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Research

Resistance Status of *Busseola fusca* (Lepidoptera: Noctuidae) Populations to Single- and Stacked-Gene Bt Maize in South Africa

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Abstract

Transgenic Bt maize expressing Cry insecticidal δ-endotoxins of *Bacillus thuringiensis* has been cultivated in South Africa for the control of *Busseola fusca* since 1998. *Busseola fusca* is resistant to Cry1Ab Bt maize at many localities throughout the maize production region. Pre-release evaluation (1994–1996) of the inherent susceptibility and post-release assessments (1998–2011) of resistance status of *B. fusca* focused on a limited number of pest populations. This study reports the current levels of susceptibility of 10 *B. fusca* populations evaluated between 2013 and 2017 and compared this data with previously reported data on the survival of this pest on Bt maize, including data of pre-release evaluations done during 1994 and 1995. Larval feeding bioassays in which plant tissue of maize events expressing either Cry1Ab or Cry1A.105+Cry2Ab2 (stacked event) proteins were conducted and survival and different life history parameters recorded. Results show a shift in levels of susceptibility of *B. fusca* to Bt maize. Pre-release evaluation of the single-gene event showed very low larval survival on Bt maize leaf tissue while studies 10 yr later and the current study reported survival of up to 40% and 100% on Cry1Ab maize, respectively. While no larvae completed their life cycle on the stacked event, higher LT50 values in this study indicate a shift in susceptibility of *B. fusca* to the stacked-gene event and highlight the importance of baseline information and monitoring of pest populations for their susceptibility to Bt maize.

Key words: Cry protein, insect resistance management, stem borer

Transgenic Bt crops that express Cry insecticidal δ-endotoxins of *Bacillus thuringiensis* are important tools in the management of crop pests. Bt crops have the potential to reduce the use of chemical pesticides (Gould 1998, Brookes and Barfoot 2016) but if pests evolve resistance to this technology, the benefits associated with Bt crops are lost (Carrière et al. 2015, Tabashnik and Carrière 2017). From the first commercialization of Bt maize, there have been concerns about resistance evolution in target pests (Gould 1998). Evolution of resistance to Bt toxins under laboratory and field conditions had already been recorded by Tabashnik (1994) in several lepidopteran families prior to the first commercial release of Bt crops. Since then numerous cases of Lepidoptera species that evolved different mechanisms of resistance to Cry proteins have been reported (Peterson et al. 2017).

The first reports of *B. fusca* resistance to Cry1Ab Bt maize (MON810) were from the Christiana and Vaalharts areas in South Africa during 2006 (Van Rensburg 2007). Since then field resistance was documented in several areas in the South African maize production region by Van den Berg et al. (2013) who reported that the increased appearance of Bt-resistant strains during the 2006–2014 period indicated that the predicted rate of resistance evolution was underestimated.

The only way in which current Cry1Ab-resistant populations can be controlled by known genetic engineering technology is through gene pyramiding. The only pyramid event available in South Africa is MON89034. Plants of this event express Cry1A.105+Cry2Ab2 proteins which provide effective control of Cry1Ab-resistant populations.

Resistance management strategies that ensure the long-term effectiveness of Bt plants depend on effective resistance monitoring that enable early detection of resistance to allow the implementation of appropriate management decisions in a timely manner (Tabashnik et al. 2014). An essential component of resistance management programs is the development and implementation of effective resistance monitoring techniques.

A critical first step in implementing resistance management programs is the establishment of a baseline of susceptibility among geographically distinct populations (Glaser and Matten 2003). Once

such information is available, changes in population susceptibility in response to selection with Bt can be reliably identified and quantified (Siegfried et al. 2005). Several methods have been used or suggested for detecting or monitoring recessive resistance alleles in pest populations (Andow and Alstad 1998, USEPA 2001).

Establishing baseline susceptibility for *B. fusca* is problematic given the lack of an adequate artificial diet that supports optimum and complete larval development and the inherent resistance of larvae to Cry1Ab proteins.

The first evaluation of various Bt maize events for control of *B. fusca* in South Africa was conducted between during 1994 and 1995 (Van Rensburg 1999). Due to difficulties of rearing *B. fusca* on artificial diets, baseline susceptibility was never determined using protein-incorporated diets in the laboratory. Efficacy data were collected by means of field and greenhouse evaluations of Bt maize under artificial infestation with the target pest. Although these methods are not ideal and do not provide any dose-response data, they do, on the basis of comparative life history parameters, provide indications of possible shifts in the levels of pest susceptibility. The successful use of whole plants in screening for pest resistance has been described by Nowatzki et al. (2008) who reported that part of the problems experienced with artificial rearing of *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) was overcome using a sublethal seedling assay. This resistance screening method involved introduction of neonate Diabrotica larvae into plant pots containing maize seedlings and comparing life history parameters of Bt-exposed and -unexposed larvae over time.

To maintain the effectiveness of Bt plants, it is necessary to detect changes in susceptibility through regular monitoring and to apply resistance management strategies to prevent or delay pest adaptation (Tabashnik et al. 2014).

The aims of this study were to evaluate a large number of *B. fusca* populations for their susceptibility to Bt maize and to collate reported data on the response of this pest to Bt maize since the release thereof during the 1994/1995 season.

Materials and Methods

All evaluations were done under laboratory conditions, using bioassays in which larvae were reared on whorl tissue of two Bt events and non-Bt maize plants (near-isogenic hybrid) grown under field conditions. Maize for use in the feeding bioassays was planted at 2-weekly intervals in two adjacent 0.1 ha blocks, for the duration of the growing season. The maize hybrids used in this study were the single-gene event MON810 (Cry1Ab) and a stacked event, MON89034 (Cry1A.105+Cry2Ab2). The life history parameters of *B. fusca* larvae were compared between populations collected at different localities and in some cases, during different cropping seasons.

Live insects sampled from Bt plants in the field can be used to estimate resistance allele frequency. This method involves collecting live insects from Bt plants in the field, rearing the field-collected insects for one or more generations to generate sufficient individuals for bioassays, followed by screening of the progeny of the field-collected strains either on Bt plants or in diet containing Bt toxins. Tabashnik et al. (2000) used this method to estimate Bt resistance allele frequencies of the pink bollworm, *Pectinophora gossypiella* (Saunders) in the United States.

All *B. fusca* populations were collected from sites located inside the main maize production area of South Africa except for the Venda population. This population was included since it was assumed that it would be comparatively more susceptible to Bt maize due to low selection pressure for resistance evolution in rural areas where smallholder farmers do not plant Bt maize.

Collection and Rearing of Different *Busseola fusca* Populations

Populations of *B. fusca* were collected in non-Bt maize fields during the 2013, 2014, or 2015 seasons at sites indicated in Fig. 1.

Larvae from the Venda region were collected from plants on farmer's fields and reared on non-Bt maize. Larvae of the other populations were collected during winter as diapausing larvae in harvested fields. Diapause larvae were collected by uprooting and dissecting the bases of dry maize stalks. Approximately 500–1,000 diapause larvae were collected at each site during the winter months of 2013 and 2014. The larvae were placed in 25-liter containers with slightly compressed dry maize leaf tissue and transported to the laboratory. Larvae were stored in insect-rearing chambers inside the mentioned leaf tissue and maintained between 10 and 12°C until spring when maize could be planted in the field to serve as food for larvae in the bioassays.

