist of the maize strain (Jacobs et al. 2018).

Conclusions

All populations were highly susceptible to maize that produces both Cry1A.105 + Cry2Ab2 proteins. The lower susceptibility of the *S. frugiperda* to Cry1Ab maize can be attributed to factors such as the Bt1 event being a low-dose event for *S. frugiperda*, and because the population which initially arrived on the continent may have carried resistance alleles to the Cry1Ab protein. Although Cry toxins are effective against many lepidopteran species, a single toxin does not necessarily imply similar levels of toxicity to other related species. Furthermore, a high-dose for one pest species is not necessarily a high-dose for a related species. Field observations showed that Cry1Ab maize provided partial control of *S. frugiperda* in field trials conducted with drought-tolerant Bt maize varieties in East Africa (Prasanna et al. 2018). The suppression of *S. frugiperda* by Cry1Ab maize, in addition to highly effective protection against stem borers should be seen as an advantage that this technology provides. Cry1Ab maize was never considered highly effective against *S. frugiperda* and the target pests of this protein were initially considered to be larvae of the Crambidae (Lepidoptera) family. While Nagoshi et al. (2017) reported that no resistance markers for Cry1F were present in West African *S. frugiperda* populations, no studies have been done on markers on the presence of resistance to Cry1Ab in Africa. It remains uncertain if cross-resistance occurs between Cry1A.105 + Cry2Ab2 and the closely related Cry1Ab toxin, and for how long the efficacy of the pyramid event will endure. Cry1Ab-expressing hybrids are still cultivated in South Africa, together with pyramided hybrids. Resistance may evolve more rapidly when crops with pyramided traits are cultivated concurrent to single-trait crops (Zhao et al. 2005, Tabashnik et al. 2009).

The levels of susceptibility of *S. frugiperda* to Bt maize should therefore be monitored and the frequency of Cry1Ab resistant alleles determined in African populations of this pest.

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4 Chapter 4: F_2 screening for resistance alleles of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to Cry1Ab and Cry1A.105 + Cry2Ab2 maize in South Africa

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Johnnie Van den Berg
Unit for Environmental Sciences and Management
North-West University
Potchefstroom, 2520
South Africa
Phone: +27-18-299 2376
E-mail: johnnie.vandenberg@nwu.ac.za

F_2 screening for resistance alleles of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to Cry1Ab and Cry1A.105 + Cry2Ab2 maize in South Africa

A. S. Botha[1], A. Erasmus[2], H. du Plessis[1], L. Raubenheimer[3], S. Ellis[3] and J. Van den Berg[1]

[1]Unit for Environmental Sciences and Management, IPM program, North-West University, Potchefstroom, 2520, South Africa.

[2]Agricultural Research Council, Grain Crops, Private Bag X1251, Potchefstroom, 2520, South Africa.

[3]Statistical Consultation Services, Unit for Business Mathematics and Informatics, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa.

Abstract

Spodoptera frugiperda (J.E. Smith) invaded Africa and is considered the number one maize pest in Africa. Bt maize that express Cry proteins has been used effectively to control this pest in the Americas. Bt maize is effective against African stem borer, *Busseola fusca* (Fuller) and *Chilo partellus* (Swinhoe), species and is expected to be approved for control of these pests in several African countries. Insect resistance management is complicated by mixed pest populations and the expectation that Bt maize will also provide effective control of *S. frugiperda*. This pest evolved resistance to single-gene Bt maize in its areas of origin and resistance to Cry1Ab was detected in South Africa. This study determined the frequency of Cry1Ab and Cry1A.105+Cry2Ab2 resistance alleles of *S. frugiperda* in South Africa. A F_2 screen was conducted and three of the 117 families that were established carried major resistance alleles against Cry1A.105+Cry2Ab2 maize, with a low overall estimated frequency of 0.0084 (95% credibility interval of 0.0023-0.0181). The frequency of Cry1Ab resistance alleles was 0.0819 (95% credibility interval of 0.0617-0.1036). This high frequency of alleles with resistance to Cry1Ab protein will cause rapid resistance evolution in areas where this single-gene event may in future be cultivated on the continent. Cry1Ab maize which holds promise for stem borer control in Africa, will only provide temporary control of *S. frugiperda*, which has overtaken stem borers as a priority pest in large parts of the continent. This study provides a base-line that facilitates monitoring for resistance in *S. frugiperda* populations.

Key words: F_2 screen, monitoring, fall armyworm, resistance

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), a native species of the Americas, invaded West and Central Africa during 2016 (Goergen et al. 2016). Due to its long-distance migratory ability this polyphagous pest managed to spread rapidly across the African continent (Day et al. 2017, Montezano et al. 2018). The destructive feeding habits of *S. frugiperda*, on maize (Zea mays L.), cause major economic losses, which are estimated at between US$2.48 billion and US$6.19 billion per annum, in 12 maize producing countries in Africa, if no control is applied (Day et al. 2017).

During 2017/2018 cropping season 71% (1.62 million hectares) of the total maize area panted consisted of Bt maize in South Africa (ISAAA 2017). Single-gene Bt maize (MON810 - Cry1Ab) has been planted in South Africa for stem borer *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae), control since 1998. *Busseola fusca* evolved resistance against Cry1Ab (Van Rensburg 2007), which was mitigated by the commercialization of pyramid Bt maize (MON89034 - Cry1A.105 + Cry2Ab2) in 2011 (Van den Berg 2017). Effective field-level control of *S. frugiperda*, with MON810 has been observed on trial plots in Kenya, Mozambique, and Uganda, as well as with MON89034 in South Africa, which is the only country on the continent that cultivates Bt maize (Prasanna et al. 2018). However, in bioassays conducted by Botha et al. (2019) in South Africa, significant levels of larval survival of *S. frugiperda* (4–35%) on maize leaf tissue of MON810 was observed. Furthermore one of 900 larvae screened on MON89034 completed its lifecycle (Botha et al. 2019), highlighting the importance of resistance monitoring.

Although *S. frugiperda* has been effectively controlled by Bt maize expressing Cry1F, Cry1Ab and Cry1A.105 + Cry2Ab2 proteins in the USA and Canada (Buntin et al. 2004, Siebert et al. 2012, Storer et al. 2012, Reay-Jones et al. 2016) and several South America countries (Buntin 2008, Storer et al. 2012, Bernardi et al. 2016), field-evolved resistance have been reported. The first case of field-evolved resistance by *S. frugiperda* was in Puerto Rico against Bt maize which expressed Cry1F proteins (Matten et al. 2008, Storer et al. 2010), followed by a report in Brazil five years after commercialization (Farias et al. 2014), and the southern states of the USA, 13 years after introduction of Bt maize (Huang et al. 2014). The most recent

report of field resistance was from Argentina, eight years after commercialization of Cry1F maize (Chandrasena et al. 2018). Field-evolved resistance to Cry1Ab maize was also reported in Brazil eight years after its introduction in 2007 (Omoto et al. 2016). In all these cases, resistance was detected through monitoring programs that were in place to determine shifts in susceptibility levels.

Moderate survival of *S. frugiperda* on Cry1Ab in South Africa (Botha et al. 2019), along with the ability of this pest to rapidly develop resistance to Cry proteins, emphasize the need to implement thorough resistance monitoring programs. Several methods have been developed to detect and determine the presence of Bt-resistant alleles in field populations of pests (Roush and Miller 1986, Andow and Alstad 1998, Gould et al. 1997, Venette et al. 2000). The F_2 screening method is commonly used to detect the presence of resistant alleles and is regarded the most appropriate method to estimate the frequency of rare recessive resistance alleles in *S. frugiperda* populations (Vélez et al. 2013, Farias et al. 2014, Huang et al. 2014, Bernardi et al. 2015, Farias et al. 2016, Li et al., 2016, Niu et al. 2016, Yang et al. 2018).

The F_2 screening method detects rare recessive alleles, since it preserves genetic variation among family lines and concentrates resistance alleles into homozygous genotypes which are more easily detected (Andow and Alstad 1998). Since the frequency of resistance alleles is a key factor in resistance monitoring, baseline data regarding the status of resistance is needed. Therefore the objective of this study was to determine the frequency of Cry1Ab and Cry1A.105 + Cry2Ab2 resistance alleles. This will facilitate monitoring of future shifts in susceptibility and ensure timely implementation of mitigation strategies to delay resistance evolution.

