TOXICITY OF COTTON VARIETIES EXPRESSING CRY PROTEINS TO DIFFERENT LARVAL INSTARS OF A BRAZILIAN *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE)

R. Azambuja
P. E. Degrande
E. P. de Souza
Universidade Federal da Grande Dourados – UFGD
Dourados, MS
D. R. Sosa-Gómez
Empresa Brasileira de Pesquisa Agropecuária - Embrapa Soja
P. R. Londrina
R. O. Santos
D. R. S. Santana
Universidade Federal da Grande Dourados – UFGD
Dourados, MS

Abstract

Bioassays using *H. armigera* caterpillars at different larval stages fed on plant structures of Bt cotton varieties were carried out to evaluate their effects on pest mortality, as well as possible sublethal toxicity on surviving individuals. The Bt varieties DP555 BGRR™ (MON531), FM975 WS ™ (MXB-13), DP1228 B2RF™ (MON88913 x MON15985), and FM940 GLT™ (GHB614 x T304-40 x GHB119) were effective in the control of *H. armigera*, reducing the survival of caterpillars; moreover, larval periods and pupal weights of surviving caterpillars fed the Bt varieties DP555 BGRR™ and FM975 WS™ were extended and reduced, respectively. The variety FM940 GLT™, which was introduced to Brazil in 2013 and expresses the insecticidal proteins Cry1Ab and Cry2Ae, induced the highest rates of caterpillar mortality in all six instars. Nowadays, combinations of at least two Bt proteins with different mechanisms of action were the most effective in controlling larval instars of *H. armigera* – Brazil and USA are expecting the commercial varieties with third generation of Bt cotton next season. The relatively high rates of survival of different instars fed on cotton varieties expressing Bt proteins that act on the same site suggests that these varieties are less effective at controlling caterpillars.

Introduction

Transgenic varieties of cotton, *Gossypium hirsutum* L., that contain genes of the bacterium *Bacillus thuringiensis* (Bt) Berliner expressing Cry proteins have been widely used since its first generation, reducing the need of insecticide sprays to control lepidopteran pests , preserving populations of beneficial arthropods (An et al., 2014; Poongothai et al., 2010). *Helicoverpa armigera* Hübner can cause great damage to vegetative and reproductive structures of cotton plants, and thus pest management strategies are needed to minimize yield losses. Cotton varieties containing Bt genes can be used as alternatives to chemical insecticides for the management of *H. armigera* populations. *H. armigera* was not included as a specific target pest for approved Bt cotton varieties in Brazil. In countries where *H. armigera* has been reported to occur, Bt cotton varieties, such as Bollgard™/Ingard™ and Bollgard II™, are known to be effective controlling this pest (Arshad et al., 2009; Knight et al., 2016). However, further studies on the effectiveness of Bt cotton varieties controlling *H. armigera* in Brazil are needed, given that the susceptibility of this pest species to Bt proteins may vary among different populations (Sebastião et al., 2015) and because of lack of data published regarding its control by the Bt cotton technologies commercialized in Brazil nowadays [WideStrike™ (WS) and TwinLink™ (TL)]. The objective of this study was to assess the effects of Bt cotton varieties cultivated in greenhouse conditions on larval instars of *H. armigera*, as well as to determine biological variables of immature stages of this Lepidoptera feeding on Bt and non-Bt cotton leaves and potential sublethal effects on surviving individuals.

Materials and Methods

Bioassays were carried out in the Laboratory of Applied Entomology, Federal University of Grande Dourados (UFGD), in the municipality of Dourados, Mato Grosso do Sul (MS), Brazil. A colony of *H. armigera* was established from caterpillars collected in soybean fields in São Gabriel do Oeste, MS, in November 2014, and identified using PCR-RFLP. Larvae were kept in 100-ml plastic containers lined with paper towels and fed non-Bt soybean leaves daily to avoid pre-imaginal conditioning. Caterpillars to be used in the bioassays were fed on leaves of the cotton variety FM982 GL™ (non-Bt, control) until the desired instar to be tested was reached. Bioassays of first, second, and third instar caterpillars were carried out using only leaves of the upper third portion of plants at 60 to 64 days after emergence (DAE). Fourth-instar caterpillars were fed on soft bolls at 79 DAE, whereas fifth- and sixth-instar caterpillars were fed on hard bolls at 115 DAE. Bioassays consisted of five