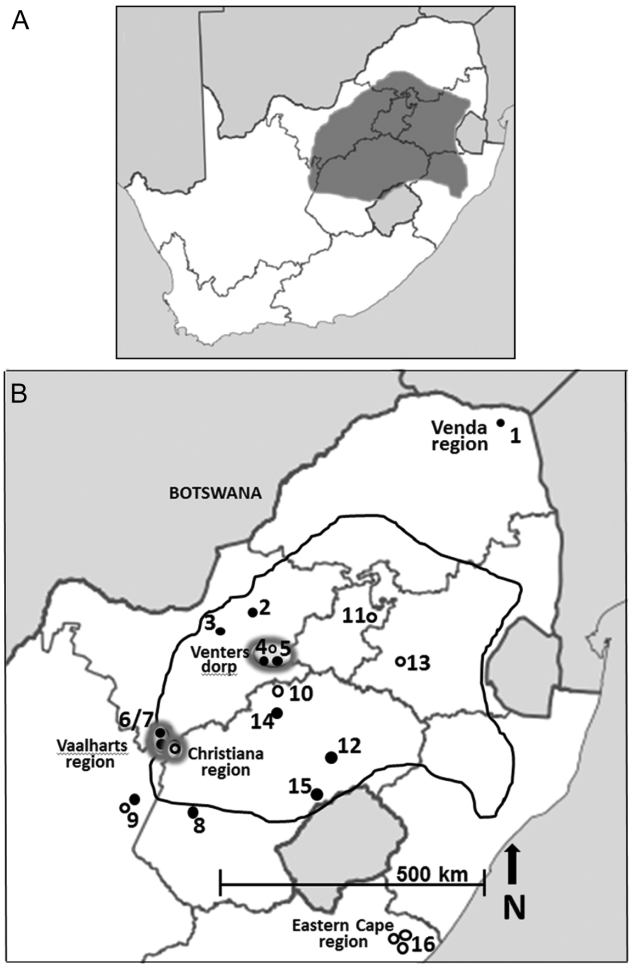

Fig. 1. (A) Map of southern Africa, indicating the maize production region of South Africa (dark area), and (B) enlargement of maize production region showing distribution of localities where *Busseola fusca* populations were sampled from for the current study (solid black circles) and previously (open circles). The Vaalharts region is considered the hot spot for Cry1Ab resistance. Oval shapes indicate the Vaalharts and Ventersdorp areas where many of the populations that were previously evaluated, were sampled. Legend: 1 = VED14, 2 = GRO14, 3 = LIC14, 4 = VEN14, 5 = RYS14, 6 = VAA14, 7 = VAA15, 8 = DOU14, 9 = Vil09Con-Bt, 10 = BRO10Con-Bt, 11 = BET14, 12 = BET08Con-Bt, 13 = BOT14, 14 = ECBt and ECRef populations.

The diapause phase of larvae was terminated following the technique developed by Van Rensburg and Van Rensburg (1993). Pupae were placed in containers until moths emerged. Male and female moths were paired in 2-liter plastic bottles to mate and lay eggs. The offspring of 20 breeding pairs of each population was used in the bioassays. This was done 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.

Larval Feeding Bioassays

Maize whorl leaves were cut from plants during the mid-vegetative growth stages (4–6 wk after seedling emergence) and used in bioassays. Larvae were inoculated onto maize whorl tissue of the two Bt maize events and non-Bt iso-hybrid. Five neonate larvae of the F1-generation of each of the 20 female moths were placed on 6- to 8-wk-old maize whorl tissue inside plastic aerated containers (50 ml) and data were recorded at regular intervals. Fresh leaf tissue was provided each time that survival was recorded. Since larvae are therefore handled many times during the assays, it is accepted that this will have an effect on mortalities that are observed and which is usually high in such feeding assays with *B. fusca*.

Larval survival and mass were determined twice a week over a period of 26 d, when pre-pupae started to form. The duration of the larval stage was also recorded. The pupae of each population were weighed and sex was determined based on the positioning of the genital scars found on sternum 8 in females and on sternum 9 in males (Harris and Nwanze 1992). Pupae were placed individually in 25-ml containers until moths emerged. Duration of the pupal period was determined from the day pupation commenced until emergence of the moth. Male and female moths were paired in aerated 2-liter plastic bottles where they mated and laid eggs on cut sections of maize stems (15 cm long × 2 cm diameter) until the female died. Moth longevity was determined as the period (days) from emergence until death.

Data Analysis

The life history data for larval survival and mass were analyzed with one-way ANOVAs (Genstat 17th edition) and *t*-tests. In cases where data were available for all three maize hybrids, a one-way ANOVA was done comparing data at the respective sampling days. In cases where data of only two treatments were available due to 100% mortality in one of the treatments, a *t*-test was used to compare data. Post hoc comparisons were made using Tukey's tests.

The number of days until 50% larval mortality (lethal time, LT50) was determined in each of the treatments by means of logistic regressions of larval survival over time. Chi-square analyses were used to determine if there were statistically significant differences in the LT50 and sex ratios between treatments of different populations. The corrected percentage mortality (corrected according to survival on the non-Bt treatment), larval duration, percentage pupation, male and female pupal mass, pupal period, and moth longevity were compared between treatments by means of Student's *t*-tests.

Results

Evaluation of Larval Survival and Growth

Larval survival of the different *B. fusca* populations over time until pre-pupae started to form is reported in Table 1. Larval survival on non-Bt maize at the end of the 26-d period ranged between 40 and 72%, with that of 7 of the 10 populations showing survival higher than 50%. Survival on the single-gene event, 26 d after inoculation of neonate larvae onto whorl leaf tissue, ranged between 3% for the Venda population (VED14) and 55% for the GRO14 and VAA15 populations (Fig. 2). No larvae survived for longer than 15 d on leaf tissue of the stacked-gene event and survival on day 12 was already very low (≤1%) (Table 1). Larvae feeding on non-Bt maize completed their life cycle and commenced forming pre-pupae on day 26 when the experiment was terminated. Pre-pupae largely formed after 26 d for larvae fed on single-gene Bt maize but the evaluations of survival and mass were terminated on day 26. Since no larvae survived on the stacked-gene Bt maize, no pre-pupae formed.

The corrected percentage mortality (Table 2) calculated for all the populations on the single-gene event ranged between 0 (VAA14) and 94% (VED14). The corrected mortality was 100% for all populations on the stacked event since no larvae survived on this event for longer than 15 d.

Mean larval mass was compared between treatments within each population after 26 d of feeding (Table 3). Larval mass 26 d after inoculation ranged between 176.3 and 289.3 mg for the different populations feeding on non-Bt maize while the mass ranged between 15.0 mg for the VED14 population and 261.6 mg for VEN14 population feeding on plants with the single-gene event (Fig. 2).

