First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize

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Reports of severe damage caused by the African stem borer, *Busseola fusca* (Fuller) to Mon810-transgenic maize (Bt) prompted a study in which the survival of progenies of diapause larvae collected from both a Bt and non-Bt planting were compared when feeding on various Bt and non-Bt hybrids. Field and greenhouse grown plants were artificially infested with neonate larvae. Larval mass was recorded at two-day intervals for three weeks. Data were subjected to simple regression analyses followed by pair wise comparison of the slopes. The two borer populations showed similar larval mass gains on non-Bt hybrids but differed in the response to Bt-hybrids. Appreciable numbers of larvae from the non-Bt derived population survived only to the eighth day. In contrast, substantial numbers of larvae of the Bt-derived population survived over the entire trial period. The mean larval mass of the Bt-derived population at the conclusion of the experiment was less on Bt-hybrids than on their susceptible counterparts. This indicates that the Bt-derived population has attained a level of resistance where some larvae are able to survive in the presence of the Bt-toxin but not without some detrimental effect on larval growth rate. Since producers are inclined to provide refugia under rain fed conditions in the immediate vicinity of irrigated plantings rather than as part of irrigated fields, the known preference of moths for high humidity could have contributed to increased selection pressure towards the development of resistance to the Bt-toxin.

Introduction

First reports on insect resistance to insecticides appeared in 1948, within six years of the introduction of DDT, the first synthetic insecticide (Hammerton & Stowell, 1963; Burgess, 2004). An abundance of scientific literature on this issue subsequently became available, most dealing with the underlying ecological, biochemical and genetic principles. Based on these, insect tolerance to a given insecticide may be expected to develop rapidly when an insect population is characterised by a high rate of development of the immature stages and a quick succession of generations, while being exposed to sublethal levels of the toxin.

Prior to 1994, microbial preparations of the entomopathogenic bacterium *Bacilllus thuringiensis* (Bt), applied as spray formulations, had been in use for decades without substantial resistance developing in field populations (Tabashnik, 1994). The diamondback moth, *Plutella xylostella* was the only insect to eventually develop resistance to Bt applied as a biopesticide (Ferré & Van Rie, 2002). However, five of 10 species of moths representing the families Noctuidae, Plutellidae and Pyralidae, selected with Bt under laboratory conditions, developed more than a 10-fold resistance, suggesting that the ability to evolve resistance to Bt is a common phenomenon among the Lepidoptera (Tabashnik, 1994). Recent laboratory studies have shown that this ability to develop resistance to Bt applies to some major agricultural pest species, including the European corn borer, *Ostrinia nubilalis* (Hübner) (Chaufaux *et al.*, 2001; Sigueira *et al.*, 2004), the pink bollworm, *Pectinophora gossypiela* (Saunders) (Tabashnik *et al.*, 2002) and the bollworm *Helicoverpa armigera* (Hübner) (Tabashnik *et al.*, 2003).

More than 62 million ha of genetically engineered Bt crops were planted worldwide between 1996 and 2002, and some pest populations are thus considered to be under pronounced selection pressure to evolve resistance (Tabashnik *et al.*, 2003). Resistance monitoring of field populations of the bollworm *H. armigera* in China, the European corn borer *O. nubilalis* and the corn earworm *Helicoverpa zea* (Boddie) in the United States (Wu *et al.*, 2002; Tabashnik *et al.*, 2003; Stodola *et al.*, 2006), in regions with high adoption of Bt-crops has however not as yet detected increases in resistance frequency. This is attributed to the high-dosage/refuge strategy adopted in the USA to delay pest resistance to Bt-crops, which involves the use of refuges of non-Bt host plants to ensure survival of susceptible individuals (Tabashnik *et al.*, 2003).