Material and methods

The F_2 screening method we followed was described by Huang et al. (2014, 2016) and Niu et al. (2016) as follows: (1) sampling larvae from field populations (minimum of 500 per population), (2) pair-mating moths derived from the field-collected larvae, (3) rearing the progeny of each parental female in separate family lines, and random mating all viable F_1 adult siblings, (4) rearing of F_2 neonates on non-Bt and Bt plant tissue, and (5) confirmation of potential positive families as true positives by means

of rescreening of the F_3 generation on whole plants. Due to the high levels of survival on Cry1Ab maize observed in this study and that of Botha et al. (2019), rescreening of F_3 larvae on Cry1Ab maize was not done. The resistance allele frequency was then calculated.

Sampling, rearing, and establishment of two-parent families

Third to fifth instar *S. frugiperda* larvae were sampled for each population in non-Bt maize fields, during January 2019. Localities were selected based on the year round production of maize and presence of *S. frugiperda* in these areas. These localities were in the districts of Groblersdal (designated GLD19; S 25° 16' 28.21", E 29° 25' 23.74") and Malelane (MLL19; S 25° 35' 42.8", E 31° 39' 54.7"), results of these two populations were pooled and an overall resistance frequency was calculated for a South African population (designated RSA19). Field-collected larvae were reared on plant tissue until the pupal stage as described by Botha et al. (2019). The pupae were collected, held in trays with filter paper and then kept in plastic cups (30 ml) until moth emergence. Parental moths, derived from the field collections, were pair-mated (one isomale and one isofemale) in 500 ml plastic cups to produce two-parent family lines as described by Farias et al. (2016). Moths were provided with a sugar solution on a soaked cotton boll. F_1 larvae were reared on meridic diet, while pupae and adults were handled as described above. Rearing was conducted in insect rearing chambers under the following conditions: 25℃ ± 1℃, 60% ± 10% RH and a 14:10 h (L:D) photoperiod. F_1 moths were used for sib-mating to generate the F_2 neonates for each of the family lines which were then used in the F_2 screening procedure.

Source of Bt and non-Bt maize plants for F_2 screening

A single-gene event of Bt maize that expresses Cry1Ab protein (DKC 80-12B) and a pyramid event that expresses both Cry1A.105 + Cry2Ab2 proteins (DKC 80-12BGEN), along with the near iso-line, a non-Bt iso-hybrid (DKC 80-10) were used during the F_2 screening process. The plant tissue provided as food for larvae was derived from the whorls of field grown maize plants of these different maize hybrids. Envirologix QuickStix strip tests were used to confirm the Bt status of the maize tissues fed to larvae.

Screening of F_2 neonates to identify potentially positive families

To determine whether a family possessed resistance alleles, 100 F_2 neonates of each two-parent family was screened on plant tissue of the above mentioned hybrids. F_2 neonates were derived from randomly mated F_1 adults. The progeny of each two-parent family were inoculated onto maize plant tissue (40 mm × 40 mm) in petri dishes (50 mm diameter × 15 mm). There were 20 replicates per family. Each replicate consisted of a petri dish into which five neonate larvae were placed (20 replicates per two-parent family × 5 larvae per replicate = 100 larvae per family). The containers with neonates were placed in insect rearing chambers maintained at the conditions described above. Survivorship was recorded four and seven days after inoculation, with larvae considered dead if they did not respond after being touched with a small paint brush. Leaf tissue was replaced with the first assessment of larval survival, four days after the experiment commenced. On day seven, when the assay was terminated, larval mass was determined and larval size was estimated as follows: small (≤2nd instar) and large (≥3rd instar) as described in Huang et al. (2014, 2016).

Defining potentially positive families possessing resistance alleles

Andow and Alstad (1998) recommended the F_2 screen as described above to identify potentially positive families that possess major resistance alleles to insecticidal proteins. Theoretically, when one of the two parents of a family possesses a major recessive resistance allele, 1/16 (6.25%) of the F_2 progeny is expected to be homozygous resistant to specific Cry proteins and would therefore be able to survive the F_2 screening process (Andow and Alstad 1998). The specifications for a family to be considered a potentially positive family (PPF) were used in the same way as Huang et al. (2016) who indicated that larvae reared on non-Bt maize for seven days were predominantly ≥3rd instars, which was similar to our study. The F_2 larvae that developed to ≥3rd instar after seven days are therefore identified as homozygous resistant, and these families are then considered positive for carrying major resistance alleles.

Confirmation of PPF on whole plants of Cry1A.105 + Cry2Ab2 maize

Based on the survivorship of the F_2 larvae on day seven, families were identified as potentially positive for carrying resistance alleles to Cry1A.105 + Cry2Ab2. To confirm whether these PPFs truly possessed resistance alleles, one family line was established from the survivors. All F_2 survivors, small (≤2nd instar) and large (≥3rd instar), were transferred to a meridic diet to increase the chance of successfully establishing the potentially positive family. Larvae, pupa and moths were treated the same as those of the parental generation. One family was established and rescreened in a greenhouse on whole plants expressing Cry1A.105 + Cry2Ab2 proteins.

For the resistance confirmation tests, five neonates per PPF were inoculated into the whorl of each plant during the V3-V6 stages. Ten plants per treatment for each family were used for resistance confirmation of the F_3 generation (50 larvae per family were rescreened). The two maize treatments were the same as described above. Larval survival, mass and plant injury, according to the 1-9 Davis scale (Davis et al. 1992), were recorded seven days after inoculation.

The presence of Cry1A.105 + Cry2Ab2 in the Bt plants and absence of Cry proteins from non-Bt control plants were confirmed by means of Envirologix QuickStix strip tests. A PPF that survived on Bt plants and developed to ≥3rd instars after seven days in the greenhouse was regarded a true positive family, with major resistance alleles (Huang et al. 2014, 2016). If larvae of a PPF did not survive the F_3 rescreen but its larvae survived the F_2 screen, they were considered to carry minor resistance alleles (Niu et al. 2016).

Statistical analysis

Two statistical analyses were conducted to examine the F_2 screening results. Firstly, the expected resistance allele frequency was calculated (equation [1] along with its 95% credibility intervals (CIs) [equation 3]), followed by the calculation of the probability of a false negative, P_{No} (i.e., probability of missing a resistance allele present in a line). The estimation of resistance allele frequencies and corresponding 95% CIs were calculated using a Bayesian analysis (Andow and Alstad 1998, 1999).

The expected resistance allele frequency was calculated as follows: let p be the probability that an iso-female line has a resistant allele and that it tests positive, given that N lines are tested and S lines have a resistance allele. The probability of a success is 4 q, where q is the frequency of the resistant allele. Each isofemale line represents one Bernoulli trial. Similar to Stodola and Andow (2004), a beta prior was put onto p, resulting in a beta posterior, $p|data \sim Beta(a+S, N-S+b)$. The resulting expected frequency of resistance will then be

$$E(p|data) = \frac{a+S}{a+b+N} \quad [1]$$

with variance

$$Var(p|data) = \frac{(a+S)(N-S+b)}{(a+b+N)^2(a+b+N+1)}. \quad [2]$$

The expected value and variance of q will then be $E(q|data) = (1/4)E(p|data)$ and $Var(q|data) = (1/16)Var(p|data)$. The posterior distribution of q will be a 4-parameter beta distribution, $q|data \sim Beta(a+S, N-S+b)$ defined on $[0, 1/4]$.

The 95% credibility interval for q can then be calculated by using the following

$$\int_0^L f(q|data)dq = 0.025 = \int_U^{1/4} f(q|data)dq, \quad [3]$$

where L is the lower limit of the interval and U is the upper limit of the interval which is calculated using the excel function BETA.INV.

An advantage of Bayesian statistics is that prior information can be incorporated and after the experiment has been completed, the posterior distribution

is used for inference. The choice of prior will thus have an influence on the posterior distribution, and the resulting credibility interval. Since this experiment is a first of its type for South African data, there was no prior data available which could contribute to the choice of the prior distribution. Non-informative (or vague) priors were therefore used for this analysis. The uniform prior is well-known and often used, if $a = b = 1$, the resulting prior will be the uniform prior.

