

Movement and survival of *Busseola fusca* (Lepidoptera: Noctuidae) larvae within maize plantings with different ratios of non-*Bt* and *Bt* seed

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Abstract

BACKGROUND: Products of plant biotechnology, for example genetically modified *Bt* maize, provide useful tools for pest management. The benefits provided by insect-resistant plants are, however, threatened by the evolution of resistance by target pest species. The high-dose/refuge insect resistance management strategy (IRM) as well as seed mixtures are globally used as IRM strategies. *Busseola fusca* (Lepidoptera: Noctuidae), the target stem borer of *Bt* maize in Africa, evolved resistance to *Bt* maize expressing Cry1Ab protein in South Africa. Owing to high larval mobility and subsequent sublethal exposure of larvae moving between non-*Bt* and *Bt* plants, more rapid resistance evolution has been proposed as a possibility with deployment of seed mixture strategies.

RESULTS: Laboratory and field studies were conducted to study *B. fusca* larval mobility. In the laboratory, different scenarios of *B. fusca* larval movement between single-gene (Cry1Ab) and stacked-trait (Cry1A.105 and Cry2Ab2) *Bt* maize were studied. Data on larval survival and mass over time indicated that Cry proteins do not kill larvae above certain developmental stages. A 2 year field study with the single gene and the stacked event was conducted using seed mixtures containing 5, 10, 15 and 20% non-*Bt* seed as well as a control treatment (non-*Bt* seed only).

CONCLUSION: Larval movement continued for 5 weeks and resulted in a significant incidence of *Bt* and non-*Bt* damaged plants, indicating that the movement behaviour of *B. fusca* is of such a nature that seed mixtures as an IRM strategy may not be effective to delay resistance evolution.

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Keywords: Cry proteins; genetically modified crops; larval movement; refuge; resistance evolution; stem borers

1 INTRODUCTION

The use of genetically modified (GM) crops with insecticidal properties is a significant step forward in pest management. These transgenic crops are modified to express *Bacillus thuringiensis* (*Bt*) Cry proteins that control specific target insects.¹ In South Africa, only single-gene events of *Bt* maize expressing Cry1Ab protein were planted until recently to control the African stem borer, *Busseola fusca* (Lepidoptera: Noctuidae). A stacked event expressing different proteins (Cry1A.105 and Cry2Ab2) has been deployed in South Africa since 2011 in an attempt to control *B. fusca*, which has become resistant to Cry1Ab produced by single-gene events.^{2,3}

As with any pesticide, resistance evolution of target pests is a concern. Insect resistance management (IRM) strategies are crucial in delaying resistance evolution and ensuring sustainability of *Bt* crop cultivation.⁴ Several IRM strategies can be used, of which the high-dose/refuge strategy is the most common. This strategy relies on a crop expressing a high dose of *Bt* proteins in order to kill as many of the target pest population as possible.⁵ Along with this high-dose expression, a separate non-*Bt* refuge of predetermined size is planted near the *Bt* crop.⁴ The non-*Bt* refuge serves as a source of susceptible adults of the target pest

that mate with the few resistant adults that survive on the *Bt* crop.⁴ This susceptible offspring is then controlled by the high dose of *Bt* protein expressed in the crop. Refuge compliance has been identified as a possible weakness of the high-dose/refuge strategy.⁶ Non-compliance to refuge requirements most likely contributed significantly to resistance development of *B. fusca* in South Africa.^{7,8}

Another IRM approach is the use of seed mixtures or the 'refuge in a bag' strategy. In this case, a single bag of seed contains a predetermined ratio of non-*Bt* to *Bt* seed, thereby eliminating the need to plant separate non-*Bt* refuges.⁹ A random refuge is therefore included within the cultivated area,⁹ thereby eliminating the problem of low or non-compliance to refuge requirements. The seed mixture strategy simplifies adherence to refuge requirements,

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with producers not needing to take responsibility for planting of a separate refuge.¹⁰ Although seed mixture products are very practical from a planting and compliance monitoring point of view, there is concern regarding its efficacy in managing resistance evolution, especially for migrating pests.¹¹

The feeding behaviour and movement of target pest insects that are highly mobile play an important role in determining the effectiveness of seed mixtures to delay resistance evolution.¹⁰ Larger larvae that move from non-*Bt* to *Bt* plants could be exposed to sub-lethal dosages of *Bt* proteins, leading to survival of heterozygous (RS) individuals and an increased rate of resistance evolution.¹² This may be even more of a problem for *B. fusca* if the *Bt* events are not high dose.¹³ A target pest species exhibiting no or limited migration between plants would probably not be affected in this way. Apart from the concern about the ability of a seed mixture strategy to delay resistance evolution, some studies suggest that deploying this strategy is still better than using no refuge at all.¹⁴ The success rate of high-dose expression, predispersal mortality rates and the rate of larval dispersal from *Bt* plants all affect a seed mixture's ability to delay resistance evolution.¹⁵ An increased risk of rapid resistance evolution may be present when seed mixtures are deployed to manage *Ostrinia nubilalis* (Lepidoptera: Crambidae).¹⁵