completely randomized treatments using four Bt cotton varieties (DP555B GRR™, FM975 WS™, DP1228 B2RF™, and FM940GLT™) and one non-Bt cultivar (FM982 GL™), with 40 replicates per treatment. Each experimental unit consisted of a plastic container (150 ml) lined at the bottom with a paper filter moistened with distilled water. The specific plant structure used in the test was placed on the paper filter, along with a caterpillar from the rearing colony. Plant structures were replaced every 2 days in order to maintain food quality. Caterpillar mortality was assessed every 24h for 7 days. Data were compared using the Kaplan-Meier test in conjunction with the Gehan-Breslow method. In bioassays involving first-instar caterpillars, duration (days) and larval-stage survival were also evaluated, as well as the duration of the pre-pupal stage, and weight and duration of the pupal stage. Because the data did not conform to assumptions of normality, the non-parametric Kruskal-Wallis test was applied, with significance level set to 5%.

Results and Discussion

Newly-hatched *H. armigera* caterpillars were highly susceptible to the Cry proteins expressed in the leaves of the four Bt cotton varieties, with mortality rates significantly higher than the non-Bt group (K-M = 85.565, df = 4, p = 0.001) (Fig. 1); the varieties DP1228 B2RF™ (Cry1Ac and Cry2Ab2) and FM940 GLT™ (Cry1Ab and Cry2Ae) presented the highest rates of caterpillar mortality (~100% within 7 days). For the variety FM975WS™ (Cry1Ac and Cry1F), caterpillar mortality was 52.5%, whereas for DP555 BGRR™ (Cry1Ac), mortality was only 17.5% among first-instar caterpillars, an indication that this variety may be ineffective at controlling *H. armigera* caterpillars.

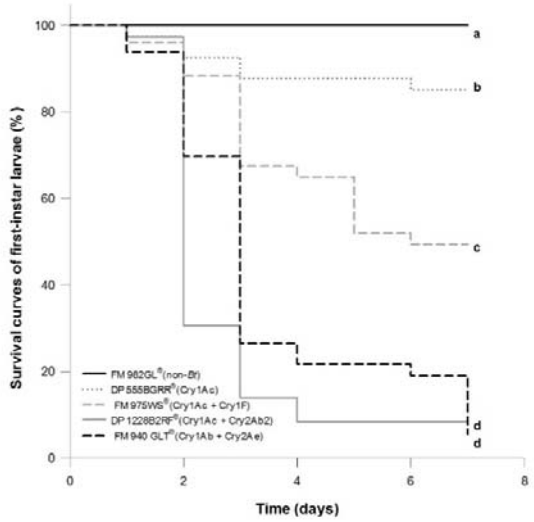

Fig. 1. Survival curves of first-instar *Helicoverpa armigera* caterpillars fed on Bt cotton leaves of the varieties DP555 BGRR™ (MON531), FM975 WS™ (MON88913 x MON15985), DP1228 B2RF™ (MON88913 x MON15985), FM940 GLT™ (GHB614 x T304-40 x GHB119), and the non-Bt variety FM982GL™ (GHB614).

Several lepidopteran species (Sivasupramaniam et al. 2008), including *H. armigera*, are known to be tolerant to Cry1Ac, and larval survival has also been observed (An et al., 2014; Gunning et al., 2005). In addition, the varieties DP1228 B2RF™ (Cry1Ac and Cry2Ab2) and FM940 GLT™ (Cry1Ab and Cry2Ae) express two Bt proteins with distinct binding sites in the midgut of lepidopterans, enhancing the control, and supporting the observed results. Thus, using plant varieties that produce combinations of Bt toxins should be considered in management plans aimed at increasing pest control efficacy, host spectrum, and hindering the development of resistance to Bt toxins (Brévault et al 2009; Roush, 1998).

In the evaluation of the sublethal effects on first-instar caterpillars, mortality rates of 100% were observed only for the Bt varieties DP1228 B2RF™ and FM940 GLT™, suggesting that the expression of two toxic proteins by the same plant can have additive effects and thus increase the pest-control efficiency (Sivasupramaniam, et al., 2008). Mortality rates of 65 and 85% were observed for the Bt varieties DP555 BGRR™ and FM975 WS™, respectively. Surviving caterpillars of *H. armigera* that fed on leaves of the varieties DP555 BGRR™ and FM975 WS™ had significantly longer larval periods and smaller pupae than those in the non-Bt-fed control group. Kranthi et al. (2005) reported that surviving caterpillars fed on different structures of Bt cotton had lower body

weights compared to those fed non-Bt cotton. Duration of the pre-pupal stage of surviving caterpillars fed the cotton variety FM975 WS™ differed significantly from that of the control group; however, pupal duration was not significantly affected by the Bt cultivars DP555 BGRR™ and FM975 WS™.