The probability of a false negative was calculated as proposed by Stodola and Andow (2004). The probability of detecting a false negative, P_{No}, depends on the number of F_1 males (M) and F_1 females (F) that contribute to the F_2 generation, the number of F_2 offspring screened per F_1 female (J), and the non-screen mortality of F_2 larvae (μ).

Rescreening of F_3 larvae to eliminate the probability of a false positive

Leaf injury rating, larval mass and instar was analysed by means of t-tests. Significant differences between the treatments were determined using the Tukey HSD test ($P < 0.05$).

Results

Two-parent families and baseline survival data

A total of 117 two-parent families (74 from GLD19 and 43 from MLL19) were screened on Cry1Ab and Cry1A.105 + Cry2Ab2 maize leaf tissue (Table 1). Survivorship of larvae reared on non-Bt maize leaves were 70.5 ± 1.1% (mean ± S.E.) after 7 days for the GLD19 population and 68.1 ± 2.1% (mean ± S.E.) for MLL. All of the surviving larvae were ≥3rd instar.

Survival of two-parent families on Cry1Ab maize leaf tissue

Larvae of all 74 families of GLD19 survived on Cry1Ab leaf tissue and 24 families were considered positive for possessing major resistance alleles. The same results were recorded for MLL19 where all 43 families had some survivors after seven days and 14 of the families were considered possessing major resistance alleles. The mean survivorship of larvae for the GLD19 families was 38.4 ± 1.88% (mean ± S.E.), whereas the larval survival of the MLL19 families was 53.3 ± 2.93% (mean ± S.E.) after seven days of feeding (data not shown). A total of 2843 of the inoculated 7400

larvae of the GLD19 population survived the F_2 screen (Table 1). Since 197 of these larvae developed beyond the ≥3rd instar after seven days, they were considered positive of possessing major resistance alleles. The remaining 2646 larvae were smaller than ≤2nd instar and were considered positive for possessing minor resistance alleles. For the MLL19 population 2290 of the 4300 inoculated larvae survived, comprising of 381 large (≥3rd instar) larvae and 1909 small (≤2nd instar) larvae (Table 1).

Survival of two-parent families on Cry1A.105 + Cry2Ab2 maize leaf tissue

Among the 74 families of the GLD19 population, a total of 202 larvae from 50 families survived on Cry1A.105 + Cry2Ab2 leaf tissue, four days after inoculation. Three days later (day seven) 34 families with a total of 95 larvae survived, which included 89 ≤2nd instar (small) larvae and six ≥3rd instar (large) larvae. Five of these six large larvae were from the same family (GLD19-32), while the other larvae were from the GLD19-5 family. For the MLL19 population, 164 larvae from 25 of the 43 families survived after four days. On day seven, 67 larvae from 15 families were still alive. Of these 67 larvae, only three were ≥3rd instar and all were from a single family (MLL19-16).

Probability of a false negative

The probability of a false negative was calculated as proposed by Stodola and Andow (2004) with an estimated female mortality rate of 0.3, based on the survival of larvae in the control treatment. The number of males and females were assumed to be equal in the F_1 progeny and ranged between 9 and 67 per line. The number of F_2 progeny screened (J) was 100 for each line. The detection probability of this F_2 screen was therefore 96 and 92% for the Groblersdal and Malelane populations, respectively (Fig. 1).

Rescreening F_3 larvae on whole plants

Based on the PPF criteria, three families were considered positive. These were two from the Groblersdal population (GLD19-5 and GLD19-32) and one from the Malelane population (MLL19-16) (Table 2). Only one of these families was able to produce F_3 progeny (GLD19-32) that we used during rescreening. The leaf injury

rating of the non-Bt and Cry1A.105 + Cry2Ab2 plants did not differ significantly (p = 0.17) with mean damage ratings of 4.9 and 3.4 respectively (Table 3). There was no significant difference (p = 0.48) between the sizes of larvae feeding on the non-Bt plants (mean instar = 3.4) and those feeding on Bt plants (mean instar = 3.1) (Table 3). Correspondingly, the larval mass of the two treatments also did not differ significantly (p = 0.72), with larvae feeding on non-Bt maize weighing 0.0079 g and those feeding on Cry1A.105 + Cry2Ab2 maize weighing 0.0075 g (Table 3). Results of the rescreening confirmed that the potential positive family (GLD19-32) possessed resistance alleles against Cry1A.105 + Cry2Ab2 proteins. The other two identified PPFs which were not rescreened (GLD19-5 and MLL19-16) were therefore also considered positive for carrying major recessive resistance alleles, due to similar observations made during the F_2 screen.

Major and minor resistance allele frequency

Cry1Ab

Alleles with resistance against Cry1Ab and Cry1A.105 + Cry2Ab2 maize were detected in both populations. The estimated frequency of major Cry1Ab resistance alleles in the GLD19 population was 0.0822 (95% CI of 0.0572 - 0.1094) and 0.0833 (95% CI of 0.0512 - 0.1189) for MLL19 (Table 4). Overlapping credibility intervals indicated that there were no significant differences in frequency of resistance alleles amongst the two populations, with an overall frequency (RSA19) of 0.0819 (95% CI of 0.0617 - 0.1036). Some of the families had surviving larvae on day seven but larvae were not able to complete their lifecycle. These families were considered positive for possessing minor resistant alleles. The minor Cry1Ab resistance allele frequency was estimated at 0.1678 (95% CI of 0.1406 - 0.1928) for GLD19 and 0.1667 (95% CI of 0.1311 - 0.1988) for MLL19 (Table 4).

Cry1A.105 + Cry2Ab2

The F_2 screen and whole-plant rescreening showed that two of the 74 families from Groblersdal and one of the 43 families from Malelane carried resistance alleles against the Cry1A.105 + Cry2Ab2 proteins in Bt maize. The estimated major resistance allele frequency for GLD19 was 0.0099 (95% CI of 0.0021 - 0.0233) whereas the frequency of major resistance alleles for MLL19 was 0.0111 (95% CI of

0.0014 - 0.0301) (Table 4). Based on their overlapping 95% credibility intervals the expected frequencies in the two populations were not significantly different. The overall expected major resistance allele frequency of the two *S. frugiperda* populations from South Africa (RSA19) was estimated at 0.0084 (95% CI of 0.0023 - 0.0181) (Table 4). GLD19 had a minor resistance allele frequency of 0.1085 (95% CI of 0.0814 - 0.1366) while the frequency of minor resistant alleles for MLL19 was 0.0833 (95% CI of 0.0512 - 0.1189) (Table 4).

Discussion

The goal of insect resistance monitoring is to detect susceptibility shifts early enough to enable proactive management and prevent control failure, thereby enhancing the sustainability of Bt crops (Huang 2006, Tabashnik et al. 2008). Several methods of resistance monitoring have been proposed to aid in the development and implementation of insect resistance management (IRM) programs. Monitoring is an essential and compulsory part of an IRM program, and the results thereof is used to evaluate the success of IRM strategies (Huang 2006). This is done by the collection of baseline data to enable future comparisons, and ultimately detection of shifts in the frequency of resistance alleles (Glaser and Matten 2003, Tabashnik et al. 2014).

In our study the F_2 screening method proposed by Andow and Alstad (1998) was used, due to its accuracy and ability to detect rare recessive resistance alleles. This method allows the detection of resistance alleles in field collected pair-mated family lines and is widely used to detect resistant alleles.

The F_2 screening method was effective for the estimation of the frequencies of alleles with resistance to Cry1Ab and Cry1A.105 + Cry2Ab2 toxins. Overall, three of the 117 families were found positive for carrying major Cry1A.105 + Cry2Ab2 resistance alleles. This high frequency of Cry1Ab resistant alleles in both populations supports the observations that this protein is not a high-dose for *S. frugiperda* (Omoto et al. 2016, Sousa et al. 2016).

The main IRM strategy applied for delaying target pests from evolving resistance to Bt crops is the high-dose/refuge strategy (Tabashnik et al. 2004). In order for the high-dose/refuge strategy to be successful, several key assumptions should be met. Firstly, inheritance of resistance should be recessive, secondly, the

initial frequency of resistance alleles should be low (<0.001), and thirdly, refuge plantings should be abundant (Tabashnik 1994, Gould 1998, Tabashnik et al. 2009).