During the testing of experimental Bt-events for control of the South African stem borer complex, which commenced in 1994, the African stem borer, *Busseola fusca* (Fuller) was shown to be more tolerant to a specific event than the spotted stem borer *Chilo partellus* (Swinhoe) (Van Rensburg, 1999). The first Bt-transgenic hybrids all employed the event Mon810 and were grown on approximately 50 000 ha during the 1998/99 growing season. No leaf feeding damage was observed during the vegetative growth stages in any of the plantings. At harvest of the 1999 crop, diapause larvae and damage to the lower stems were noticed at a number of localities, involving various hybrids. Subsequent studies indicated a window of approximately 14 days in the period immediately before and after tasseling in which relatively late infestations may result in increased larval survival (Van Rensburg, 2001). This was attributed to larvae feeding on the silks, of which a high water content contributes to a reduced concentration of the Bt-protein, allowing survival of some larvae until completion of the first two instars, and subsequent successful penetration of the stems.

South African production of Bt-maize has increased from 77 000 ha (2.8 % of total area under maize) in 2000 to 943 000 ha (34.9 %) in 2006 (James, 2006). During the 2004/05 season severe damage to Bt-plantings was caused by *B.*

fusca at a number of localities during the mid-whorl stage of plant development. Various hybrids from different seed companies were involved. All affected plantings were grown under irrigation and all fields had a history of continuous Bt-production. Eliza tests confirmed the presence of the Bt-gene (M. Potgieter, Pioneer Hi-bred RSA, personal communication) and it became apparent that some populations of the insect may be in the process of evolving resistance to the Bt-toxin. This study reports on the level of resistance recorded in a population collected from a Bt-planting compared to that from a non-Bt area.

Material and methods

Two thousand diapause larvae were collected during the winter of 2006 from stubble in a Bt-planting (cultivar Phb33A14) in which leaf feeding damage had occurred during the late vegetative stages. The site was in an irrigation area with a history of Bt-use, near Christiana (27°57'S, 25°05'E) in the North-West Province. To serve as a susceptible standard, larvae were also collected from stubble in a rain fed non-Bt planting near Ventersdorp (26°15'S, 27°20'E), 320 km distant. This is an area where Bt-maize has not been adopted. Larvae from the two sites were kept in separate batches in cold storage (12°C) for approximately four months until the onset of the 2006/07 season. Termination of diapause and the subsequent handling of moths, eggs and neonate larvae were done in accordance with established methods for maintaining a laboratory colony (Van Rensburg & Van Rensburg, 1993), keeping pupae, moths and eggs of the two populations separate.

Since moths emerged over a period of several weeks, with eggs collected twice a week, the use of neonate larvae for artificial infestation of plants necessarily required various planting dates in order to infest plants at approximately the same growth stage.

In a field experiment conducted during October/November 2006 the Bt-transgenic hybrids Phb33A14 and PAN 6012 (both Mon810) as well as their non-Bt counterparts Phb33A13 and PAN 6084 were grown in adjacent blocks of six rows each. Rows were 40 m long and 1.5 m apart, with a total of approximately 300 plants per hybrid. Rows were split into two subunits of 20 m length for artificial infestation with larvae from the two populations. Second and third plantings were done at seven-day intervals. Plants were artificially infested with neonate larvae three to four weeks after emergence, using camel hair brushes. In order to limit larval migration induced by overcrowding at later developmental stages, only two larvae were applied per plant. Every two days for a period of 18 days a number of plants per hybrid and per insect population were randomly selected and dissected. Average larval mass per plant was recorded. In order to ensure reliable estimates of larval mass at later stages of the experiment, more plants had to be evaluated at later stages, due to larval mortality. The number of plants dissected during each evaluation increased from 33 to 60 per hybrid and population. At the conclusion of the experiment five plants were randomly selected from each hybrid and the presence or absence of the Bt-gene confirmed by means of lateral flow strip tests.

In a greenhouse experiment conducted during October

2006, 250 plants of each of the hybrids Phb33A14 (Bt-transgenic, Mon810) and its susceptible counterpart Phb33A13, were hydroponically grown at temperatures of 25°/18° (day / night) and natural daylight. Plants of each hybrid were divided into two groups of 125 plants each, spatially separated to avoid lateral movement of larvae between populations. Artificial infestation and evaluation were conducted as described above. Fourteen plants per hybrid and per insect population were dissected every two days for 18 days. Average larval mass per plant was recorded.

Data from the greenhouse and field trials were analysed separately, using pooled data over planting dates for the field trial. For purposes of illustration, means and standard errors of larval mass were plotted over time. The data were subjected to simple regression of the model $Y = a + bX$, using $\log_{10}$-transformation of the dependent variable. Slopes of the regression lines for each hybrid and population were pairwise compared using Student's t-tests for difference at $P = 0.05$ (Genstat for Windows, 2007).