Studies of the effects of *Bt* maize seed mixtures on survival and migration of *B. fusca* larvae have not been done before. Suitable IRM strategies are needed for *B. fusca*, as it was one of the first in the world to develop resistance against the Cry1Ab protein expressed in *Bt* maize.¹⁶ Furthermore, IRM faces challenges in African smallholder farming systems because the planting of separate refuges of non-*Bt* maize is not practical in these systems.

The objectives of the study were to determine (i) to what extent migrating *B. fusca* larvae of different ages are effectively controlled by *Bt* plants using the seed mixture strategy, and (ii) if there is potential for using this strategy to manage resistance evolution by *B. fusca*.

2 EXPERIMENTAL METHODS

Two *Bt* maize events and their near-isogenic, non-*Bt* isoline were used in this study. These were the single-gene (Cry1Ab) event (referred to below as *Bt1*) and the stacked event (Cry1A.105 + Cry2Ab2) (referred to below as *Bt2*). The larvae used to inoculate plants in the laboratory and field trials were the F1 generation of diapause larvae collected from maize in the Ventersdorp area (26° 20' 14.0" S, 26° 45' 22.6" E), North-West Province, South Africa. This population was previously shown to survive on *Bt* maize, although not at very high levels, which makes it a *Bt*-tolerant population. At the time of this study, no susceptible population of *B. fusca* could be found in South Africa, and the population used was the most susceptible of which sufficient numbers were available to conduct this study.

2.1 Laboratory experiments

Two experiments were conducted, one with maize whorl tissue and the other with maize ear tissue. The experimental designs (completely randomised) were similar for these trials. In these experiments, the migration of larvae of different ages (3, 9 and 21 days old) was simulated between non-*Bt* maize and the *Bt1* and *Bt2* events. The treatments were designed to simulate different migration scenarios and were as follows: transferring larvae from non-*Bt* to *Bt1*, non-*Bt* to *Bt2* and non-*Bt* to non-*Bt* as a control treatment.

For larvae reared on *Bt1* and *Bt2* maize, similar treatments combinations were used, i.e. *Bt1* → *Bt1*, *Bt1* → *Bt2* and *Bt1* → non-*Bt*, as well as *Bt2* → *Bt2*, *Bt2* → *Bt1* and *Bt2* → non-*Bt*.

Maize was grown under field conditions, and whorl and ear tissues were removed as needed for the laboratory study.

The experiment commenced by inoculating whorl and ear tissue with first-instar larvae and allowing larvae to feed for either 3, 9 or 21 days before transfer to feed on tissue of the different treatments of maize. Larvae were weighed and placed on the whorl tissue or ear tips in plastic containers (100 mL). This was replicated at least 10 times with five larvae per container (container representing a replicate), depending on the numbers of larvae that survived on plants they were initially reared on before the first transfer. Larvae were then allowed to feed for 7 days, after which larval survival and mass were determined. These experiments were maintained at 25 ± 2 °C, 50–60% humidity and a 10:14 D:L photoperiod.

2.2 Field trial

The field study was conducted over two growing seasons (2011/2012 and 2012/2013) at the Agricultural Research Council – Grain Crops Institute, Potchefstroom, North-West Province, South Africa.

The experimental layout consisted of a randomised block design with five treatments planted to different ratios of *Bt* and non-*Bt* seeds for both *Bt* events, *Bt1* and *Bt2*. The five treatments were 5, 10, 15 and 20% non-*Bt* to *Bt* seed ratios, and a control treatment containing non-*Bt* seed only. Each treatment was replicated 4 times, and plots consisted of a 7 × 5 m area, with five rows planted at an interrow spacing of 0.9 m and an intrarow spacing of 17 cm. At planting, the position of each non-*Bt* and *Bt* seed was recorded inside each plot, and non-*Bt* seeds were marked with a trial marker. A map was created of the positions of *Bt* and non-*Bt* plants in each treatment plot in order to distinguish between damage to *Bt* and non-*Bt* plants in the experiment.

A single non-*Bt* plant, in the middle row of each treatment, was inoculated with 50 neonate *B. fusca* larvae 4 weeks after seedling emergence. Fifty larvae per plant were used, as this is within the range of egg batch size of this species. The levels of natural infestation in both experiments were determined 6 weeks after seedling emergence, on a block of non-*Bt* maize planted adjacent to the experiment. Monitoring of stem borer damage commenced 3 days after inoculation, and the incidence of damaged plants was recorded thereafter at weekly intervals for 9 weeks.