Studies have shown that when the assumptions around the high-dose/refuge strategy are met, resistance evolution is delayed or may even be undetectable (Hutchison et al. 2010, Huang et al. 2011, Campagne et al. 2016). The aim of our study was to estimate the frequency of resistance alleles of *S. frugiperda* in South Africa, to assess whether the assumption of rarity of resistance alleles is met. Frequency of resistance alleles, more specifically the functionality (dominant or recessive) of resistance alleles, is one of the major influences affecting the rate of evolution of resistance to Bt crops. The frequency of alleles with resistance to Cry1A.105 + Cry2Ab2 of GLD19 was estimated at 0.0099 and for MLL19 at 0.0111, with the overlapping 95% credibility intervals indicating similarity between populations. Both these frequencies are higher than the expected initial frequency of 0.001. This higher frequency of resistance alleles indicates a high risk of resistance evolution. Similar and higher resistance frequencies have been observed in *Ostrinia nubilalis* (<0.015), *Helicoverpa armigera* (<0.0225), *Diatraea saccharalis* (<0.0328) and *Heliothis virescens* (<0.0263) without the occurrence of control failure (Andow et al., 1998, Liu et al. 2008, 2010, Xu et al. 2009, Blanco et al. 2009, Engels et al. 2010, Huang et al. 2012, Kukanur et al. 2018). Although the rate of resistance evolution is influenced by genetic and behavioral characteristics of pests (Pannuti et al. 2016, Zhu et al. 2019) and compliance to IRM requirements, control failure usually occurs within a few generations if the frequency of resistance is >0.1 (Tabashnik and Croft 1982, Roush and Miller 1986). Examples thereof occurred in Florida (USA) and Bahia (Brazil) where reduced efficacy of Cry1F Bt maize against *S. frugiperda* was reported, where these populations had resistant allele frequencies of 0.293 and 0.192, respectively (Huang et al. 2014, Farias et al. 2016).

The high frequencies of resistance alleles to Cry1Ab and Cry1A.105 + Cry2Ab2 observed in this study may indicate the possibility of previous exposure to Bt proteins with the same mode of action. Since *S. frugiperda* invaded South Africa only in 2017 (du Plessis et al., 2018), the brief exposure to locally cultivated Bt maize is an unlikely explanation for these high frequencies of resistance alleles. *Spodoptera frugiperda* populations in West Africa, where the pest was first detected

in 2016 (Goergen et al. 2016), as well as populations that subsequently established throughout sub-Saharan Africa could also not have been exposed to Bt maize since South Africa is currently the only country on the continent where Bt maize is cultivated. Studies conducted by Huang et al. (2016) and Niu et al. (2016) on Cry1A.105 and Cry2Ab2 respectively, showed that resistant alleles were present in *S. frugiperda* populations in Florida in the USA. Resistance allele frequencies in different populations of *S. frugiperda* ranged between 0.0021-0.0868 for Cry1A.105 and 0.0035-0.0766 for Cry2Ab2. The frequencies of resistance alleles recorded in this study are largely similar to that reported by Huang et al. (2016) and Niu et al. (2016) in the Florida region, from where it is speculated that this pest could have invaded West Africa (Nagoshi et al. 2017, 2018, 2019, Nagoshi 2019). The only studies done on the Cry1A.105 + Cry2Ab2 pyramid event were eight and five years ago by Yang et al. (2013) and Li et al. (2016) respectively, on different North-American populations. Since none of the 361 families that were screened by the latter authors were found to be resistant, resistance allele frequencies were very low compared to the frequency of 0.0084 recorded in this study.

Fatoretto et al. (2017) indicated that most of the Bt maize hybrids cultivated in Brazil were no longer effective against S*. frugiperda,* three years after commercial release in Brazil. The climate in tropical and sub-tropical Africa is similar to that in Brazil where an average of eight generations of *S. frugiperda* occurs per annum under field conditions. Although the period of potential exposure of this pest to Cry1Ab maize in South Africa seems short (3 growing seasons), environmental conditions in the areas where *S. frugiperda* populations persist during winter, together with the reproductive nature of *S. frugiperda* (Barros et al. 2010) allows for development of many generations per annum. It is estimated that this pest has completed approximately 24 generations (≈ 8 generation per year) in some of these areas since its first report in South Africa in January 2017. Fortunately, due to very low (<0°C) minimum winter temperatures in the main maize production regions of South Africa, *S. frugiperda* overwinters only in low lying areas in the far north and eastern areas of the country (du Plessis et al. 2018) where maize is not the major crop and cultivation of Bt maize is limited (Strydom et al. 2019, Kotey et al. 2017, Assefa and Van den Berg 2015).

Monitoring is essential for both the implementation and evaluation of IRM programs (Huang et al. 2006). Detection of susceptibility shifts as early as possible will enable proactive mitigation measures to be implemented (Huang 2006, Tabashnik et al. 2008). The early implementation of IRM strategies have been the success of Bt crops in Australia (Downes et al. 2007, Downes et al. 2016, Tabashnik et al. 2013). These strategies were additional to the high-dose/refuge strategy and included the rotation of Bt proteins with different modes of action, or pyramiding of Bt proteins prior to the detection of resistance (Roush 1998, Bates et al. 2005). *Spodoptera frugiperda* is known for its ability to develop resistance against single-gene Bt maize events (Storer et al. 2010, Huang et al. 2014, Omoto et al. 2016). Since single-gene Bt maize failed to control *S. frugiperda* in Brazil and the United States, second generation pyramids such as Cry1A.105 + Cry2Ab2 and Cry1F + Cry1Ab + Vip3Aa20 were deployed (Buntin et al. 2004, Siebert et al. 2012, Reay-Jones et al. 2016). Although Vip proteins holds promise in terms of *S. frugiperda* control and IRM, resistance alleles have been reported in the USA (Vip3A and Vip3Aa51) (Yang et al. 2018, 2019) and Brazil (Vip3Aa20) (Amaral et al. 2019). Second-generation Bt maize produces two or more distinct Bt toxins, expressed at a high dose, with all the proteins being equally active against the same target pests, resulting in complete 'redundant killing' of susceptible insects (Comins 1986, Gould 1986, Carrière et al. 2016).

Niu et al. (2013) and Bernardi et al. (2015) emphasized that proteins incorporated into Bt pyramids should be carefully selected on the basis of their toxicity, for this strategy to be effective against resistant populations. Since Cry and Vip proteins bind to different receptors in the midgut of susceptible insects, these proteins are recommended for pyramiding in transgenic crops to improve resistance management (Roush 1998, Sansinenea 2012). Sena et al. (2009), Vélez et al. (2013), Yang et al. (2013), Niu et al. (2014). Santos-Amaya et al. (2015) reported that Vip3A is highly effective against susceptible and Cry1 resistant *S. frugiperda* larvae, and that should therefore be combined with Cry1 proteins to counter and delay resistance evolution. Both Huang et al. (2016) and Niu et al. (2016) suggested that pyramiding Cry1A.105 and Cry2Ab2 proteins will contribute to a sustainable IRM strategy, due to the differences in mode of action of these proteins.

Conclusions

This study is the first to provide information on the frequency of *S. frugiperda* alleles with resistance to Bt maize in Africa and will contribute to future monitoring for shifts in susceptibility to Bt proteins. The frequency of alleles conferring resistance to Cry1Ab is high and control failure of this single-gene Bt maize will most probably occur within a few generations. The higher than expected frequencies of alleles conferring for resistance to Cry1A.105 + Cry2Ab2 maize in *S. frugiperda*, is possibly because of the presence of resistance alleles which initially arrived on the continent. However, the frequency of resistance to the pyramid event was lower and it may provide control for a longer period before resistance is detected.

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Table Captions:

Table 1. Number of F_2 families screened and survivorship of F_2 larvae, four and seven days after inoculation onto maize leaf tissue Groblersdal (GLD19) and Malelane (MLL19).

Table 2. Mean mass and survival of *Spodoptera frugiperda* larvae of potential positive families (PPF) with resistance alleles to Cry1A.105 + Cry2Ab2 proteins. Data were recorded after seven days of feeding on Bt maize leaf tissue in the F_2 screen assay.

Table 3. Leaf injury ratings and performance of F_3 larvae of family GLD19-32 during whole plant assays for rescreening of *Spodoptera frugiperda* for resistance to Cry1A.105 + Cry2Ab2. Data were recorded seven days after inoculation of neonate larvae onto Bt maize.