Survival rates of second-instar *H. armigera* caterpillars declined only in individuals fed on the varieties DP 1228 B2RF™ (Cry1Ac and Cry2Ab2) (39% survival) and FM940 GLT™ (Cry1Ab and Cry2Ae) (15% survival) compared to the non-Bt control group (K-M = 68.379, df = 4, $p = 0.001$) (Fig. 2). Sivasupramaniam et al. (2008), in an assessment of *H. zea* second-instar caterpillar mortality, also observed that caterpillars fed the cultivar Bollgard II™, had higher rates of mortality and lower larval weights than caterpillars fed Bt cotton varieties expressing only Cry1Ac; moreover, mortality of *H. zea* caterpillars was higher in cotton expressing the protein Cry1Ac than in the non-Bt control group. In our study, on the other hand, larval mortality of *H. armigera* caterpillars was lower when fed the varieties FM975 WS™ (Cry1Ac and Cry1F) and DP555 BGRR™ (Cry1Ac) and did not differ significantly from that of the non-Bt group.

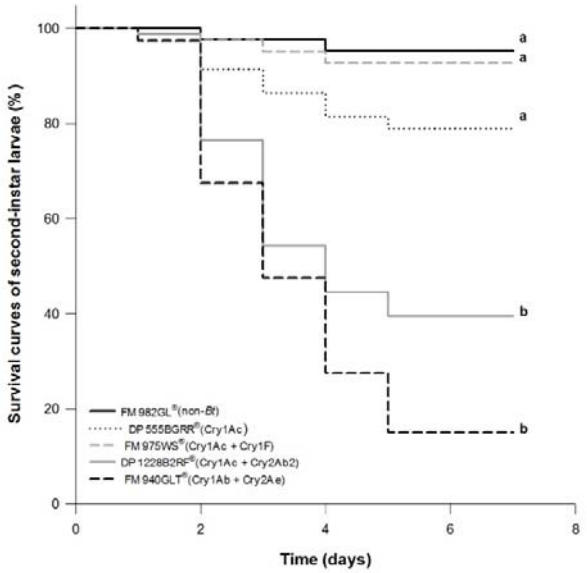

Fig. 2. Survival curves of second-instar *Helicoverpa armigera* caterpillars fed on Bt cotton leaves of the varieties DP555 BGRR™ (MON531), FM975 WS™ (MON88913 x MON15985), DP1228 B2RF™ (MON88913 x MON15985), FM940 GLT™ (GHB614 x T304-40 x GHB119), and the non-Bt variety FM982GL™ (GHB614).

Bioassays with third-instar caterpillars revealed that survival rates were lower for the varieties DP1228 B2RF™ and FM940 GLT™ (K-M = 35.285, df = 4, $p = 0.001$) (Fig. 3), whereas survival rates of caterpillars fed on the varieties FM975 WS™ and DP1228 B2RF™ were not significantly different. The highest rate of mortality observed for this instar was 57.5% for caterpillars fed the variety FM940 GLT™, with mortality rates lower than 50% observed for the remaining Bt varieties. Expression levels of the Bt proteins may have influenced the survival of caterpillars, given that the variability in the efficacy of Bt cotton against target insect pests is attributed mainly to changes in the expression of Bt proteins due to cultivar, stage, plant matrix, and environmental conditions (Poongothai et al., 2010; Knight et al., 2013; Kranthi et al., 2005).

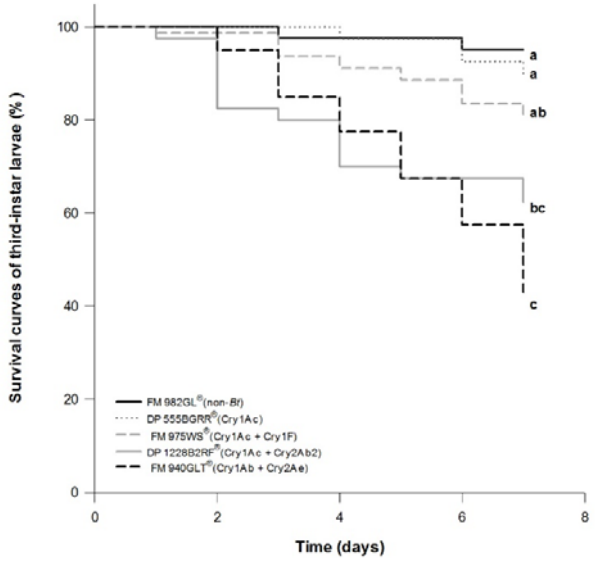

Fig. 3. Survival curves of third-instar *Helicoverpa armigera* caterpillars fed on Bt cotton leaves of the varieties DP555 BGRR™ (MON531), FM975 WS™ (MON88913 x MON15985), DP1228 B2RF™ (MON88913 x MON15985), FM940 GLT™ (GHB614 x T304-40 x GHB119), and the non-Bt variety FM982GL™ (GHB614).