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

2.3 Data analysis

For the laboratory experiment, larval survival and mass were analysed by means of ANOVA followed by Fisher protected tests. Data on the incidence of stem borer damage, expressed as percentage stem-borer-damaged plants per plot over time in the field experiment, were analysed using the Table Curve 2D v.5.01 statistical program to generate curves that described the incidence of damage over time. The incidence of damaged plants in the different

Table 1. Survival and mass of *B. fusca* larvae (3, 9 and 21 days old) 7 days after being transferred between whorl tissue of different maize types^a

	Three-day-old larvae		Nine-day-old larvae		21-day-old larvae	
	Survival (%)	Mass (mg)	Survival (%)	Mass (mg)	Survival (%)	Mass (mg)
non-Bt → non-Bt	62.0 c	9.67 d	70.9 b	38.79 b	85.5 c	154.90 b
non-Bt → Bt1	62.0 c	6.88 cd	80.0 b	21.89 b	87.3 c	140.30 b
non-Bt → Bt2	0 a	0 a	20.0 a	1.55 a	78.2 bc	40.10 a
Bt1 → non-Bt	78.2 c	5.92 bcd	92.0 b	30.76 b	50.1 ab	152.80 b
Bt1 → Bt1	52.0 c	2.42 ab	78.0 b	21.09 b	30.2 a	166.40 b
Bt1 → Bt2	22.0 ab	2.35 ab	2.0 a	1.48 a	20.3 a	14.20 a
Bt2 → non-Bt	50.0 bc	3.72 abc	–	–	–	–
Bt2 → Bt1	50.0 bc	0.98 a	–	–	–	–
Bt2 → Bt2	3.3 a	0.17 a	–	–	–	–
F	18.72	11.48	45.31	10.99	15.95	21.10
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

^a Significance $P < 0.05$.

treatments was compared using Tukey's tests obtained from multi-factor ANOVA. The incidence (%) of damaged Bt and non-Bt plants per plot was also calculated and compared between treatments by means of ANOVA. Larval mass and survival on plants in the reference plots were also determined. ANOVA was done using GenStat 17th edition¹⁷ (GenStat, 2014).

3 RESULTS

3.1 Laboratory experiment

3.1.1 Larval growth and survival on maize whorl tissue

Larval survival after the initial 7 day feeding period was high (52–92%) in all movement scenarios (Table 1). Larval survival after transfer to whorl tissue of the respective treatments did not differ significantly between the control and Bt1 treatments. Significantly lower survival was recorded between treatments where larvae were transferred to whorl tissue of the stacked event (non-Bt → Bt2 and Bt2 → Bt2) (Table 1). Survival of nine-day-old larvae after feeding for another 7 days on the non-Bt control treatment and on Bt1 maize was high (70 and 80% respectively) and did not differ significantly between the different scenarios (Table 1).

Larval mass was the greatest for the following movement scenarios: non-Bt → non-Bt, non-Bt → Bt1 and Bt1 → non-Bt, and did not differ significantly between these treatments after the 7 day feeding period (Table 1). Larval mass was very low after the 7 day feeding period in all treatments involving exposure to Bt2 maize tissue. None of the larvae reared on Bt2 from the start survived longer than 9 days, and therefore no evaluation of survival of nine-day-old larvae could be conducted.

Many 21-day-old larvae survived the 7 day period in nearly all treatment scenarios, although survival was significantly lower for larvae that were reared on Bt1 maize for the whole period or for those that were transferred to Bt2 after 21 days. No larvae survived for 21 days on Bt2 whorl tissue, and no evaluation of a migration scenario from Bt2 to non-Bt or Bt1 maize could therefore be conducted. The lowest larval mass was recorded when larvae were transferred from non-Bt and Bt1 maize tissue to Bt2 (Table 1).

3.1.2 Larval growth and survival on maize ear tissue

The rate of survival of larvae reared for 3 days on any of the types of maize followed by a 7 day feeding period on non-Bt and Bt1 maize ears was high and in most cases did not differ significantly from the control (Table 2). Larval survival was only significantly

Table 2. Survival and mass of *B. fusca* larvae (3, 9 and 21 days old) 7 days after being transferred between ears of different maize types^a