Table 4. Estimated frequency of resistance to Cry1Ab and Cry1A.105 + Cry2Ab2 maize and corresponding 95% credibility intervals for two populations of *Spodoptera frugiperda* collected from Groblersdal (GLD19) and Malelane (MLL19) in South Africa.

Figure caption

The probability (P_{No}) of not detecting a recessive resistance allele in a two-parent family line that produces F_1 families with 10, 20, 30, 50, 60, 75 males (M) and females (F) and F_2 families with (J) progeny per F_1 female. A mortality rate of 0.3 for larvae for other reasons was used.

Table 1. Number of F_2 families screened and survivorship of F_2 larvae, four and seven days after inoculation onto maize leaf tissue Groblersdal (GLD19) and Malelane (MLL19).

Population code	No. of F_2 families screened	Survival after 4 days		Survival after 7 days			
		No. of positive families	No. of larvae	No. of positive families	No. of larvae ≤2nd instar	No. of larvae ≥3rd instar	Total No. of larvae
Cry1Ab							
GLD19	74	74	3740	74	2646	197	2843
MLL19	43	43	3186	43	1909	381	2290
Cry1A.105 + Cry2Ab2							
GLD19	74	50	202	34	89	6	95
MLL19	43	25	164	15	64	3	67

Table 2. Mean mass and survival of *Spodoptera frugiperda* larvae of potential positive families (PPF) with resistance alleles to Cry1A.105 + Cry2Ab2 proteins. Data were recorded after seven days of feeding on Bt maize leaf tissue in the F_2 screen assay.

Family code	Family No.	≤2nd instar larvae		≥3rd instar larvae	
		No. of larvae	Mean mass (g/larvae)	No. of larvae	Mean mass (g/larvae)
GLD19-5	5	13	0.0014	1	0.0091
GLD19-32	32	11	0.0022	5	0.0079
MLL19-16	16	10	0.0015	3	0.0080

Table 3. Leaf injury ratings and performance of F_3 larvae of family GLD19-32 during whole plant assays for rescreening of *Spodoptera frugiperda* for resistance to Cry1A.105 + Cry2Ab2. Data were recorded seven days after inoculation of neonate larvae onto Bt maize.

Hybrid	Leaf injury rating (mean ± S.E.)	Larval instar (mean ± S.E.)	Larval mass (g/larvae) (mean ± S.E.)
Control	4.9 ± 0.89	3.4 ± 0.20	0.0079 ± 0.0007
Cry1A.105 + Cry2Ab2	3.4 ± 0.51	3.1 ± 0.31	0.0075 ± 0.0011
t-value	1.44	0.73	0.36
P-value	0.17	0.48	0.72

Table 4. Estimated frequency of resistance to Cry1Ab and Cry1A.105 + Cry2Ab2 maize and corresponding 95% credibility intervals for two populations of *Spodoptera frugiperda* collected from Groblersdal (GLD19) and Malelane (MLL19) in South Africa.

Population code	No. of F_2 families screened	No. of potential positive families		Estimated frequency of		95% credibility intervals for	
		Minor resistant alleles	Major resistant alleles	minor resistant alleles	major resistant alleles	minor resistant alleles	major resistant alleles
Cry1Ab							
GLD19	74	50	24	0.1678	0.0822	0.1406 - 0.1928	0.0572 - 0.1094
MLL19	43	29	14	0.1667	0.0833	0.1311 - 0.1988	0.0512 - 0.1189
RSA19	117	79	38	0.1681	0.0819	0.1464 - 0.1883	0.0617 - 0.1036
Cry1A.105 + Cry2Ab2							
GLD19	74	32	2	0.1085	0.0099	0.0814 - 0.1366	0.0021- 0.0233
MLL19	43	14	1	0.0833	0.0111	0.0512 - 0.1189	0.0014 - 0.0301
RSA19	117	46	3	0.0987	0.0084	0.0773 - 0.1210	0.0023 - 0.0181

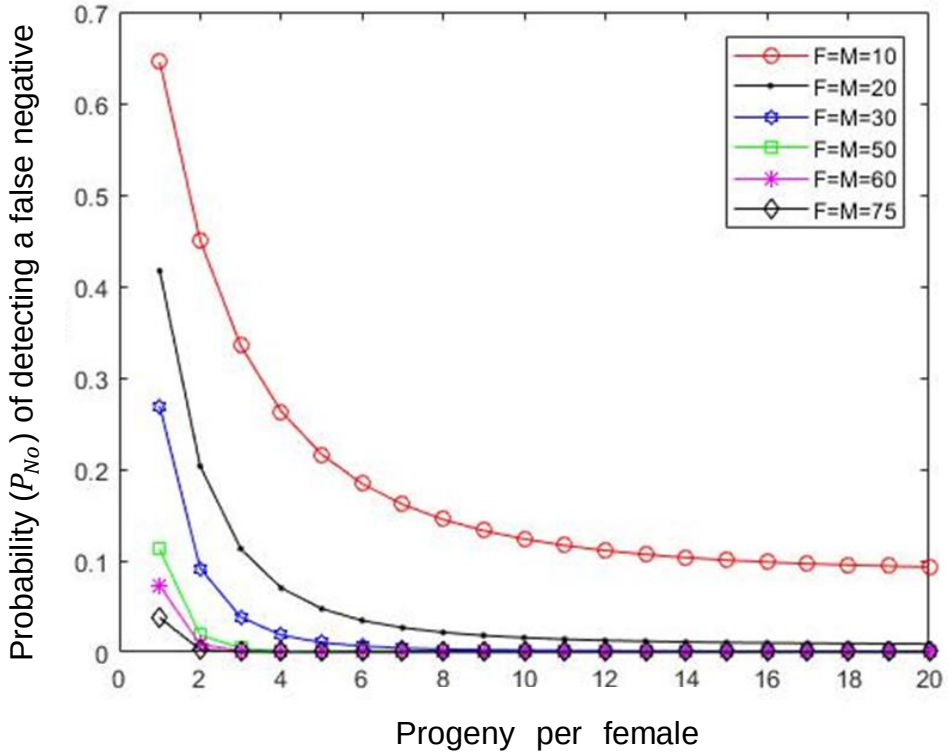

Figure 1.

The probability (P_{No}) of not detecting a recessive resistance allele in a two-parent family line that produces F_1 families with 10, 20, 30, 50, 60, 75 males (M) and females (F) and F_2 families with (J) progeny per F_1 female. A mortality rate of 0.3 was used.

5 Chapter 5: Conclusion

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae), a highly polyphagous pest which prefers to feed on plants of the Poaceae family (Montezano *et al*., 2018), invaded Africa during 2016 (Goergen *et al*., 2016). This pest is now regarded a major pest of maize in Africa and requires prime attention regarding effective and sustainable control methods for smallholder farmers. Bt maize is one of the few environmentally friendly ways of controlling target pests. Target pests do however have the ability to develop resistance against Bt proteins (Van Rensburg, 2007; Storer *et al*., 2010; Dhurua & Gujar, 2011; Dively *et al*., 2016; Omoto *et al*., 2016), which threatens the longevity and durability of this control tactic (Tabashnik *et al*., 1994; Gould, 1998; Carrière *et al*., 2010). Several cases of field evolved resistance against Bt crops have been reported worldwide (Tabashnik & Carrière, 2017), mostly due to poor compliance to resistance management strategies (Kruger *et al*., 2009; Tabashnik *et al*., 2013; Farias *et al*., 2014b).

Insect resistance management (IRM) strategies are a compulsory component of stewarding biotechnological pest control products (Huang, 2006; Alacalde *et al*., 2007). Although IRM strategies used for different Bt crops and in different geographical regions may differ due to differences in the biology of pests, farming practices, and farmer literacy levels and experiences (Head, 2004), the basic elements required to develop and implement an IRM plan remains the same (Matten *et al*., 2008). In order to constantly adapt and improve developed IRM programs to specific conditions, monitoring is needed to facilitate effective resistance management (Huang, 2006). These studies are usually initiated by the collection of baseline data, followed by further monitoring studies to evaluate the effectiveness of implemented IRM strategies (Alacalde *et al*., 2007). In developing countries, where farming is largely dominated by smallholder farmers (Aheto *et al*., 2013), stewardship practices should be implemented by the technology developers rather than individual farmers, in order to ensure high levels of compliance (Head, 2004; Head & Greenplate, 2012).