The LT50 values, which indicate the number of days until 50% mortality of larvae, are provided in Table 4. In the single-gene treatment, the LT50 ranged between 5 d (VED14) and 25 d GRO14). In the stacked-gene treatments, the LT50 was between 4 d (LIC14; VAA14 and VEN14) and 7 d (BOT14) (indicated by fiducial limits, Table 4).

Duration of the larval period was, in most cases, significantly shorter on non-Bt maize than on plants of the single-gene event (Table 5). On non-Bt maize the larval duration ranged between 33 and 46 d compared to 32 and 55 on Cry1Ab maize. On the latter, both the VAA14 and VAA15 populations had the shortest larval development periods compared to other populations on Bt maize as well as the non-Bt treatments.

The highest incidence of pupation observed on the non-Bt maize was 39.2% while it was 19.6% on the single-gene event (data not shown). No pupation was recorded on the stacked-gene treatment. Results on pupal mass indicated a significant difference between populations but no statistical significant difference between treatments. The duration of the male pupal stage for larvae that were reared on non-Bt maize ranged between 12 and 15 d compared to 13 and 15 d for those reared on plants of the single-gene event. Female pupal period on both the control and single-gene event treatments lasted 13–14 d. No significant differences were observed in the sex ratio between the Bt and non-Bt treatments in the populations where pupae formed and data did not deviate from the expected sex ratio of 1:1 (data not shown). There was no statistical significant difference in moth longevity between the non-Bt and Bt treatments in any of the populations (data not shown). Mean male moth longevity on the control and single-gene event treatments ranged between 5 and 7 d while that of female moths lasted 6 and 8 d on the control and single-gene event, respectively.

Discussion

Different levels of resistance, ranging from susceptible to highly resistant, are evident from this evaluation of 10 *B. fusca* populations. The only population (VED14) that was susceptible to both Bt maize events was the one collected in the smallholder farming area in the Venda region of South Africa, which had a very low larval survival percentage (<3%) and mean mass. Results from a recent study by Kotey et al. (2017) also showed very low survival (<1.5%) for larvae of three different populations collected in rural farming areas in the

Table 1. Comparison of percentage larval survival of different *Busseola fusca* populations over time on non-Bt maize, single-gene and stacked-gene Bt maize whorl tissue

	Day 5			Day 8			Day 12			Day 15			Day 19			Day 22			Day 26		
Populations	Con[a]	810[a]	89[a]	Con	810	89	Con	810	89	Con	810	89	Con	810	89	Con	810	89	Con	810	89
BET14	83a	91a	60b	52b	79a	27c	48b	73a	–	45a	50a	–	43a	23b	–	41a	18b	–	40a	17b	–
	$F = 12.9$; $P < 0.01$			$F = 29.1$; $P < 0.01$			$t = -3.7$; $P < 0.01$			$t = -0.9$; $P = 0.4$			$t = 3.9$; $P < 0.01$			$t = 4.0$; $P < 0.01$			$t = 4.2$; $P < 0.01$	
BOT14	93a	88a	74b	81a	80a	40b	75a	68b	1c	71a	38b	0.4c	70a	20b	–	68a	14b	–	67a	14b	–
	$F = 17.9$; $P < 0.01$			$F = 113.1$; $P < 0.01$			$F = 482.3$; $P < 0.01$			$F = 299.6$; $P < 0.01$			$t = 20.2$; $P < 0.01$			$t = 17.6$; $P < 0.01$			$t = 17.9$; $P < 0.01$	
DOU14	97a	82b	48c	87a	73b	4c	73a	60a	0.4b	64a	45b	0.4c	59a	39b	–	51a	30b	–	47a	27b	–
	$F = 71.2$; $P < 0.01$			$F = 279.0$; $P < 0.01$			$F = 82.4$; $P < 0.01$			$F = 58.0$; $P < 0.01$			$t = 3.3$; $P = 0.01$			$t = 4.8$; $P < 0.01$			$t = 6.1$; $P < 0.01$	

	Day 5			Day 8			Day 12			Day 15			Day 19			Day 22			Day 26		
Populations	Con[a]	810[a]	89[a]	Con	810	89	Con	810	89	Con	810	89	Con	810	89	Con	810	89	Con	810	89
GRO14	91a	90a	69b	86a	83a	9b	80a	78a	1b	76a	66b	0.4c	73a	63b	–	70a	58b	–	68a	55b	–
	$F = 9.85$; $P < 0.01$			$F = 543.04$; $P < 0.01$			$F = 492.91$; $P < 0.01$			$F = 371.69$; $P < 0.01$			$t = 3.66$; $P < 0.01$			$t = 3.71$; $P < 0.01$			$t = 3.82$; $P < 0.01$	
LIC14	91a	70b	17c	87a	55b	–	82a	46b	–	77a	37b	–	75a	31b	–	74a	30b	–	72a	30b	–
	$F = 304.19$; $P < 0.01$			$t = 7.88$; $P < 0.01$			$t = 7.61$; $P < 0.01$			$t = 7.72$; $P < 0.01$			$t = 8.55$; $P < 0.01$			$t = 8.21$; $P < 0.01$			$t = 7.30$; $P < 0.01$	
RYS14	94a	94a	44b	89a	85a	10b	82a	72a	–	77a	64b	–	75a	58b	–	69a	53b	–	61a	48b	–
	$F = 88.03$; $P < 0.001$			$F = 360.16$; $P < 0.01$			$t = 2.18$; $P = 0.06$			$t = 3.3$; $P = 0.01$			$t = 5.17$; $P < 0.01$			$t = 3.50$; $P < 0.01$			$t = 2.91$; $P = 0.02$	
VAA14	83a	89a	19b	64a	69a	4b	58a	66a	1b	53a	61a	1b	50a	55a	–	48a	51a	–	40a	40a	–
	$F = 281.77$; $P < 0.01$			$F = 176.45$; $P < 0.01$			$F = 173.69$; $P < 0.01$			$F = 185.98$; $P < 0.01$			$t = 1.11$; $P = 0.30$			$t = -0.63$; $P = 0.55$			$t = 0.0$; $P = 1.0$	

	Day 5			Day 8			Day 12			Day 15			Day 19			Day 22			Day 26		
Populations	Con[a]	810[a]	89[a]	Con	810	89	Con	810	89	Con	810	89	Con	810	89	Con	810	89	Con	810	89
VAA15	86a	86a	81a	78a	74a	4b	76a	66b	–	75a	63b	–	72a	60a	–	69a	57a	–	66a	55a	–
	$F = 0.98$; $P = 0.41$			$F = 854.61$; $P < 0.01$			$t = 3.05$; $P = 0.02$			$t = 2.73$; $P = 0.03$			$t = 2.19$; $P = 0.06$			$t = 2$; $P = 0.08$			$t = 1.91$; $P = 0.09$	
VED14	83a	45b	38b	73a	8b	2b	70a	6b	–	65a	5b	–	63a	4b	–	58a	4b	–	55a	3b	v
	$F = 13.01$; $P < 0.01$			$F = 178.2$; $P < 0.01$			$t = 12.29$; $P < 0.01$			$t = 12.77$; $P < 0.01$			$t = 14.41$; $P < 0.01$			$t = 10.80$; $P < 0.01$			$t = 12.41$; $P < 0.01$	
VEN14	86a	71b	10c	72a	54b	4c	67a	45b	1c	64a	38b	–	61a	36b	–	59a	33b	–	58a	31b	–
	$F = 303.27$; $P < 0.01$			$F = 111.15$; $P < 0.01$			$F = 86.86$; $P < 0.01$			$t = 6.50$; $P < 0.01$			$t = 8.31$; $P < 0.01$			$t = 7.61$; $P < 0.01$			$t = 9.99$; $P < 0.01$	

[a]Treatments: Control (Con) (non-Bt); MON810 (810) (single-gene); MON89034 (89) (stacked-gene).