	Three-day-old larvae		Nine-day-old larvae		21-day-old larvae	
	Survival (%)	Mass (mg)	Survival (%)	Mass (mg)	Survival (%)	Mass (mg)
non-Bt → non-Bt	53.3 bc	6.67 c	98.3 b	68.09 b	100.0 a	173.9 b
non-Bt → Bt1	46.7 bc	6.47 c	90.9 ab	68.55 b	100.0 a	181.0 b
non-Bt → Bt2	7.5 a	0.05 a	80.0 ab	9.35 a	86.0 a	133.5 ab
Bt1 → non-Bt	73.3 c	5.35 c	90.0 ab	61.59 b	96.0 a	151.0 ab
Bt1 → Bt1	50.0 bc	4.27 bc	83.6 ab	53.13 b	96.0 a	151.7 ab
Bt1 → Bt2	10.0 a	0.15 a	76.0 a	9.32 a	94.0 a	115.4 a
Bt2 → non-Bt	52.0 bc	0.98 ab	–	–	–	–
Bt2 → Bt1	25.0 a	0.85 ab	–	–	–	–
Bt2 → Bt2	0.1 a	0.02 a	–	–	–	–
F	10.32	12.73	3.05	19.14	2.34	3.11
P	<0.001	<0.001	<0.016	<0.001	0.054	0.015

^a Significance $P < 0.05$.

lower in migration scenarios where larvae were transferred from non-Bt and Bt1 maize to Bt2 maize. None of the larvae reared on Bt2 survived 7 days after inoculation. Mass of three-day-old larvae feeding for another 7 days on the respective maize tissues was highest in the following migration scenarios: Bt1 → non-Bt, non-Bt → non-Bt, non-Bt → Bt1 and Bt1 → Bt1. Larvae transferred to Bt2 were very small and non-viable after 7 days of feeding on the latter tissue (Table 2).

Survival of nine-day-old larvae was high after feeding for another 7 days on non-Bt and Bt1 maize ear tissue (Table 2). Survival of nine-day-old larvae reared on non-Bt and Bt1 maize ear tips and transferred to Bt2 ear tips for another 7 days was high (>76%), while that of 21-day-old larvae in a migration scenario from non-Bt to Bt2 and Bt1 to Bt2 maize was also high (86 and 94% respectively) (Table 2). While mass of nine-day-old larvae transferred from non-Bt and Bt1 to Bt2 ear tips was significantly lower than that in other movement scenarios, the mass of 21-day-old larvae was largely similar between treatments.

3.2 Field trials

Natural infestation during both seasons was low, with 5 and 3% of plants showing symptoms of larval feeding damage 8 weeks after crop emergence during seasons 1 and 2 respectively. The reduction in larval numbers on a per plant basis over time in the reference plot illustrates the migration of *B. fusca* larvae off plants over time (Fig. 1). A proportion of the reduction could, however, also be ascribed to a degree of predation on young larvae that could take place under field conditions. The mean percentage larvae recovered per plant in the reference plot decreased over 4 weeks to 26 and 18% respectively in the two seasons. The first prepupae were observed in the samples taken on day 35 (5 weeks) after inoculation (data not shown), and larval numbers were not recorded further.

Recorded larval mass on non-Bt maize in the laboratory study was similar to that of the reference block of non-Bt maize in growing season 2 (Fig. 2). Larval mass of 28-day-old larvae feeding on non-Bt and Bt1 maize whorl tissue in the laboratory study ranged between 140.3 and 166.4 mg larva⁻¹ (Table 1), while mean larval mass of 28-day-old larvae under field conditions was 130 mg larva⁻¹. The mass of a 21-day-old larva 7 days after being transferred from non-Bt to Bt2 (40.1 mg) was approximately the same as that of a 14–16-day-old larva that fed on non-Bt maize under field conditions, while mass of a 21-day-old larva transferred from Bt1 to Bt2 was the same as that of a 14-day-old larva on non-Bt maize.

The incidence of plants exhibiting stem borer feeding damage in whorls (during vegetative growth stages) and on stem and ear tissue (during reproductive stages) in the different treatment plots over the two seasons is shown in Fig. 2. The overall mean incidence of damaged plants was in the same range across the two seasons (Fig. 2). The increase in damage over time is indicative of the movement of larvae to neighbouring plants. The incidence of damaged plants increased over the first 6 weeks after inoculation, after which it levelled off. There was a high rate of *B. fusca* movement in non-Bt plots until pupation commenced. In general, the incidence of damaged plants in the Bt1 seed mixture treatments (Figs 2a and b) was similar to that of the control treatment for season 1, but the incidence of damage was lower than in the non-Bt control in season 2.