Results

Field trial

Temporal mass gains by larvae in each stem borer population on the non-Bt hybrids are shown in Figure 1. The increase in mean larval mass was exponential in both populations, whereas differences between growth curves at each time interval appeared to be marginal. The respective tendencies pertaining to each population on the Bt-hybrids are shown in
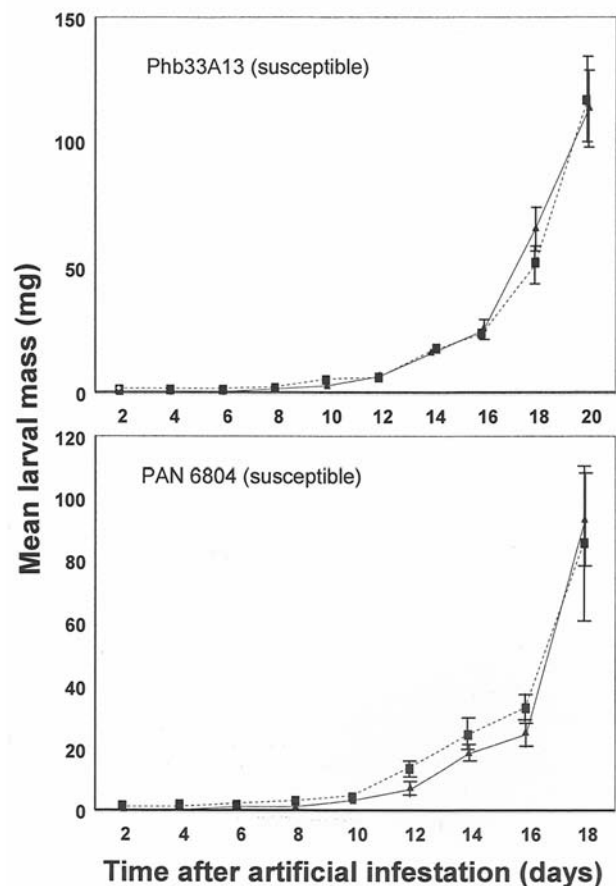
Figure 1 Larval mass gain at two-day intervals of two *B. fusca* populations on two non-Bt hybrids (Field trial). (Solid line = non Bt-population; broken line = Bt-population).
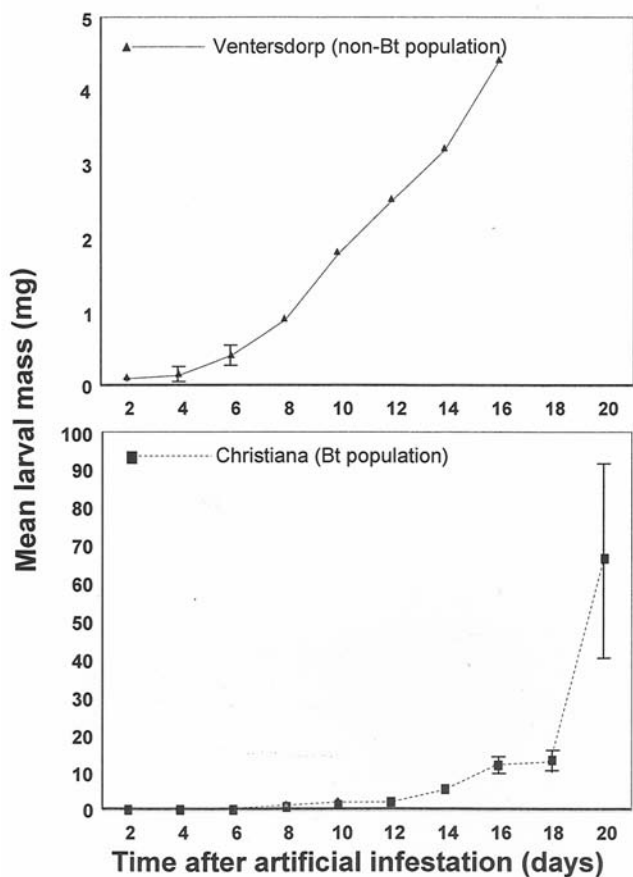
Figure 2 Larval mass gain at two-day intervals of two *B. fusca* populations on the Bt-hybrid Phb33A14 (Field trial).
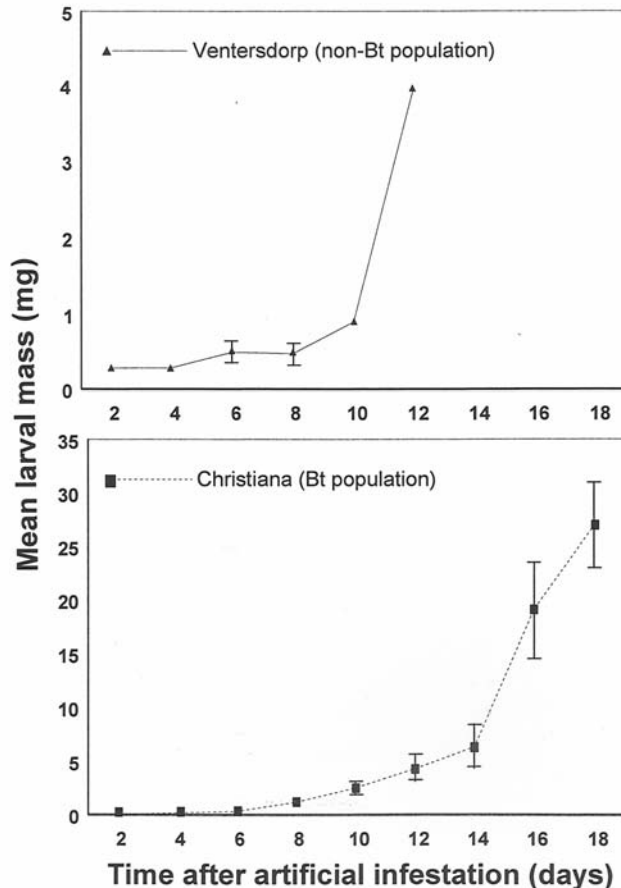
Figure 3 Larval mass gain at two-day intervals of two *B. fusca* populations on the Bt-hybrid PAN 6012 (Field trial).