Baseline data of the response of *S. frugiperda* to Bt maize in Africa is required to assess its status of susceptibility and to facilitate monitoring for resistance, which is important for IRM. The aim of this study was to develop a baseline dataset by

assessing the susceptibility of South African populations of *S. frugiperda* to Bt maize. This study provides valuable information for future monitoring studies, to evaluate whether shifts in susceptibility occurs. This study had two objectives, firstly to evaluate the effect of single- and pyramid-gene Bt maize on *S. frugiperda,* and secondly, to estimate the frequency of alleles with resistance to single- and pyramid-gene Bt maize. The objectives of this study were accomplished by conducting two separate methods of screening. The first, a phenotypic screen, evaluated the influence of Bt on the overall survival and life history parameters of this pest, while the second, a genotypic screen, estimated the frequency of resistant alleles by means of an F_2 screen.

The results of this study will contribute to development and management of IRM strategies. The high-dose/refuge strategy is considered a solely sufficient IRM strategy when all assumptions of this strategy are met (Hutchison *et al*., 2010; Huang *et al*., 2011a; Campagne *et al*., 2015). The three main assumptions for this IRM strategy to be effective are: (1) initial resistance allele frequencies are low, (2) these alleles are functionally recessive, and (3) a refuge area is available and capable of producing susceptible individuals in vast numbers (Gould, 1998; Bates *et al*., 2005).

During the first part of this study, nine different populations of *S. frugiperda* were sampled and screened on single and pyramid-gene Bt maize. All of the populations showed moderate levels of survival (4-35%), with between 4 and 35% of the F_1 larvae which completed their lifecycle on single-gene Bt maize. Although moderate levels of survival occurred, life history parameter indicators of all populations showed high levels of susceptibility, based on significantly lower larval and pupal masses, lower levels of pupation, higher levels of growth inhibition, and increased developmental times. While all of the life history parameters were adversely affected, the impact of Cry1Ab seemed to diminish as the life-cycle proceeds. Amongst the nine populations screened, larvae that were able to survive and adults that were able to reproduce (laid eggs) were recorded in seven populations. The lower susceptibility of the *S. frugiperda* to Cry1Ab maize can be attributed to factors such as the MON810 event being a low-dose event for *S. frugiperda* (Omoto *et al*., 2016; Sousa

et al., 2016), and because the population which initially arrived on the continent may have carried resistance alleles to the Cry1Ab protein.

Regarding pyramid gene Bt maize, high to complete levels of susceptibility were observed, with only one larva that completed its life-cycle. Although the survival of one individual indicated that resistance alleles are present, the frequency of resistant alleles was unknown. The frequencies of resistance alleles, which influence the rate of resistance evolution, are one of the most important factors that determine the efficiency of IRM strategies. It is therefore important to determine resistance allele frequencies for monitoring purposes, since monitoring is a mandatory part of IRM (Glaser & Matten, 2003; Huang, 2006; Alacalde *et al*., 2007; Lecoq *et al*., 2007). The F_2 screening method, commonly used to monitor resistance levels of field-collected pest populations (Andow *et al*., 1998; Bentur *et al*., 2000; Andreadis *et al*., 2007; Downes *et al*., 2007; Blanco *et al*., 2009; Engels *et al*., 2010; Huang *et al*., 2011b; Yang *et al*., 2013a; 2013b; 2018; 2019; Kukanur *et al*., 2018; Amaral *et al*., 2019; Kumar *et al*., 2019), was used to determine the frequency of resistance alleles.

Spodoptera frugiperda is the only pest species that managed to develop resistance over a relatively short period of time to several Cry proteins in Puerto Rico, Brazil, Southern States of North America and Argentina (Matten *et al*., 2008; Storer *et al*., 2010; Farias *et al*., 2014a; Huang *et al*., 2014; Omoto *et al*., 2016; Chandrasena *et al*., 2018). These findings emphasize the importance of collecting baseline data such as the frequency of resistant alleles. Baseline data is the first contribution to a monitoring program, and shifts in frequency of resistance alleles may serve as early warning sign for the implementation of proactive IRM practices (Tabashnik *et al*., 2013; 2014). Since the results of our first objective indicated that resistant alleles were present in South African *S. frugiperda* populations, a resistance monitoring study was initiated.

Several methods have been developed to detect and determine the presence of Bt-resistant alleles in field populations of pests (Roush & Miller, 1986; Andow & Alstad, 1998; Gould *et al*., 1997; Venette *et al*., 2000). The F_2 screening method is most widely adopted due to this methods' ability to accurately estimate the frequency of rare recessive resistance alleles in field-derived pest populations (Andow & Alstad, 1998).

Two separate populations in South Africa were collected in the districts of Groblersdal and Malelane. These localities were selected based on year-round production of maize and presence of *S. frugiperda* in these areas (Glaser & Matten, 2003). The overall estimated frequency of alleles conferring for resistance against single-gene Bt maize, was 0.0819. One of the assumptions of IRM strategies is that initial frequencies of resistance alleles are low (<0.001). The high frequency of resistance alleles to Cry1Ab found in our study is either due to previous exposure of the source population of *S. frugiperda* to Bt crops that expresses Cry1 proteins, or it supports the fact that MON810 is not a high-dose event for this pest. This indicates the discerning of not registering MON810 as a high-dose against this pest. When Cry toxins are expressed at a low-dose, selection pressure allows not only homozygous resistant individuals to survive but heterozygous individuals, and the survival of heterozygous individuals is the driving force of resistance evolution. Since our F_2 results for the single-gene Bt maize is the first ever regarding the frequencies of Cry1Ab resistance alleles of *S. frugiperda*, and the fact that this is baseline data, made it difficult to compare the frequencies to those reported by others. Since no other F_2 screening studies regarding Cry1Ab resistance allele frequencies have been done, we compared our results to resistance allele frequencies of other Cry toxins. These comparisons highlight the fact that resistance allele frequencies nearing 0.1 are high, and product failure may occur within a few generations (Huang *et al*., 2014; Farias *et al*., 2016).

Based on the assumptions of the high-dose/refuge strategy, the frequencies of alleles conferring resistance to Cry1A.105 + Cry2Ab2 Bt maize found in South African populations are higher than what the initial frequency should be (<0.001). This high frequency can probably be ascribed to previous selection pressure and exposure of the source population to Bt crops. In this study the frequency of alleles conferring resistance to pyramid Bt maize was 0.0084. This higher than the expected frequency of resistance alleles does not indicate immediate product failure, but rather a potential risk of resistance evolution, which poses significant challenges for IRM strategies to ensure sustainable use of this technology for as long as possible (Head, 2004).

The high-dose/refuge strategy is capable of continuously controlling pest populations when the recessive resistance allele frequencies of the population are rare (<0.001) and refuges are planted. If these assumptions are not met, resistance will develop exponentially. Considering the ability of *S. frugiperda* to develop resistance to Bt maize, several cases have shown that the high-dose/refuge strategy alone is not sufficient in controlling this pest. This strategy should therefore be combined with other control tactics in an integrated pest management (IPM) strategy, especially in developing countries. The placement of the Bt crop within a larger framework of IPM is important for several reasons. One being that IPM will reduce the selection pressure of Bt crops, when integrated effectively, thereby delaying the rate of resistance evolution (Head & Greenplate, 2012). It is necessary to understand the potential risk of resistance evolution to any Bt crop in any country (Head, 2004). It is therefore important to collect information regarding the agricultural system, the biology of target pests, behavior of growers, and general local information on product performance to know what sort of IRM options may be feasible. This study provides information on two of the above mentioned topics namely the biology of the target pest and product performance.