The percentage of damaged plants in all Bt1 seed mixture treatments was higher than in the non-Bt plants in the different plots, except for the 20% ratio, 11 weeks after inoculation (Table 3). The mean overall incidence (%) of damaged plants did not differ

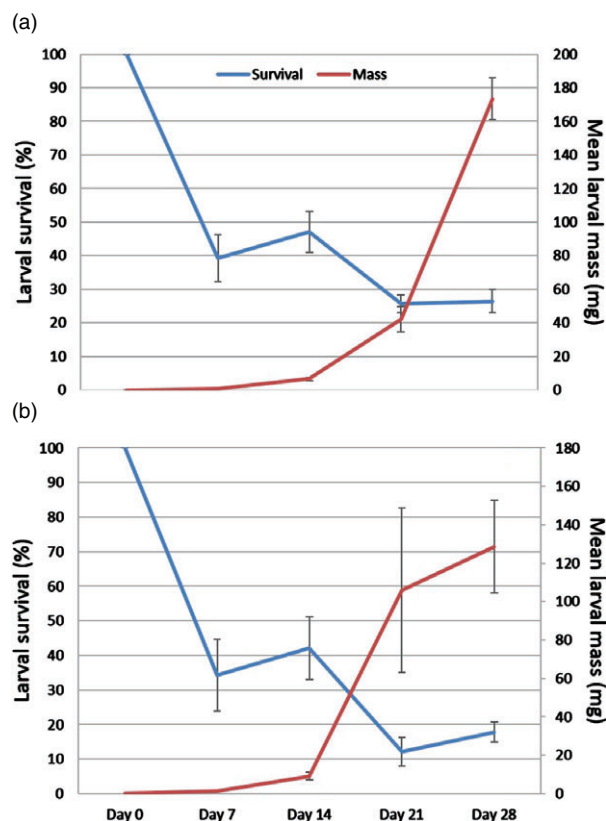


Figure 1. The mean percentage larvae recovered per plant from the reference plot as well as mean larval mass over time: (a) season 1; (b) season 2. Bars represent standard errors (SE).

between any of the Bt1 treatments in either season (Table 3). The incidence of damaged non-Bt plants per plot was, however, significantly higher in the 15 and 20% seed mixture treatments. The overall incidence of damaged plants per plot in the Bt2 treatments was low and ranged between 4.4 and 12.3% in the first season, and between 2.4 and 5.4% in the second season. In both seasons the Bt2 plots with 5% non-Bt seed mixtures suffered the lowest incidence of damaged plants, which in most cases differed significantly from the other treatments with Bt2. The incidence of Bt2 plants with damage in the different plots was negligible, ranging between 0.0 and 0.6% in season one, while no damage to Bt2 plants was recorded in season 2.

4 DISCUSSION

The level of larval survival when introduced to Bt1 or Bt2 plant tissue increased as larvae became older. The level of survival of larvae was also higher on maize ears than on maize whorl tissue.

The stacked event (Bt2) was highly effective against this Bt-resistant population of *B. fusca*, showing its current high susceptibility to Bt maize expressing Cry1A.105 and Cry2Ab2 proteins. The fact that larval survival and damage to plants was similar between the Bt1 and non-Bt treatments at the different assessment times in the laboratory experiment indicates a high level of resistance in this population. Although larval mass was initially significantly lower when larvae fed on Bt1 maize whorl tissue, larvae that survived the 21 day period and the additional 7 days on Bt1 plant tissue were similar in size to those that were reared on non-Bt maize only. Larval mass was similar between