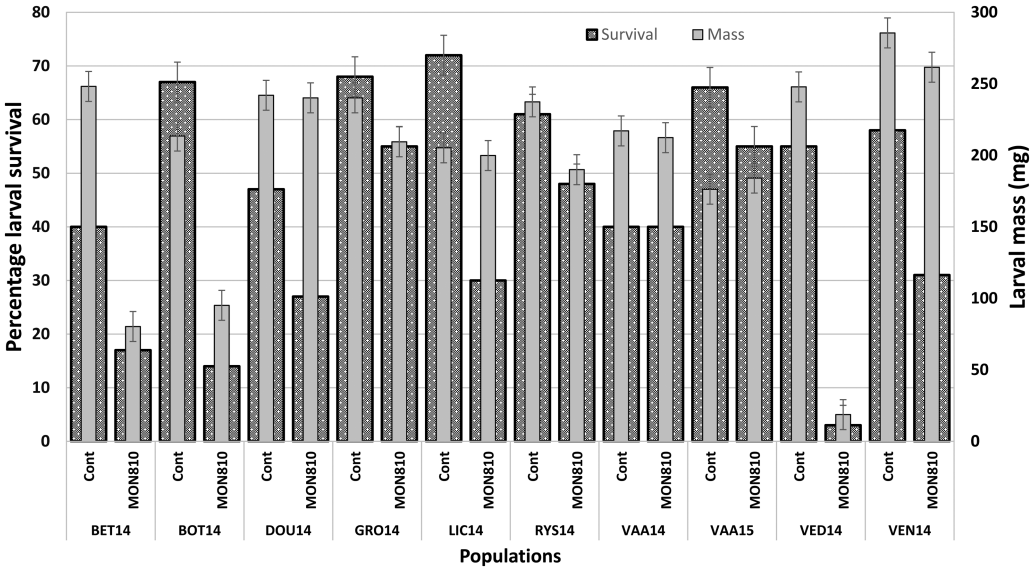

Fig. 2. Comparison of percentage larval survival and mass of different *Busseola fusca* populations on day 26 on non-Bt maize, single-gene and stacked-gene Bt maize whorl tissue.

Eastern Cape Province of South Africa. Two of the three populations from the Eastern Cape Province, designated ECBt001 (30.87372°S, 29.62144°E), ECBt002 (31.08722°S, 29.53661°E), are situated in areas where limited planting of Bt maize has been done while only non-Bt open-pollinated varieties are planted at the locality where population ECRef001 was collected (31.08271°S, 29.32504°E) (Fig. 1). It therefore seems as if there is a difference in the susceptibility of *B. fusca* populations that were collected in commercial farming systems where cultivation of Bt maize is common compared to rural farming systems where little or no Bt maize is cultivated.

Populations that showed high levels of resistance were those from BET14, BOT14, DOU14, and LIC14. Larvae from these populations survived for the whole trial period of 26 d and little or no differences were observed between larval mass on the non-Bt and Cry1Ab maize. The GRO14 and RYS14 populations can be considered resistant but larval fitness was adversely affected since mean larval mass of these populations was lower than that of the non-Bt treatments.

Table 2. Corrected percentage mortality of *Busseola fusca* larvae calculated for each population on the different Bt maize treatments

Population	Corrected % mortality[a]		*t*-value df (4)	*P*-value
	Single-gene	Stacked-gene		
BET14	58de	100	−6.33	<0.0001
BOT14	79ef	100	−7.90	<0.0001
DOU14	42bcd	100	−13.88	<0.0001
GRO14	19ab	100	−22.68	<0.0001
LIC14	58de	100	−14.30	<0.0001
RYS14	21abc	100	−12.48	<0.0001
VAA14	0a	100	−10.52	<0.0001
VAA15	16ab	100	−10.31	<0.0001
VED14	94f	100	−2.36	0.08
VEN14	46cd	100	−16.61	<0.0001
	F-value *P*-value	*F*-value *P*-value		
	29.59 <0.001	– –		

[a]Corrected percentage mortality is based on mortality observed in the non-Bt control treatment.

Larvae of the VEN14 population used in this study, 7 yr after a similar study by Van Rensburg (2007), responded differently to the single-gene event and were able to grow and survive successfully. The concerning results from this study are that larval mass on non-Bt and Cry1Ab maize was largely similar for these four populations, indicating no difference in fitness. Larvae of these four populations are therefore able to complete their life cycles on Cry1Ab maize and produce fit resistant offspring. Larval mass is an important parameter to determine whether a population can be regarded as resistant and it is a good indicator of fitness of a population (Kruger et al. 2014). Larger larvae are more fit and will develop into large reproducing adults that can give rise to a greater number of offspring.

The *B. fusca* population in the Vaalharts area of the Northern Cape Province is known to be resistant to Bt maize that express Cry1Ab proteins (Van Rensburg 2007; Kruger et al. 2011, 2014). The VAA15 2014/2015 population in this study had very high larval survival (55%) on Cry1Ab maize and did not show a significant difference in larval mass between non-Bt maize and Cry1Ab maize treatments. Larvae collected in Vaalharts in the 2013/2014 season are also regarded as highly resistant since there were no significant differences between either larval survival or mass between on the non-Bt and Cry1Ab treatments. Large numbers of larvae were able to survive and develop into fit reproductive adults that are capable of passing on the resistant gene to following generations. No larvae were able to complete their life cycles on leaf tissue of the stacked-gene event expressing Cry1A.105+Cry2Ab2 proteins. However, there were differences in the time periods that larvae from different populations survived on the stacked event. While larvae from the LIC14 population feeding on leaf tissue of the stacked event survived for only 5 d, those from BOT14, DOU14, and VAA14 were able to survive for 15 d.