The findings of this study and the environmental conditions in Africa, which are similar to those of Brazil, emphasize the importance of resistance monitoring, in order to aid the development and implementation of appropriate IRM strategies and to prevent the same shortcomings of IRM strategies in Brazil (Fatoretto *et al*., 2017). Data regarding the response of *S. frugiperda* to Bt maize and the frequencies of Bt resistance alleles should be monitored continuously. South Africa is considered the leading country regarding biotechnology in Africa, since it is the only country where cultivation of Bt maize has been approved on the continent (ISAAA, 2017). This study therefore serves as a 'case-specific' monitoring study for South Africa (Alacalde *et al*., 2007), and it also provides 'general surveillance' information regarding Bt maize for other African countries (Lecoq *et al*., 2007). Although this serves as a general surveillance of GM crops study for other African countries, Head (2004) mentioned that cropping system characteristics also play a role in development of the ideal IRM program. Considering that cropping systems and farmer behavior greatly affect IRM, and the fact that farming practices and systems in the main maize production region in South Africa differ from other African

countries, additional studies should be conducted to develop appropriate IRM strategies for different parts of the African continent.

During the 2017/2018 cropping season 1.62 million hectares of Bt maize were planted in South Africa (71% of the total maize area). Single-gene Bt maize (MON810 - Cry1Ab) has been planted in South Africa for stem borer control since 1998 and pyramid Bt maize (MON89034 - Cry1A.105 + Cry2Ab2) since 2011 (Van den Berg, 2017). Although *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) have been controlled effectively, this pest evolved resistance to single-gene Bt maize (Kruger *et al*., 2014), this problem was successfully addressed after the approval of pyramid Bt maize during 2011. Several studies (Omoto *et al*., 2016; Sousa *et al*., 2016) has shown that Cry1Ab will not be effective in controlling high pest pressure of *S. frugiperda*, and although *B. fusca* will most probably be effective controlled by Cry1Ab in other African countries, we recommend that single-gene events (e.g. Cry1Ab) should not be deployed in Africa. The reason for this is that both *B. fusca* and *S. frugiperda* have the ability, and are highly likely to develop resistance to first-generation Bt maize, due to critical assumption on which IRM strategies are based not being met, i.e. low-dose toxin expression (Sousa *et al*., 2016), high initial frequencies of resistance (this study), non-compliance to refuge requirements and absence of wild host plants that could serve as refugia (Van den Berg, 2017).

Although single-gene Bt maize will control target pests for a short period, it will not be good stewardship practice to protect pyramid Bt crops. The concurrent planting of single-gene events with low levels of efficacy or against which resistance has evolved jeopardizes the resistance traits in pyramid events since resistance may evolve more rapidly when crops with pyramided traits are cultivated concurrent to single-trait crops (Zhao *et al*., 2005; Tabashnik *et al*., 2009). Second-generation Bt maize produces two or more distinct Bt toxins, with all the proteins supposedly being equally active against the same target pest because all toxins are expressed at a high dose. The latter is known as ‘complete redundant killing’ of susceptible insects (Comins, 1986; Gould, 1986; Carrière *et al*., 2016). In the case of *S. frugiperda,* cross resistance have been observed amongst Cry1 proteins, such findings indicate that these proteins should not be pyramided (Hernández-Rodríguez *et al*., 2013; Bernardi *et al*., 2015), due to the effects it might have on the ‘redundant killing’ of

pyramid events that contain Cry1 proteins. Although the mode of action of certain Vip and Cry proteins seems to be similar regarding the activation, binding and cell lysis of Vip3 toxins (Yu *et al*., 1997), their binding sites and ionic channels are different than those of Cry1A toxins (Lee *et al*., 2003). The fact that there are low to no levels of cross resistance between Cry1 and Vip3 proteins (Vélez *et al*., 2013; Huang *et al*., 2014; Niu *et al*., 2014; Bernardi *et al*., 2015; Li *et al*., 2016), due to different mode of actions and separate binding receptors (Sena *et al*., 2009; Hernández-Rodríguez *et al*., 2013), indicates that these proteins are ideal to be combined in pyramid events (Storer *et al*., 2012). It is therefore recommended that pyramid events in which Vip proteins are expected to be deployed for managing lepidopteran pests of maize in Africa.

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6 Appendix A

Instructions to authors (excerpt): *Journal of Economic Entomology*

Formatting

For new submissions, our formatting requirements are simple—just make sure your paper has the following items:

- Continuous line numbers
- Double-spaced lines
- A title page and abstract in the main document
- Tables in a Word document (we cannot accept Excel files, unless they are supplementary files)
- Figure and table legends in the main document
- All co-authors entered into the online review system (email addresses required)

Please note there are more formatting guidelines for revised versions, as those are closer to being accepted (see the Revised Versions section of these author instructions).

- References listed in alphabetical order, cited by author and year in the text (not numbered)
- Figures and tables at the end of the main document after the references, or uploaded as separate files. Figure legends should be included at the end of the main text after the references, and table legends should be next to their corresponding tables
- Text is single-column

Language

- English. A second abstract in a second language is permitted. Authors are responsible for the accuracy of non-English abstracts.
- Manuscripts with poor English that would not be understandable for reviewers will be withdrawn before review. Those authors are encouraged to pursue English-language assistance from a native speaker or editing service before resubmitting their paper. Having a paper in good English makes it easier for editors and reviewers to focus on the scientific merits of the paper. For more information on language editing, please see the Language Editing section of these author instructions.

Statistics and sample size

Statistics should be fully reported (i.e., F-value, both degrees of freedom [treatments and replicates], and exact P-value [unless it's less than 0.001]). Furthermore, the

paper will be withdrawn if Duncan's Multiple Range Test is used for papers that do not deal with plant resistance. For more information on statistics, please see the Statistics section of these author instructions.

Papers that have insufficient sample sizes (e.g., only a single year of data collected at one location for either insect surveys, pesticide studies, or other field data) are immediately withdrawn. The duration and size of trials/sampling must be biologically significant.

Abbreviations

Abbreviations should be used sparingly. Standard abbreviations for measurements according to Scientific Style and Format, 8th edition, are acceptable, as well as common abbreviations that improve the readability of a manuscript (e.g., DNA, PCR). All other abbreviations used should be defined at the first use.

Article Types

Research

Research articles report original observations and experiments, the results of the experiment, and a discussion of the significance of the results. There is no word limit for research articles.

Title Page

The title page should include:

1. **Corresponding author:** Include full name, mailing address, telephone number, and email address.
2. **Title:** Should be as short as possible. Only include common names that are listed in the ESA Common Names of Insects & Related Organisms. Do not include authors of scientific names. Insert "([Order]: [Family])" immediately after the name of the organism.
3. **Author list:** Include all authors in the order the names should be published.
4. **Affiliation line:** Include full addresses of all authors. If there are multiple affiliations, designate through numbered footnotes.
5. **Abstract:**
    a) 250 words or less.
    b) Give scientific name and authority at first mention of each organism.
    c) Do not cite references, figures, tables, probability levels, or results.
    d) Refer to results only in the general sense.
    e) A second abstract in a second language is permitted.

6. **Keywords:**
    a) Below the abstract, provide three to five keywords, separated by commas.
    b) Do not use abbreviations, combined keywords, or species names.

Body

Introduction

Clearly state the basis of your study along with background information and a statement of purpose.

Materials and Methods

Include a clear and concise description of the study design, experiment, materials, and method of statistical analysis.

Results

Clearly present the results. Do not include interpretation of results or interpretation of statistical analysis—simply present the results of the experiment and the results of the statistical analysis. Data listed in tables should not be listed in the results; instead, refer to the table.

Discussion

Interpret and discuss results of the study and their implications. Include suggestions for direction of future studies, if appropriate.

Acknowledgments

Place the acknowledgments after the text. Organize acknowledgments in paragraph form in the following order: persons, groups, granting institutions, grant numbers, and serial publication number.

References

- EndNote style is “Environmental Entomology,” and Reference Manager style is “Journal of Medical Entomology.”
- References should be in alphabetical order. If multiple references from the same author are cited, those references should be in chronological order.
- Abbreviate journal titles according to the most recent issue of BIOSIS Serial Sources.
- For non-English titled journals that are cited in the references, the title of the journal should be spelled out.

- Systematics-related articles may specify that all serial titles be spelled out for final publication.

Sample reference styles

Journal Articles

Evans, M. A. 2000. Article title: subtitle (begin with lowercase after colon or dash unless first word is a proper noun). J. Abbr. 00:000–000.
Evans, M. A. 2001a. Article title. J. Abbr. 00: 000–000.
Evans, M. A., and R. Burns. 2001. Article title. J. Abbr. 00: 000–000.
Evans, M. A., and A. Tyler. 2001. Article title. J. Abbr. 00: 000–000.
Evans, M. A., A. Tyler, and H. H. Munro. 2000. Article title. J. Abbr. 00: 000–000.
Evans, M. A., R. Burns, and A. A. Dunn. 2001. Article title. J. Abbr. 00: 000–000.