Figure 2 (Phb33A14) and Figure 3 (PAN 6012). Note the difference in Y-axis scale for the two populations. On Phb33A14, appreciable numbers of larvae of the non-Bt population (Ventersdorp) survived only to day eight. Single surviving larvae were found until day 16 and had attained a mass of 4.4 mg. In contrast, substantial numbers of larvae of the Bt population (Christiana) survived over the entire trial period, attaining a mean mass of 66.2 mg on the 20th day. Similar results were obtained with PAN 6012 (Figure 3). The bulk of the non-Bt population survived only until day eight with single surviving larvae being found until day 12, with a final mass of 3.95 mg. The Bt population survived over the full trial period, reaching a mean larval mass of 27.2 mg on the 18th day (larvae in the fourth instar). At the conclusion of the experiment the mean larval mass of the Bt population appeared to be less on the Bt-hybrids than on their susceptible counterparts (49.3 and 85.0 mg respectively).

Results from regression analyses are provided in Table 1. Comparison of the slopes of the regression lines are provided in Table 2. Only the meaningful comparisons and not the full

Table 1 Regression analyses of larval mass (mg, Log_{10}-transformed) of two *B. fusca* populations on time (days after infestation) using two Bt-transgenic hybrids and their susceptible counterparts (Field trial 2006)

| Population	Hybrid	Intercept	Slope	F	P	R
Non-Bt	Phb33A13	-1.1224	0.1612	442.76	<0.0001	0.992
(Ventersdorp)	Phb33A14	-1.1325	0.1192	40.23	0.0007	0.933
	PAN 6084	-1.0046	0.1573	420.70	<0.0001	0.992
	PAN 6012	-0.9162	0.1028	15.65	0.0167	0.745
Bt	Phb33A13	-1.3194	0.1712	423.79	<0.0001	0.992
(Christiana)	Phb33A14	-1.2785	0.1397	155.38	<0.0001	0.978
	PAN 6084	-1.0207	0.1657	556.31	<0.0001	0.994
	PAN 6012	-1.1594	0.1472	478.10	<0.0001	0.993