In fourth-instar caterpillars fed on soft bolls, mortality rates differed significantly from that of the control group for caterpillars fed on the variety FM940 GLT™, for which the mortality rate was 57.5% (K-M = 60.230, df = 4, $p = 0.001$) (Fig. 4). Caterpillar survival was higher than 80% for the remaining varieties (i.e., mortality rates below 20%), demonstrating their low effectiveness in the control of fourth-instar caterpillars of *H. armigera* and indicating the need for field-control strategies specific to this larval instar.

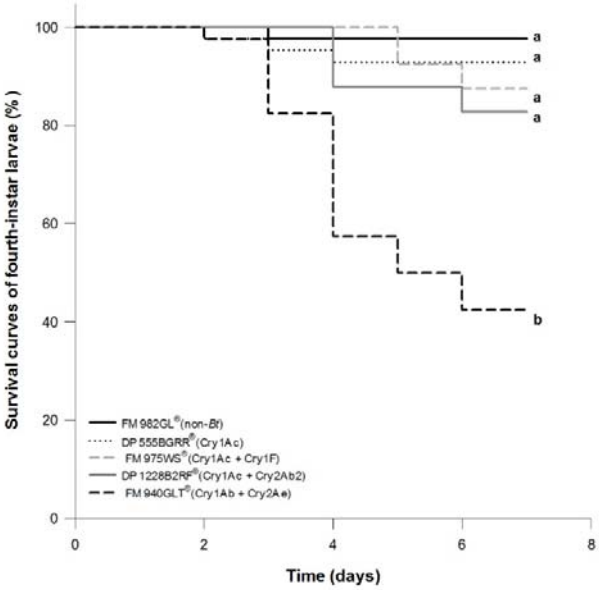

Fig. 4. Survival curves of fourth-instar *Helicoverpa armigera* caterpillars fed on Bt cotton soft bolls of the varieties DP555 BGRR™ (MON531), FM975 WS™ (MON88913 x MON15985), DP1228 B2RF™ (MON88913 x MON15985), FM940 GLT™ (GHB614 x T304-40 x GHB119), and the non-Bt variety FM982GL™ (GHB614).

The survival of fifth-instar caterpillars of *H. armigera* fed hard bolls was significantly affected by all of the Bt cotton varieties compared to the non-Bt group, with 100% or near 100% mortality in all treatments (K-M = 80.329, df = 4, $p = 0.001$) (Fig. 5).

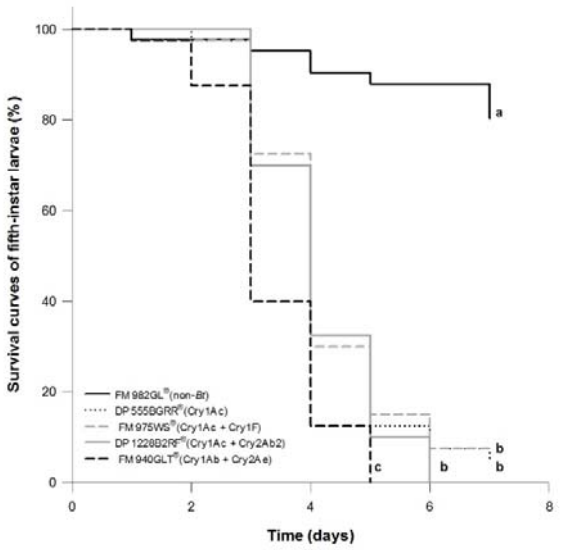

Fig. 5. Survival curves of fifth-instar *Helicoverpa armigera* caterpillars fed on Bt cotton hard bolls of the varieties DP555 BGRR™ (MON531), FM975 WS™ (MON88913 x MON15985), DP1228 B2RF™ (MON88913 x MON15985), FM940 GLT™ (GHB614 x T304-40 x GHB119), and the non-Bt variety FM982GL™ (GHB614).