The duration of the period until 50% of a population was killed (LT50) differed between populations indicating differences in the level of tolerance to the Cry1Ab protein. It can be assumed that in populations where there were no differences between the LT50 in the non-Bt and single-gene event treatments that the larvae of the different treatments were equally fit and that the moths will be able to produce offspring successfully. Kruger et al. (2014) observed no differences in fecundity of moths that survived on Bt and non-Bt

Table 3. Comparison of larval mass (mg) of *Busseola fusca* populations over time on non-Bt maize, single-gene and stacked-gene Bt maize whorl tissue

	Day 5			Day 8			Day 12			Day 15			Day 19			Day 22			Day 26		
Populations	C[a]	810[a]	89[a]	C	810	89	C	810	89	C	810	89	C	810	89	C	810	89	C	810	89
BET14	0.7	0.6	0.04	5.9	2.4	0.07	45.7	16.8	–	110.4	16.3	–	200.6	20.6	–	242.8	38.4	–	248.2	80.3	–
	$F = 61.0$; $P < 0.01$			$F = 80.6$; $P < 0.01$			$t = 8.9$; $P < 0.01$			$t = 11.6$; $P < 0.01$			$t = 19.0$; $P < 0.01$			$t = 17.8$; $P < 0.01$			$t = 10.0$; $P < 0.01$		
BOT14	1.7	0.7	0.003	9.0	2.7	0.01	41.7	20.2	0.01	99.7	16.0	0.01	171.2	22.1	–	218.0	50.1	–	213.5	95.1	–
	$F = 10.6$; $P < 0.01$			$F = 377.2$; $P < 0.01$			$F = 66.1$; $P < 0.01$			$F = 535.6$; $P < 0.01$			$t = 45.8$; $P < 0.01$			$t = 29.7$; $P < 0.01$			$t = 10.2$; $P < 0.01$		
DOU14	1.5	1.0	0.10	12.7	6.3	0.15	38.2	38.1	0.04	93.7	72.2	0.06	181.8	146.0	–	242.2	217.9	–	242.0	240.2	–
	$F = 155.1$; $P < 0.01$			$F = 493.8$; $P < 0.01$			$F = 10.3$; $P < 0.01$			$F = 85.0$; $P < 0.01$			$t = 1.9$; $P < 0.09$			$t = 0.9$; $P = 0.41$			$t = 0.1$; P =0.94		
GRO14	0.8	0.6	0.04	4.2	3.5	0.09	20.0	16.8	0.08	87.6	78.5	0.04	156.3	119.1	–	204.1	157.6	–	240.2	209.5	–
	$F = 50.2$; $P < 0.01$			$F = 110.7$; $P < 0.01$			$F = 84.5$; $P < 0.01$			$F = 172.7$; $P < 0.01$			$t = 5.0$; $P < 0.01$			$t = 4.4$; $P < 0.01$			$t = 2.6$; $P = 0.03$		
LIC14	0.9	0.9	0.08	11.4	4.3	–	42.7	20.3	–	76.3	60.9	–	136.3	111.5	–	161.9	191.9	–	205.3	199.9	–
	$F = 39.4$; $P < 0.01$			$t = 15.7$; $P < 0.01$			$t = 8.9$; $P < 0.01$			$t = 1.6$; $P = 0.16$			$t = 1.8$; $P = 0.10$			$t = -0.001$; $P = 1.00$			$t = 0.4$; $P = 0.71$		
RYS14	1.9	1.3	0.05	9.5	5.4	0.10	66.7	35.5	–	133.4	80.9	–	173.1	121.2	–	230.0	165.0	–	237.4	190.0	–
	$F = 80.4$; $P < 0.001$			$F = 54.4$; $P < 0.01$			$t = 6.8$; $P < 0.01$			$t = 7.0$; $P = 0.01$			$t = 5.8$; $P < 0.01$			$t = 5.1$; $P < 0.01$			$t = 6.1$; $P < 0.01$		
VAA14	1.5	1.0	0.09	5.5	3.6	0.16	23.5	24.4	0.1	66.6	63.5	0.1	118.4	128.6	–	183.8	194.2	–	217.1	212.4	–
	$F = 572.2$; $P < 0.01$			$F = 255.4$; $P < 0.01$			$F = 193.8$; $P < 0.01$			$F = 125.0$; $P < 0.01$			$t = -1.1$; $P = 0.29$			$t = -1.2$; $P = 0.26$			$t = 0.3$; $P = 0.75$		
VAA15	0.4	0.6	0.03	6.7	5.7	0.10	27.5	18.2	–	69.4	67.4	–	141.3	128.9	–	169.1	162.7	–	176.3	184.1	–
	$F = 225.6$; $P < 0.01$			$F = 35.8$; $P < 0.01$			$t = 5.0$; $P < 0.01$			$t = 0.4$; $P = 0.73$			$t = 1.7$; $P = 0.12$			$t = 1.1$; $P = 0.29$			$t = -1.3$; $P = 0.22$		
VED14	0.9	0.2	0.06	3.5	0.3	0.08	20.4	0.6	–	59.7	1.4	–	128.0	2.4	–	204.8	7.9	–	247.9	15.0	–
	$F = 107.9$; $P < 0.01$			$F = 26.8$; $P < 0.01$			$t = 11.6$; $P < 0.01$			$t = 16.4$; $P < 0.01$			$t = 24.1$; $P < 0.01$			$t = 27.9$; $P < 0.01$			$t = 20.2$; $P < 0.01$		
VEN14	1.3	0.7	0.26	6.0	2.1	0.27	51.5	12.5	0.3	128.2	44.0	–	220.4	127.8	–	271.3	199.7	–	285.6	261.6	–
	$F = 62.2$; $P < 0.01$			$F = 110.7$; $P < 0.01$			$F = 50.0$; $P < 0.01$			$t = 14.3$; $P < 0.01$			$t = 8.8$; $P < 0.01$			$t = 4.1$; $P < 0.01$			$t = 1.2$; $P = 0.27$		

[a]Treatments: Control (Con) (non-Bt); MON810 (810) (single-gene); MON89034 (89) (stacked-gene).

Table 4. LT50 values of the different populations of *Busseola fusca* feeding on Bt and non-Bt under laboratory conditions

Population	LT50 (days)					
	MON810 (95% fiducial limits)	Chi-square	*P*-value	MON89034 (95% fiducial limits)	Chi-square	*P*-value
BET14	15 (14.62–16.35)	190.03	<0.0001	6 (5.68–6.45)	143.23	<0.0001
BOT14	14 (13.60–14.98)	131.39	<0.0001	7 (6.67–7.26)	59.08	0.016
DOU14	16 (14.87–17.37)	212.74	<0.0001	5 (3.64–6.06)	1285.9	<0.0001
GRO14	25 (23.25–27.13)	110.89	<0.0001	6 (5.33–6.37)	346.60	<0.0001
LIC14	14 (11.89–15.11)	261.94	<0.0001	4 (3.67–4.02)	14.42	1.00
RYS14	22 (20.88–24.53)	190.95	<0.0001	5 (4.76–5.27)	77.83	<0.0001
VAA14	21 (19.03–22.80)	201.62	<0.0001	4 (–6.65–6.73)	3545.3	<0.0001
VAA15	24 (21.68–28.30)	235.74	<0.0001	6 (5.89–6.17)	25.44	0.941
VED14	5 (2.49–7.12)	1422.7	<0.0001	5 (4.47–4.83)	61.71	0.009
VEN14	14 (12.18–15.62)	267.73	<0.0001	4 (–0.058–5.38)	2447.3	<0.0001

Table 5. Mean duration of the larval period (days) of *Busseola fusca* populations on non-Bt and Bt treatments until pupation