Table 2 Pair wise comparisons of slopes of regression lines (Field trial, 2006; F = 69.58; P < 0.001; R^2 = 93.9). Bt and non-Bt refer to insect populations

| Comparisons		F prob
Bt vs. non-Bt	Phb33A13	0.882
	PAN 6804	0.702
	Phb33A14	0.090
	PAN 6012	0.023
Phb33A13 vs. Phb33A14	Bt	<0.0001
	Non-Bt	0.009
PAN 6804 vs. PAN 6012	Bt	<0.0001
	Non-Bt	0.001

matrix is shown. The t-statistics confirmed that the two populations did not differ significantly on either of the non-Bt hybrids. On the Bt-hybrids the non-Bt population differed significantly from the Bt population on PAN 6012 but not on Phb33A14. Comparison of the slopes of each population on each Bt-hybrid and its susceptible standard differed significantly for all four combinations.

Greenhouse trial

Although based on fewer larvae than the field trial, the greenhouse experiment provided similar results. The two populations showed similar larval mass gains on the non-Bt hybrid (Figure 4) but differed in the response to the Bt-hybrid (Figure 5). A single surviving larva of the non-Bt population had attained a mass of 8.3 mg on day 14. The mean larval mass of the Bt population on the Bt-hybrid was 26.1 mg on day 18, compared to a mean mass of 79.9 mg on the non-Bt hybrid. Regression analyses are provided in Table 3. Pair-wise comparison of the slopes confirmed a significant difference between the two populations on the Bt-hybrid (t = 0.008), with no differences on the susceptible hybrid (t = 0.354) (F = 75.6; P <0.001; R^2 = 94.1).
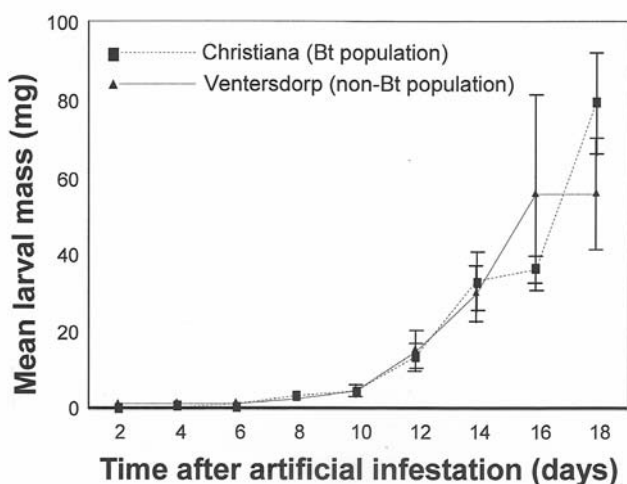
Figure 4 Larval mass gain at two-day intervals of two *B. fusca* populations on the non-Bt hybrid Phb33A13 (Greenhouse trial).
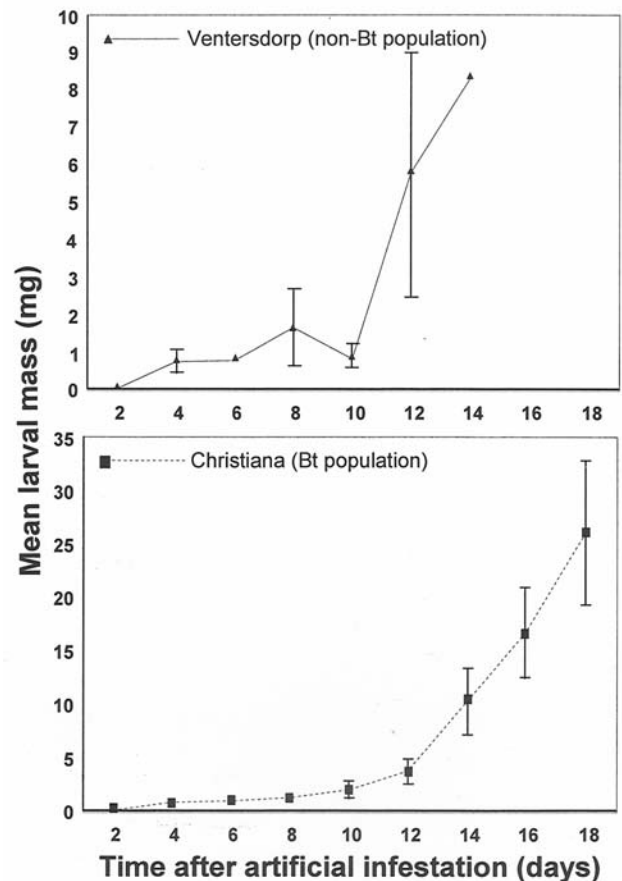
Figure 5 Larval mass gain at two-day intervals of two *B. fusca* populations on the Bt-hybrid Phb33A14 (Greenhouse trial).