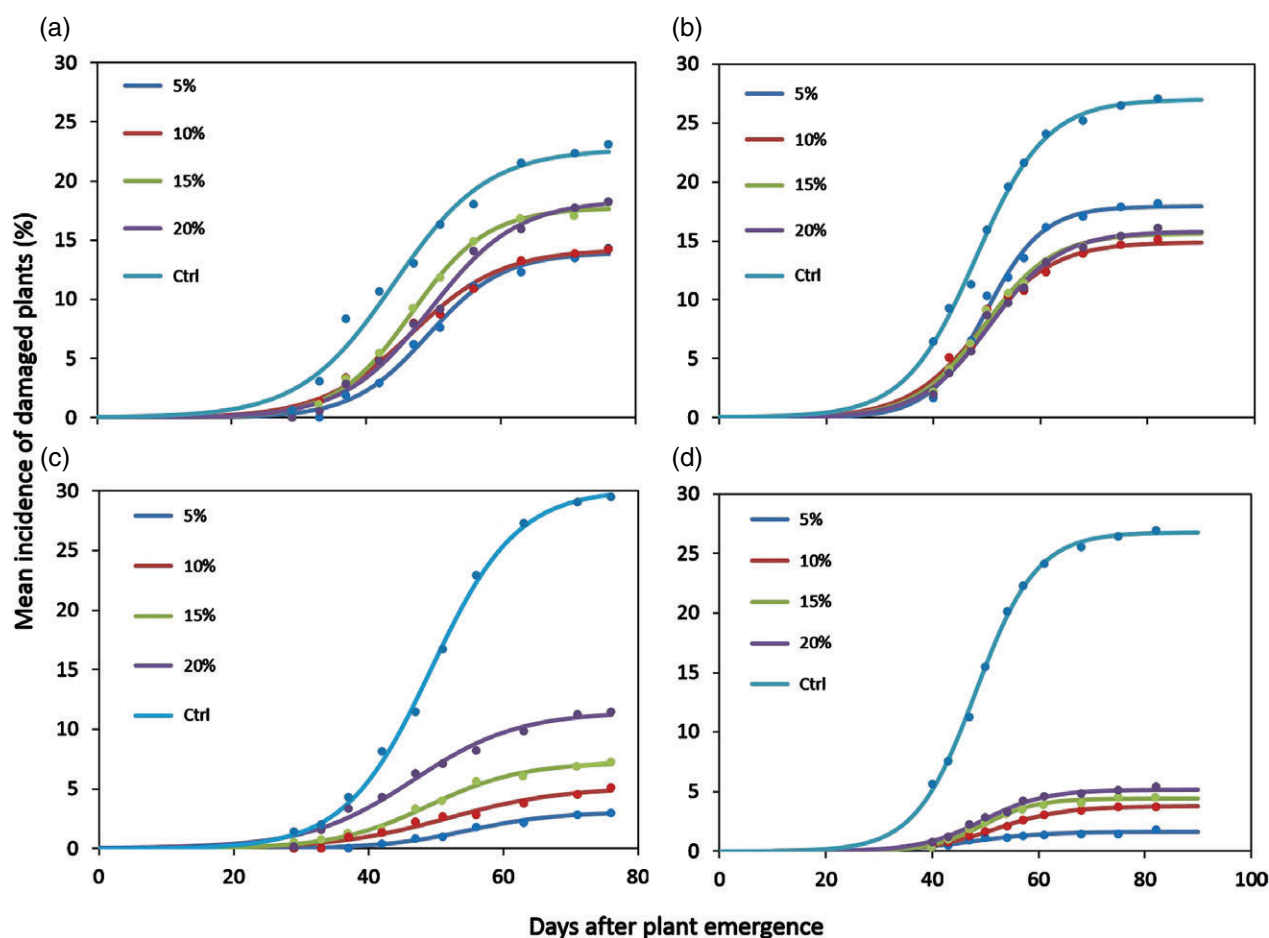


Figure 2. Mean incidence of damaged plants over time after inoculation of plots in the field with different ratios of *Bt* and non-*Bt* seed: (a) *Bt1*, season 1; (b) *Bt1*, season 2; (c) *Bt2*, season 1; (d) *Bt2*, season 2.

Bt1 and non-*Bt* maize ears on each of the respective assessment stages, indicating that this resistant strain of *B. fusca* did not suffer any fitness costs.

The use of a Cry1Ab-resistant strain of this pest in a study such as this would have defeated the purpose of studying the effect of exposure of larvae to Cry1Ab maize in seed mixtures. However, as this *Bt*-resistant population was still highly susceptible to the stacked event, results from the *Bt1* component of the study are useful in illustrating larval migration and damage patterns in seed mixture plantings. Results from *Bt1* plots are therefore only used to illustrate the migratory potential of larvae, similar to what it would be in non-GM maize.

The long period during which *B. fusca* larvae migrate is evident from the increase in incidence of damaged plants over the 5–7 week period after infestation (Fig. 2). This was especially evident in non-*Bt* control plots and *Bt1* treatments, which could technically also serve as control plots for the field component of this study.

The high levels of migration observed in non-*Bt* plots during the period of approximately 4–5 weeks provides a challenge to IRM strategies for this pest. Older larvae migrating from non-*Bt* to *Bt* plants will be exposed to sublethal dosages of *Bt* protein, thereby increasing potential risk of resistance development. Previous studies indicated that seed mixtures were not the optimum IRM strategy for pests with high migratory behaviour.⁴ Using the strategy to manage resistance evolution in *B. fusca* can therefore be questioned.

Larvae of this species were previously described to migrate over long distances.^{18,19} Field studies also indicated that *B. fusca* larvae from a single egg batch could migrate up to 3.6 m away from the natal plant, implying that the larvae from a single egg batch could potentially infest plants over an area of 40 m².²⁰ In grain sorghum it was observed that up to seven plants were infested after larval migration off the natal plant.²¹ *B. fusca* has a long larval phase that may range between 31 and 50 days, with migration taking place throughout all larval stages.^{8,22,23}