Mortality rates were also high among sixth-instar caterpillars fed on hard bolls, ranging between 80% and 97% for all treatments, which were significantly from that of the non-Bt group (K-M = 51.520, df = 4, $p = 0.001$) (Fig. 6). Variations in Cry protein expression among different plant parts in different seasons is one factor that affected the survival of *H. armigera* larvae; in addition, concentrations of Cry2Ab may increase in large bolls toward the end of the growing season (Knight et al., 2013), and might be another contributing factor to the rise in mortality of caterpillars fed on hard bolls observed in our study.

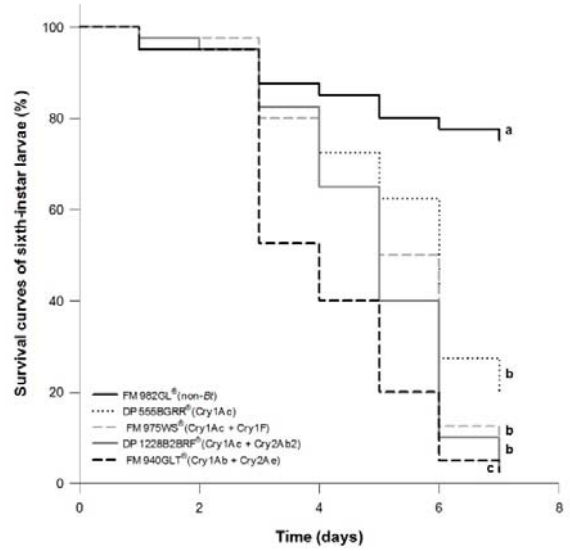

Fig. 6. Survival curves of sixth-instar *Helicoverpa armigera* caterpillars fed on Bt cotton hard bolls of the varieties DP555 BGRR™ (MON531), FM975 WS™ (MON88913 x MON15985), DP1228 B2RF™ (MON88913 x MON15985), FM940 GLT™ (GHB614 x T304-40 x GHB119), and the non-Bt variety FM982GL™ (GHB614).

Non-Bt factors may also have contributed to the increase in mortality of fifth- and sixth-instar *H. armigera* caterpillars fed on hard bolls of more developed cotton plants. According to Sivasupramaniam et al. (2008), *H.*

zea caterpillars fed on bolls of non-Bt cotton exhibited lower body weights and higher mortality rates than caterpillars fed leaves and other younger plant structures; the authors further suggested that non-Bt cotton potentially influenced tissue bioactivity toward the end of the plant cycle, due to the amount of plant tissue consumed and variations in the nutritional quality of different plant tissues.

The variety FM940 GLT™ mainly differed from the other Bt varieties on that since it promoted higher mortality of fifth and sixth instars over a shorter period of time. The Bt varieties DP555 BGRR™ (Cry1Ac), FM975 WS™ (Cry1Ac and Cry1F), DP1228 B2RF™ (Cry1Ac and Cry2Ab2), and FM 940 GLT™ (Cry1Ab and Cry2Ae) were effective controlling *H. armigera* by reducing caterpillar survival rates. Caterpillar mortality was higher in the varieties DP1228 B2RF™ (Cry1Ac and Cry2Ab2) and FM940 GLT™ (Cry1Ab and Cry2Ae), both of which produce two Bt proteins that act on distinct midgut binding sites. In addition, larval stage durations were extended and pupal weights reduced in surviving caterpillars fed the Bt varieties DP555 BGRR™ and FM975 WS™, resulting in decreased feeding by caterpillars. Fifth and sixth instars of *H. armigera* caterpillars fed on hard bolls were highly susceptible to the four Bt cotton varieties tested. Mortality rates in all six instars were highest for caterpillars fed the TwinLink™ cotton variety FM940 GLT™, which produces the proteins Cry1Ab and Cry2Ae. This variety started to be cultivated in Brazil in 2013, the same year that *H. armigera* was first reported to be present in the country.

Summary

This is a first public data on assessing the efficacy of different Bt cotton varieties on *H. armigera* in Brazil The cotton varieties that produced at least two Bt proteins that act at distinct binding sites were more effective in controlling larval instars of *H. armigera* (Bollgard II™ and TwinLink™ technologies). Furthermore, the maintenance of structured refuges that cover at least 20% of crop fields is needed, as well as alternative refuge areas and other host plants, in order to reduce the use of chemical insecticides and increase the density of natural enemies. Implementing such practices will reduce negative environmental impacts and enhance the sustainability of available Bt technologies.

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