Population	Larval duration (number of days) (SE)[a]		*t*-value (df)	*P*-value
	Non-Bt	Single-gene		
BET14	44 (1.24)	49 (1.22)	–1.980 (59)	0.05
BOT14	36 (1.38)	[b]	17.771 (30)	<0.001
DOU14	33 (0.88)	47 (2.73)	–5.910 (70)	<0.001
GRO14	44 (1.08)	52 (1.88)	–3.924 (97)	<0.001
LIC14	41 (0.86)	40 (0.96)	1.010 (65)	0.32
RYS14	41 (1.12)	46 (1.69)	–2.639 (113)	0.01
VAA14	33 (0.77)	33 (1.28)	0.112 (36)	0.91
VAA15	46 (2.66)	32 (5.66)	2.572 (9)	0.03
VED14	39 (0.79)	[b]	18.15 (83)	<0.001
VEN14	36 (0.63)	36 (0.95)	–0.500 (132)	0.62

[a]SE = standard error.
[b]No data available.

maize. A study done by Kruger et al. (2011) on larvae collected from the Vaalharts area (resistant; VAA08Bt-Bt) and Viljoenskroon (susceptible; Vil09Con-Bt) showed that the susceptible population had an LT50 of 4 d on Bt maize compared to the resistant population that had a LT50 of 9 d. In the current study, conducted 2 and 3 yr later, the LT50 of the two populations collected in the Vaalharts region (VAA14; VAA15) was between 21 and 25 d on the single-gene event and between 5 and 7 d on the stacked event. Although no larvae survived for the whole trial period on leaf tissue of the stacked-gene event, the 5- to 8-d period until death of all larvae, compared to the 80–90% mortality levels after only 3–4 d observed in pre-release studies with Cry1Ab maize (Table 6) (Van Rensburg 2001) with susceptible *B. fusca*, raises concern about a possible shift in susceptibility of this pest. For example, 27% and 17% of *B. fusca* larvae of the BET14 and LIC14 populations, respectively, survived on the stacked-gene event after 8 and 5 d (Table 1). The high levels of larval mortality (between 22 and 72%) recorded in the non-Bt maize treatments indicated that significant numbers of larvae in the Bt treatments also died of causes other than the Cry proteins. This would skew results toward lower LT50 values, indicating higher than actual susceptibility levels. These LT50 values could therefore be much higher, indicating higher levels of resistance to be present in nearly all populations evaluated in this study. The high levels of mortality observed on non-Bt maize in this study are similar the approximately 45% mortality after only 3–4 d of larval feeding on non-Bt in similar experimental setups, reported by Van Rensburg (2001).

Several studies on the levels of resistance of *B. fusca* to Cry1Ab maize have been done since Bt maize was introduced into South Africa in 1998. Results from the literature as well as from this study are summarized in Table 6. Results from the current study were compared with studies done by Van Rensburg (1999, 2011) and Kruger et al. (2011, 2012b). Van Rensburg (1999) first evaluated different Bt maize events for the control of *B. fusca* during the growing seasons of 1995/1996 and 1996/1997 using larvae collected at Ventersdorp. Baseline susceptibility of *B. fusca* on protein-incorporated diets has however never been determined because of difficulties rearing this pest in artificial diets. Efficacy data have in the past been collected through field and greenhouse evaluations using artificial infestation of Bt maize with neonate larvae, similar to the methods used in this study. These efficacy evaluations were conducted using plants under optimal growing conditions in greenhouses and field plots (Van Rensburg 1999). Van Rensburg (1999) reported data from greenhouse studies (season 1995/1996) with inbred lines of MON810, in which larval survival of 31 and 25% was observed after 11 d of feeding (Van Rensburg 1999). However, on hybrids of MON810 (season 1996/1997), larval survival of *B. fusca* of the Ventersdorp population was significantly lower, with 2% after 10 d of feeding on plants under greenhouse conditions. Under field conditions (season 1996/1997), the levels of survival of *B. fusca* on the same MON810 hybrids were between 1.2 and 1.9% after early infestations and between 0.4 and 0.8% after late infestations (Van Rensburg 1999).

Van Rensburg (2007, 2011) also provided data on the status of resistance of different *B. fusca* populations to Bt maize that express Cry1Ab and Cry1A.105+Cry2Ab2 proteins. Van Rensburg (2007, 2011) conducted a bioassay with Bt-transgenic hybrids during the 2006/2007 season in which a Ventersdorp population was used as a susceptible (control) population to compare its survival and life history parameters with that of a resistant population from Christiana (2006/2007). The Ventersdorp population was chosen as a susceptible control because Bt maize had at that time not been widely cultivated in that area, whereas Christiana had a history of Bt maize use and a resistant *B. fusca* population has previously been reported from this locality (Van Rensburg 2007). Van Rensburg (2011) reported larval survival for 12–16 d on Bt maize that expresses Cry1Ab for the susceptible population (Ventersdorp) while larvae of the resistant population (Christiana 2006/2007) survived for the trial period of 20 d. Mean larval mass of the Ventersdorp 2006/2007 population was very low (between 3.95 and 4.4 mg) compared with that of the Christiana 2006/2007 population (between 27.2 and 66.2 mg). Another Bt-susceptible population (Ventersdorp 2006/2007) also evaluated in the 2006/2007 season showed that larvae did not survive for longer than 13 d on the single-gene event and 9 d on the

Table 6. Summary of results available from published data and the current study on development and survival of *Busseola fusca* on Bt maize in South Africa since the first evaluations were done during 1995