Clear patterns in the intraseasonal progression of larval infestations have been described.²⁴ After hatching underneath the leaf sheath, *B. fusca* neonate larvae ascend to the whorl, where they either commence feeding deep inside the whorl or disperse via 'ballooning off'.²⁵ Approximately 4% of larvae leave the natal plant immediately after hatching.¹⁸ This dispersal phenomenon is generally density dependent and might also be influenced by host plant quality. From the third instar onwards, larvae migrate to the lower parts of the plant where they penetrate the stem. Larvae can remain in plant whorls, especially in older (6–8-week-old) plants, up to the fourth instar.^{18,25} Migration does not cease after larvae commence feeding inside maize stems, but continues until the sixth instar. It has been reported that, towards the end of their cycle, larvae occurred singly in plant whorls and stems, indicating their highly active migration behaviour during the 4–5 weeks post-hatching.¹⁸ Furthermore, a significant positive relationship has been reported between plant stand and the

Table 3. Incidence (%) of stem-borer-damaged *Bt*- and non-*Bt* maize plants (mean number of damaged plants in brackets) in plots planted with different ratios of *Bt* and non-*Bt* maize seed, 11 weeks after inoculation with *B. fusca* larvae (*Bt1* = single-gene event; *Bt2* = stacked event)^a

Treatment	Season 1			Season 2		
	Mean overall % damaged plants	Mean % non- <i>Bt</i> plants that were damaged	% <i>Bt</i> plants damaged (number of plants)	Mean overall % damaged plants	Mean % non- <i>Bt</i> plants that were damaged	% <i>Bt</i> plants damaged (number of plants)
<i>Bt1</i>						
Control – non- <i>Bt</i>	24.1 a (27.0)	–	–	29.5 b (53.2)	–	–
5% seed ratio – <i>Bt1</i>	15.4 a (19.0)	2.7 a (3.3)	12.7 a (15.8)	19.0 a (33.0)	1.0 a (1.7)	18.0 a (31.3)
10% seed ratio – <i>Bt1</i>	16.3 a (20.3)	4.8 ab (6.0)	11.5 a (14.3)	15.5 a (28.0)	1.2 a (2.2)	14.3 a (25.8)
15% seed ratio – <i>Bt1</i>	20.8 a (27.0)	8.5 b (10.8)	12.3 a (16.3)	16.3 a (30.8)	2.8 ab (5.2)	13.6 a (25.7)
20% seed ratio – <i>Bt1</i>	19.1 a (27.0)	8.7 b (12.3)	10.4 a (14.8)	16.4 a (29.7)	3.6 b (6.3)	12.8 a (23.3)
<i>F</i>	1.21	8.70	0.17	9.66	6.92	1.79
<i>P</i>	0.349	0.002	0.917	<0.001	0.002	0.182
<i>Bt2</i>						
Control – non- <i>Bt</i>	29.1 c (36.3)	–	–	27.5 b (52.2)	–	–
5% seed ratio – <i>Bt2</i>	4.4 a (5.5)	3.8 a (4.8)	0.6 a (0.8)	2.4 a (4.3)	2.4 a (4.3)	–
10% seed ratio – <i>Bt2</i>	6.2 a (8.0)	6.2 a (8.0)	0.0 a (0)	4.2 b (7.8)	4.2 b (4.2)	–
15% seed ratio – <i>Bt2</i>	7.9 ab (11.5)	7.5 ab (11.0)	0.4 a (0.5)	5.3 b (9.3)	5.3 b (9.3)	–
20% seed ratio – <i>Bt2</i>	12.3 b (19.0)	12.1 b (18.8)	0.2 a (0.3)	5.4 b (9.5)	5.4 b (9.5)	–
<i>F</i>	52.81	9.57	0.89	90.98	10.37	–
<i>P</i>	<0.001	0.002	0.476	<0.001	<0.001	–

^a Significance $P < 0.05$.

number of larvae that migrate successfully and survive on maize plants.²⁰

Larval movement patterns in maize are affected by plant architecture at the time of oviposition, i.e. whether infestation takes place during the vegetative growth stages or during reproductive stages of plant growth. In late-infested maize, first-instar larvae may commence feeding on silk of ears, panicles or in young emerging panicles before migrating and commencing feeding inside ears or stems. It was reported that there is no clear preference for first instars to feed on ears of late-infested maize, and that occurrence of first instars on ears was most likely a function of plant growth stage at the time of oviposition and larvae searching for soft tissue and shelter.²⁴

Results from the field plots planted with *Bt2* seed mixtures in this study can be used to assess the potential effect of seed mixtures on the rate of larval movement and exposure to *Bt* plants. Although the stacked event effectively controlled migrating larvae, which is evident from the laboratory results and lower incidence of damaged plants observed in the *Bt2* field plots, the overall incidence of plants showing borer damage in the first season was high (4.4–12.3%) from an agronomical standard. The action threshold that triggers the application of insecticides for control of *B. fusca* is when 10% of plants in a field show symptoms of stem borer damage. Although the incidence of damaged plants for the 5% seed mix ratio in both seasons was low (<5%), farmers may not accept such damage levels. Further analysis of the results, separating damage to *Bt* and non-*Bt* plants, did, however, reveal that the incidence of damage to *Bt2* plants within plots was negligible. None of the treatments exhibited a higher incidence of damaged plants than the proportion of non-*Bt* plants that were present inside the respective plots. The highest incidence of damaged *Bt* plants was in the treatment with 20% *Bt* and non-*Bt*. This shows that there was a high larval movement.