Discussion

It appears that the Bt population has attained a level of resistance where some larvae are able to survive in the presence of the Bt-toxin but not without some detrimental effect on larval growth rate. The result is consistent with previous observations on *O. nubilalis* (Bourguet *et al.*, 2000), *P. gossypiella* (Liu *et al.*, 2001) and *Sesamia nonagroides* (Eizaguirre *et al.*, 2005), where a delay in larval development was observed with resistant individuals feeding on Bt-crops or on sub-lethal levels of the toxin. This observation has the added implication that the efficacy of the refuge strategy may be decreased, since moths derived from Bt-maize have less probability of mating with those from non-Bt fields, as was shown with *O. nubilalis* (Bourguet *et al.*, 2000).

The observed increase in the resistance level of the Bt population could be a result of selection pressure derived from continuous exposure of larvae of the second seasonal moth flight to sub-lethal levels of the toxin at late plant growth stages. Alternately, moths of *B. fusca* are sensitive to low humidity, as reflected in the tendency of population magnitude to vary in accordance with the rainfall cycle (Van Rensburg *et al.*, 1987). For practical reasons some maize producers are reluctant to include refugia inside irrigated plantings and regard susceptible plantings provided under rain fed conditions in the immediate vicinity of irrigated plantings as refugia. Moths possibly give preference to irrigated maize, which could have contributed to increased selection pressure towards the development of resistance to the Bt-

Table 3 Regression analyses of larval mass (mg, Log_{10}-transformed) of two *B. fusca* populations on time (days after infestation) using a Bt-transgenic hybrid and its susceptible counterpart (Greenhouse trial 2006)

Population	Hybrid	Intercept	Slope	F	P	R
Non-Bt	Phb33A13	-0.4693	0.1283	103.06	<0.0001	0.968
(Ventersdorp)	Phb33A14	-1.0464	0.1408	44.28	0.0012	0.948
Bt	Phb33A13	-0.9689	0.1674	179.21	<0.0001	0.981
(Christiana)	Phb33A14	-0.9647	0.1343	132.93	<0.0001	0.975

toxin.

The Bt population used in this study was identified as being resistant due to moderate leaf feeding damage, which was observed at relatively late vegetative growth stages. This was, however, a different site from that observed during 2004 in which more severe damage was noticed at earlier growth stages. It is therefore possible that other populations with higher levels of resistance may exist in the area. The extent of resistance throughout the maize production area needs further investigation, with emphasis on irrigation areas with a high adoption rate of Bt-maize, including those at Vaalharts, the Orange and Riet river irrigation schemes near Douglas, and irrigated plantings of the upper Vaaldam area.

A previous study indicated that laboratory colonies of *O. nubilalis* resistant to Cry1Ab also had decreased susceptibility to a number of other Bt-toxins, including Cry1Ac (Sigueira *et al.*, 2004). It is therefore important that future evaluations of resistance with *B. fusca* include hybrids transgenic with Bt11, the only other Bt-event currently available in South Africa. New Bt-events should also be subject to testing for efficacy of control of resistant populations.

A possible long term solution to the development of resistant populations could be to introgress more than one Bt-event into the same hybrids (gene stacking). In the short term, rotation of Bt-maize under irrigation with susceptible hybrids and a concomitant use of insecticides should be encouraged, while the inclusion of refugia within the same Bt-planting should be vigorously enforced.

Acknowledgements

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