		Single-gene maize		Stacked-gene maize		Mean larval mass (mg)[a]		LT50 (days)		
Population	Type of study	Day[b]	% survival	Day[b]	% survival	Single-gene	Stacked-gene	Single-gene	Stacked-gene	Source
Ventersdorp 1995/1996	Greenhouse	11	2–3	–	–	–	–	–	–	Van Rensburg (1999)
Ventersdorp 1996/1997	Greenhouse	10	0.6–0.9	–	–	0.10	–	–	–	Van Rensburg (1999)
Ventersdorp 1999/2000	Laboratory[c]	4	10	–	–	–	–	<1	–	Van Rensburg (2001)
Ventersdorp 1999/2000	Laboratory[d]	2	20	–	–	–	–	<1	–	Van Rensburg (2001)
Ventersdorp 1999/2000	Field	14	0.9	–	–	–	–	<1	–	Van Rensburg (2001)
Ventersdorp 2006/2007	Field 1	12	1[e]	–	–	3.95	–	–	–	Van Rensburg (2007)
Ventersdorp 2006/2007	Field 2	16	1[e]	–	–	4.40	–	–	–	Van Rensburg (2007)
Ventersdorp 2006/2007	Laboratory	13	0	9	0	–	–	–	–	Van Rensburg (2011)
Ventersdorp 2007/2008	Greenhouse	16	20	9	10	5.00	0.1	–	–	Van Rensburg (2011)
VEN14	Laboratory	26	31	26	0	261.6	–	13.89	3.72	Current study
VAA08Bt-Bt	Greenhouse	12	1.1	–	–	2.4	–	3.27	–	Kruger et al. (2011)
VAA08Ref-Bt	Greenhouse	12	2.2	–	–	0.8	–	3.41	–	Kruger et al. (2011)
VAA09Ref-Bt	Laboratory	30	12	–	–	186.5	–	6.84	–	Kruger et al. (2011)
VAA09Bt-Bt	Laboratory	30	14.8	–	–	201.8	–	8.96	–	Kruger et al. (2011)
BET08Con-Bt	Greenhouse	12	0	–	–	–	–	2.22	–	Kruger et al. (2011)
Vaalharts 2009/2010	Greenhouse	13	40	5	45	6.80	0.75	–	–	Van Rensburg (2011)
Vil09Con-Bt	Laboratory	6	0	–	–	–	–	3.02	–	Kruger et al. (2011)
VAA10Bt-Bt	Greenhouse	66	39	–	–	25.0	–	35.64	–	Kruger et al. (2014)
VAA14	Laboratory	26	40	15	1	212.35	–	20.69	4.02	Current study
VAA15	Laboratory	26	55	8	4	184.08	–	24.30	6.03	Current study
Christiana 2006/2007	Field 1	18	–	–	–	27.2	–	–	–	Van Rensburg (2007)
Christiana 2006/2007	Field 2	20	–	–	–	66.2	–	–	–	Van Rensburg (2007)
Christiana 2006/2007	Laboratory	18	–	–	–	26.1	–	–	–	Van Rensburg (2007)
CRH08Con-Bt	Greenhouse	12	6	–	–	–	–	2.41	–	Kruger et al. (2011)
Douglas 2007/2008	Laboratory	2	30	6	5	0.2	0.4	–	–	Van Rensburg (2011)
DOU14	Laboratory	26	27	26	0	240.19	–	16.08	5.00	Current study
Rysmierbult 2007/2008	Laboratory	30	2	4	0	50	–	–	–	Van Rensburg (2011)
RYS14	Laboratory	26	48	26	0	189.96	–	22.48	5.02	Current study
BRO10Con-Bt	Laboratory	66	9	–	–	17	–	0.55	–	Kruger et al. (2014)
VED14	Laboratory	26	3	26	0	14.97	–	5.15	4.67	Current study
BET14	Laboratory	26	17	26	0	80.26	–	15.47	6.07	Current study
BOT14	Laboratory	26	14	26	0	95.07	–	14.29	7.01	Current study
GRO14	Laboratory	26	55	26	0	209.53	–	24.94	5.58	Current study
LIC14	Laboratory	26	30	26	0	199.94	–	13.50	3.85	Current study
ECBt001 2015/2016	Laboratory	21	1	7	0	2.8	–	–	–	Kotey et al. (2017)
ECBt002 2015/2016	Laboratory	21	1	7	0	4.27	–	–	–	Kotey et al. (2017)
ECRef001 2015/2016	Laboratory	21	1.5	7	0	7.48	–	–	–	Kotey et al. (2017)

[a]Mean larval mass as recorded on the day mentioned in the day (trial duration) column.

[b]Percentage larval survival was recorded on the day indicated under column heading 'Day'.

[c]Experiment conducted with furl tissue of 3-wk-old plants.

[d]Experiment conducted with whorl tissue of 6- to 7-wk-old plants.

[e]Single surviving larvae as noted in article.

stacked-gene event (Van Rensburg 2011). During the 2007/2008 season, Van Rensburg (2011) recorded larval survival of approximately 20% for a field-collected population from Ventersdorp after 16 d on tissue of the single-gene event with larvae also surviving for 9–13 d on the stacked-gene event. In the current study, the Ventersdorp population survived (31%) for the trial period of 26 d on the single event with a mean larval mass of 261.6 mg. Larval survival of this population on the stacked-gene event was recorded until day 12 but no significant weight gain was observed. These results indicate a decrease in susceptibility in the Ventersdorp population over a time frame of 19 yr since the first trial was conducted with larvae collected in the same area (Ventersdorp population 1995/1996).

Kruger et al. (2011) evaluated the susceptibility of populations from Christiana (CHR08Con-Bt) and Bethal (BET08Con-Bt) and observed 100% mortality within 12 d. A susceptible population from the Viljoenskroon area (Vil09Con-Bt) feeding on tissue of the single-gene event also had 100% mortality after 6 d of feeding (Kruger et al. 2011). The Venda population that was screened in the current study showed similar results although surviving larvae were recorded on the single-gene event after 26 d. These larvae had no mass increase, which indicates this population's susceptibility. From day 12 onwards no survival of larvae of the Venda population was recorded on the stacked-gene event.

The population collected in Rysmierbult (Van Rensburg 2011) was reported to be highly susceptible to Bt maize with mortality of 98% and 100% on the single- and stacked-gene events, respectively, after 4 d. The Rysmierbult population (RYS14) in the current study feeding on tissue of the single-gene event had a larval survival of 48% on day 26 and successfully gained mass over the trial period. In the current study, larvae of the RYS14 population fed for a period of 8 d on tissue of the stacked-gene event before 100% mortality was observed. However, although this was not compared statistically, the time that larvae survived in the latter study was twice the time it took until 100% mortality was reached in the Rysmierbult population reported by Van Rensburg (2011).

A Douglas population evaluated by Van Rensburg (2011) was also highly susceptible to both the single and stacked events and did not survive for more than 4 and 9 d, respectively. The DOU14 population monitored in the current study showed some concerning results. A significant number of larvae feeding on the single-gene event survived (27.2%) for the duration of the trial period and there was no significant difference in larval mass between the control (non-Bt) and single-gene treatments, which is a clear indication of a shift in susceptibility. Larvae from this population also survived on the stacked-gene for 15 d with a survival of 0.4%.

Evaluation of a Vaalharts 2009/2010 population during that season showed 45% survival after feeding for 5 d on the stacked-gene event with a mean larval mass of 0.75 mg (Van Rensburg 2011). Larvae that were reared on the single-gene event survived 40% at 10 d after commencement of feeding. In the current study with two Vaalharts populations (VAA14 and VAA15), larval survival was recorded at 40% (2013) and 55% (2014) after 26 d. Larvae gained mass faster compared to those reported by Van Rensburg (2011) (186.5 mg in single-gene treatments after 35 d and a mean larval mass of 184.1 mg and 212.35 mg after 26 d). VAA14 larvae from this study survived on MON89034 for a period of 12 and 19 d, respectively. Therefore, over a period of 4 yr, larvae were able to survive 10 d longer on the stacked-gene event when compared to the study conducted by Van Rensburg (2011) during the 2009/2010 season.

The results reported by Van Rensburg (1999) and trends indicted by Kruger et al. (2012a,b, 2014) regarding larval survival and mass gain on the single-gene event are similar to those observed in this study on the stacked event. For example, Kruger et al. (2012a,b, 2014) reported increased survival and LT50 periods compared to the initial observations by Van Rensburg (1999) during the mid-1990s. The observed trend in survival and mass gain of larvae of the different populations indicate a shift in susceptibility. Some populations that were observed as susceptible in the past are now showing longer periods of survival and a comparative increase in mean larval mass on both Bt maize events.