The presence of larvae inside stems was not determined in this study, and no information can be provided on the number of larvae that successfully completed their life cycles on *Bt* or non-*Bt* maize. However, the negligible number of damaged *Bt2* plants in all plots suggests that no larvae survived on *Bt2* plants. This is supported by results from the laboratory study in which different migration scenarios were evaluated. As the incidence of plant damage between 5 and 20% seed mixtures did not differ significantly, a 20% ratio might be the better option to reduce selection pressure and generate more susceptible adults. However, the agronomical disadvantage of increased larval survival in 20% seed mixture plantings could be offset by higher numbers of RS individuals to mate with resistant individuals.^{10,12} A concern, however, with a 20% seed mixture is the potential exposure of migrating larvae to sublethal dosages of *Bt* proteins, as indicated in this study, which may eventually lead to survival of some individuals.

A study of dispersal and movement of *O. nubilalis* larvae on *Bt* and non-*Bt* maize showed that larvae detected *Bt* proteins within the first hour, and that larvae dispersed from *Bt* plants earlier than from non-*Bt* plants.⁵ Movement of *O. nubilalis* larvae from *Bt* to non-*Bt* plants was identified as a potential contributing factor to resistance development that could have adverse implications for a seed mixture strategy.⁵ A study conducted with *Helicoverpa zea* (Lepidoptera: Noctuidae) indicated that there was no significant difference in the occurrence of larvae on and damage to maize ears between plantings of pure *Bt* maize and seed mixtures.²⁶ Numbers of larvae and damage were, however, significantly higher in plantings with only non-*Bt* seed.²⁶ The possibility of increased dispersal under low larval density conditions owing to the presence of the toxin in *Bt* plots was observed.²⁶ This could indicate increased movement between plants if *Bt* proteins are detected by feeding larvae, similarly to observations on *O. nubilalis* dispersal behaviour.⁵

It has been suggested that *Bt* events controlling third-instar *O. nubilalis* larvae could be considered high dose, and, if fourth-instar larvae are controlled, the *Bt* event could be regarded as ultra-high dose.²⁷ If this principle is applied to 21-day-old *B. fusca* larvae transferred to *Bt2* maize, it could be concluded that this event is also low dose for this species. Therefore, it was concluded that *Bt1* was a low-dose event against *B. fusca*.^{13,28}

Whether initial infestation occurs on a *Bt* or a non-*Bt* plant in a seed mixture planting, premigration feeding behaviour will influence larval growth, survival and migration. While these aspects were not addressed in this study, results from the laboratory assays indicated that, if oviposition were to occur on *Bt2* plants and were to be followed by primary migration to non-*Bt* plants 9 days thereafter, the likelihood of survival would be high. Any secondary migration from the non-*Bt* to other *Bt2* plants would most likely not result in a sufficiently high level of mortality necessary to ensure efficacy of a seed mixture IRM strategy. This would be particularly true for late infestations, where neonate larvae commence feeding on ear and silk tissue where toxin expression levels are low. Neonate *O. nubilalis* dispersal was significantly greater from *Bt* plants than from non-*Bt* plants, and this could influence the efficacy of a seed mixture strategy.²⁹ For *B. fusca*, predispersal feeding behaviour on *Bt* maize could also play a role in the efficacy of seed mixtures. This aspect of *B. fusca* biology has, however, not been studied before.

5 CONCLUSIONS

The efficacy of a seed mixture strategy depends on structured high-dose/refuge strategies with the production of sufficient numbers of SS individuals and the ability of the *Bt* event to kill nearly all the heterozygote RS individuals. Survival of larvae in the seed mixture plantings will depend largely on the initial establishment on the non-*Bt* plants, movement and whether the *Bt* event is high dose. This study has indicated that *B. fusca* migrates extensively over its whole larval life cycle, over long distances, and that significant larval survival is possible if larvae get the opportunity to feed on non-*Bt* plants for a short period before migration to a *Bt* plant. Whether or not moths lay their eggs on non-*Bt* plants in such seed mixtures will also affect the likelihood of sublethal exposure.

The 5% seed mixtures seem to be the most efficient in controlling migrating larvae. While this may be acceptable in terms of an IRM strategy, this may not be practical under field conditions where farmers use a 10% action threshold for the control of *B. fusca*. A 5% mixture could, however, increase the selection pressure of *Bt* proteins on larvae while potentially exposing a greater number of resistant adults to each other during the mate-finding and mating process.

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