In Press

Evans, M. A. 2002. Article title. J. Econ. Entomol. (in press).

Books

Burns, R. 2001. Title (initial cap only): subtitle (no initial cap after colon). Publisher, city, state abbreviation or country.
Evans, M. A. 2001. Colorado potato beetle, 2nd ed. Publisher, city, state abbreviation or country.
Tyler, A. 2001. Western corn rootworm, vol. 2. Publisher, city, state abbreviation or country.

Article/Chapter in Book

Tyler, A. 2001. Article or chapter title, pp. 000–000. In T.A.J. Royer and R. B. Burns (eds.), Book title. Publisher, city, state abbreviation or country.
Tyler, A., R.S.T. Smith, and H. Brown. 2001. Onion thrips control, pp. 178–195. In R. S. Green and P. W. White (eds.), Book title, vol. 13. Entomological Society of America, Lanham, MD.

No Author Given

(USDA) U.S. Department of Agriculture. 2001. Title. USDA, Beltsville, MD.
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Patents

Harred, J. F., A. R. Knight, and J. S. McIntyre, inventors; Dow Chemical Company, assignee. 1972 Apr 4. Epoxidation process. U.S. patent 3,654,317.

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Theses/Dissertations

James, H. 2001. Thesis or dissertation title. M.S. thesis or Ph.D. dissertation, University of Pennsylvania, Philadelphia.

Software

SAS Institute. 2001. PROC user's manual, version 6th ed. SAS Institute, Cary, NC.

Online Citations

Reisen, W. 2001. Title. Complete URL (protocol://host.name/path/file.name) and/or DOI (Digital Object Identifier)

Tables

- Tables should be editable tables in a Word document.
- If a table continues on more than one page, repeat column headings on subsequent page(s).
- All columns must have headings.
- Leave no space between lowercase letters and their preceding values (e.g., 731.2ab).
- Do not footnote the title—use the unlettered first footnote to include general information necessary to understand the title (e.g., define terms, abbreviations, and statistical tests).
- Use approved abbreviations or abbreviations already defined in the text and define others in the general footnote.
- Use the following abbreviations in the body or column headings of tables only: amt (amount), avg (average), concn (concentration), diam (diameter), exp (experiment), ht (height), max (maximum), min (minimum), no. (number), prepn (preparation), temp (temperature), vs (versus), vol (volume), wt (weight) Jan (January), Feb (February), Mar (March), April, May, June, July, Aug (August), Sept (September), Oct (October), Nov (November), and Dec (December).

Figures

- Figures should be at least 300 dpi, or 1200 dpi for line graphs.
- The quality in which figures are submitted is the quality in which they will print—please ensure figures are high quality.
- The following file types of figures are accepted: tif (preferred), eps (preferred), rtf, ppt/pptx, pdf, ps, psd, ai, gif, png. Figures should be in their native format for best quality.
- Figures should be prepared in CMYK color.
- Maximum height: 240 mm.
- Maximum width (one-column figure): 82 mm.
- Maximum width (two-column figure): 171 mm.
- All authors are required to pay additional charges for color figures. Authors may elect to publish in grayscale in print and in color online for no charge.
- For more information on preparing figures, see OUP's Author Resource Centre on figures.

Authors are encouraged to submit a graphical abstract as part of the article, in addition to the text abstract. The graphical abstract should clearly summarize the focus and findings of the article, and will be published as part of the article online and in PDF. The graphical abstract should be submitted for peer review as a separate file, selecting the appropriate file-type designation in the journal's online submission system. The file should be clearly named, e.g. graphical_abstract.tiff. See this page for guidance on appropriate file format and resolution for graphics. Please ensure graphical abstracts are in landscape format.

Species Authority, Order, Family, and Common Names

- Authors should provide the authority, order, and family for all organisms that are central to the paper (including plants, bacteria, and other non-arthropod organisms) at the first mention of the organism. It is the author's responsibility to provide accurate authority, order, and family information. Organisms mentioned in passing or whose importance to the paper is limited do not need to have full authority, order, and family listed, nor do mentions of common names of groups (e.g., mosquitoes, beetles, ticks, etc.).
- If a species name is included in the title of a paper, either the ESA-approved common name or the Latin name, the order and family should also be provided. If the order and family of an organism is provided in the title of the paper, it does not need to be provided again in the abstract and main text.

- If an organism is not listed in the title but is central to the paper, the order and family should be provided at first mention of the organism in both the abstract and the main text.
- The taxonomic authority of an organism that is central to the paper should be given the first time the organism is mentioned in both the abstract and the main text, but not in the title. For tables that include lists of species, authority should be given for each species if it is the first time it is being mentioned in the paper.
- If multiple organisms in the same genus are central to the paper, order and family only need to be provided for the first species mentioned in the genus. If multiple organisms are central to the paper, are in different genera, but are in the same order and family, order and family should be provided at first mention of each organism.
- If two organisms that share the same order and family are mentioned in the title or listed in the text of the paper together, the order and family should be placed after the first species listed and does not need to be repeated after the second species.
- A genus can be abbreviated after the first mention (except to start a sentence, in which case the genus should be spelled out). If two species belong to different genera but the genera start with the same letter, the first two letters can be used for abbreviations.
- Only ESA approved common names should be used. Common names are lower case, except for proper nouns and their derivations.

Statistics

All data reported (except for descriptive biology) must be subjected to statistical analysis. Results of statistical tests may be presented in the text, in tables, and in figures. Statistical methods should be described in Materials and Methods with appropriate references. Descriptions should include information such as sample sizes and number of replications. Only t-tests, Chi square, and analyses of variance require no citation. Cite the computer program user's manual in the References Cited.

Analysis of Variance or t-test

When presenting the results of analysis of variance or a t-test, specify F (or t) values, degrees of freedom, and P values. This information should be placed in parentheses in the text. Example: ($F = 9.26$; $df = 4, 26$; $P < 0.001$). If readability of the text is affected by the presence of repeated parenthetical statistical statements, place them in a table instead.

Model Analysis, Guidelines, Equations, and Computer Code

Model Analysis

At the beginning of the manuscript, authors should state clearly the goals of their model construction and analysis. Evaluation by reviewers depends upon these goals and the type of model. Authors should attempt to describe the main conclusions, limitations, and sensitivity of results to assumptions. For stochastic models, describe the variability in the results.

Modeling Guidelines

- The following guidelines pertain to any mathematical model calculated for purposes other than statistical analysis.
- Authors must adequately describe both model structure and model analysis.
- Authors must explain and justify original equations and computer programs or justify the selection of a published software package used in the computation of models.
- Model structure and steps in the analysis must be described in the Materials and Methods section.
- Without presenting extensive computer code, the text must permit an understanding of the model that would allow most mathematically inclined scientists to duplicate the work.
- Present all equations that represent the biology of the system being modeled.
- Unless their derivation is self-evident, show how the equations were derived and mention the underlying assumptions.
- Express how the equations are solved over time and space.
- Provide references for standard techniques (e.g., matrix manipulation, integration).
- Define all variables and parameters in each equation and describe their units (e.g., time, space, and mass).
- In the Materials and Methods or Results section, present the range of parameter values included in the model, and describe the uncertainty in or range of validity of these values.

Equations

Consult Mathematics into Type for correct formatting of equations and mathematical variables. Italicize all mathematical variables.

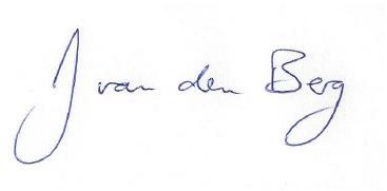
7 Appendix B

Declaration of language editing

Language editing statement

To whom this may concern,

I, Prof. Johnnie Van den Berg, hereby declare that the thesis titled: “Susceptibility of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to Bt maize in South Africa” by Andries Sarel Botha has been edited for language correctness and spelling by some of the supervisors. No changes were made to the academic content or structure of this work.



21 October 2019

Prof. Johnnie Van den Berg Date