The first report of field-evolved resistance was in 2006 (Van Rensburg 2007), 8 yr after the first planting of MON810 in 1998. Subsequently, the first signs of survival of *B. fusca* on Bt maize were recorded in the field (Van Wyk et al. 2008). The current study, 3 yr after the commencement of cultivation of the stacked-gene event in South Africa, indicates increased levels of tolerance of *B. fusca* larvae to this event.

Several factors played a role in the evolution of resistance of *B. fusca* to Cry1Ab maize in South Africa. One of the main contributing factors was the lack of refuge compliance in the region where resistance was first reported in South Africa (Kruger et al. 2009, 2011, 2014). The latter authors did however also report increased levels of compliance from 2007 onwards and the South African seed industry has since then introduced a stewardship program through training and field inspections to improve compliance levels (Van den Berg et al. 2014). It could therefore be expected that poor compliance to refuge requirements will not play a significant role in resistance evolution of *B. fusca* to the pyramid gene event in South Africa. The effectiveness of the high dose/refuge strategy can be questioned since Kruger et al. (2011) reported that resistant larvae were also present in refuge areas where larvae were sampled. Furthermore, the high-dose requirement was not attained for *B. fusca* (Van Rensburg 1999). Tabashnik et al. (2009) and Campagne et al. (2013) also reported that pre-commercialization data implied that the Cry1Ab-expressing maize deployed in South Africa was a low-dose event and did not meet requirements that would have been set for such an event in the United States (USEPA 1998).

The rate of resistance evolution of *B. fusca* to the stacked pyramid traits currently cultivated in South Africa will therefore depend on whether these events meet the requirements of the high dose needed for the high-dose/refuge strategy to be effective. A high dose should in theory be 25 times the dose needed to kill 99% of susceptible pest individuals (Roush 1994; Gould 1998; USEPA 1998, 2001; Glaser and Matten 2003). No pre-release data are available on the response of *B. fusca* to the stacked-gene event and the high-dose status of this event. Van Rensburg (2011) reported that no larvae survived for longer than 9 d on the pyramid and 90% mortality was recorded in some cases after only 4 d on this event but provided no information on the high-dose status of this event for *B. fusca*. With the small numbers of borer larvae used in those and the current bioassays, it is impossible to determine the resistance allele frequency (Cohen et al. 2000). The 100% larval mortality in a typical bioassay such as those reported in this study therefore does not indicate that a Bt event has a high dose of toxin, as defined in terms of the high-dose/refuge strategy (Cohen et al. 2000). The survival and LT50 values reported for *B. fusca* on the single-gene event by Kruger et al. (2011) (Table 6) are in a similar range those observed on the pyramid in the current study, indicating that the pyramid is most likely not a high-dose event. While no studies have been conducted on the dominance of *B. fusca* resistance to Cry1A.105 and Cry2Ab2 but data reported by Van Rensburg (2011) and in this study imply that this combination Cry proteins kill 99.99% of larvae (Table 6).

Bt crop pyramids, which produce two or more toxins that are active against the same pest, can delay evolution of resistance relative to single-toxin plants, particularly if insects resistant to one toxin are killed by other toxins in the pyramid (Roush 1998, Carrière et al. 2015). However, the durability of pyramids can be reduced by cross-resistance, which occurs when selection of a pest population with one Bt toxin causes a genetically based decrease in susceptibility to other toxins (Tabashnik et al. 2014). Mathematical modeling as well as laboratory and greenhouse experiments indicated that resistance to pyramids evolves faster when single-toxin plants that produce one of the toxins that are produced in the pyramid co-occur with two-toxin plants (Zhao et al. 2005, Onstad and Meinke 2010). In cases where a pest is resistant to one toxin that occurs in a two-toxin plant, the plant does not act as a pyramid. Toxins with similar amino acid sequences in domain II also show significant cross-resistance (Welch et al. 2015). While the importance of amino acid sequence similarity in cross-resistance was already suggested two decades ago in a study with Diamondback moth (Tabashnik et al. 1996), significant recent evidence (Zhao et al. 2005, Brévault et al. 2013, Carrière et al. 2015) confirm that there is strong cross-resistance between toxins with similar amino acid sequences in domain II. For example, Welch et al. (2015) showed that resistance to Cry1Ac and the observed cross-resistance to other Bt toxins could accelerate evolution of *Helicoverpa zea* (Boddie) resistance to pyramided Bt crops. Similarly, Carrière et al. (2016), even before commercialization of eCry3.1Ab, predicted that there would be strong cross-resistance between mCry3Aa and eCry3.1Ab, used against *D. v. virgifera*.

Pyramids are therefore more durable when they precede or rapidly replace single-toxin crops and are introduced when pest populations are still susceptible to all of the toxins in the pyramid (Carrière et al. 2016). Weak or strong cross-resistance will however accelerate evolution of resistance in pests that have inherently low susceptibility to Bt (Carrière et al. 2015). The inherent low susceptibility of *B. fusca* to Cry proteins (Van Rensburg 1999, Tabashnik et al. 2009) together with the fact that Cry1Ab maize expresses a low dose against this species (Tabashnik et al. 2009, Campagne et al. 2016) creates an environment for rapid resistance evolution to pyramid maize in South Africa. The replacement of single-gene Bt maize with the pyramid in South Africa unfortunately took several years after single-gene resistance was reported during 2006. Cocultivation of both events has been common since 2011 when the pyramid was approved for cultivation (Van den Berg et al. 2014) and still continues.

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

A similar situation is faced with the occurrence of Cry1F resistance in *Spodoptera frugiperda* (G.E. Smith) (Lepidoptera: Noctuidae) across Brazil, and the cross-resistance to Cry1Ab and Cry1A.105 (Bernardi et al. 2015, Santos-Amaya et al. 2015). This highlights the fact that resistance monitoring needs to be done on a continuous basis, and even more so in cases where single-gene and stacked-gene hybrids are cultivated concurrently.

The occurrence of Cry1Ab resistance in *B. fusca* in South Africa and the shift in susceptibility observed over time indicate that Cry1-based maize hybrids face a challenge in managing this pest in Africa and highlight the importance of effective insect resistance management for these technologies.

Conclusions

While high levels of resistance of *B. fusca* populations to Cry1Ab maize were observed in this study, no population showed resistance against the stacked event that expresses Cry1A.105+Cry2Ab2 proteins. However, although no larvae completed their life cycle on the stacked-gene event, the increased numbers of days until 50% mortality was reached are comparatively longer than those reported earlier which could show a shift in susceptibility. This study confirms resistance of *B. fusca* to Bt maize that expresses Cry1Ab proteins and highlights the importance of continuous monitoring of the resistance status of this pest. This study can be used as a baseline against which pest resistance can be compared to in future for both the single-gene and stacked-gene maize events.

This study further highlights the importance of a resistance monitoring program to provide updated and relevant information regarding the shift in susceptibility of *B. fusca* to maize expressing Cry1A.105+Cry2Ab2 proteins.

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