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Relationships between Cry1Ac and Cry2Ab protein expression in field-grown Bollgard II® cotton and efficacy against *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae)

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ABSTRACT

Seven-day laboratory bioassays with first-instar *Helicoverpa armigera* and *Helicoverpa punctigera* larvae were conducted using leaves from field-grown Bollgard II® cotton during the 2009/10 season and larval mortality and development recorded. Leaves were from three farms in the St George region and two fields on one farm at Emerald in eastern Australia. The Cry1Ac and Cry2Ab content of leaves from the same samples was determined (see Knight et al., 2013) and the relationships between Cry protein content and larval mortality and development were examined. The Cry1Ac protein content was between 3.98 and 12.08 μg/g during the growing season, while Cry2Ab content ranged between 300.6 and 953.3 μg/g. Cry1Ac and Cry2Ab content of leaves were highly correlated ($r = 0.8276$, $P < 0.001$). Seven-day mortality of *H. punctigera* larvae was close to 100% throughout the season. *H. armigera* mortality was close to 100% early in the season, but fell to ~65% by mid-to late February in the laboratory bioassays. Fitting three-dimensional non-linear models associating Cry1Ac and Cry2Ab content with *H. armigera* and *H. punctigera* mortality elucidated the relative importance of the two proteins in determining larval mortality; for this analysis, data were pooled with data from an isoline study to provide better sampling of the three-dimensional surface being modelled. For both *Helicoverpa* species, the fitted mortality response to the Cry1Ac protein was close to its maximum at protein concentrations above ~3 μg/g Cry1Ac. For *H. punctigera*, response to the Cry2Ab protein was close to maximal once Cry2Ab was greater than ~200 μg/g. In contrast, the fitted *H. armigera* mortality response to Cry2Ab increased steadily with concentration up to ~1200 μg/g Cry2Ab. These responses led to markedly different response surfaces for the two species; *H. punctigera* mortality was close to 100% at most places on the response surface, while for *H. armigera* the response surface showed stronger increases in mortality with concentration for Cry2Ab than for Cry1Ac. These results can be interpreted as meaning that at the plant-expressed range of concentrations in Bollgard II cotton the two proteins are approximately equally important for *H. punctigera* but that changes in Cry2Ab content more strongly influences changes in larval mortality in *H. armigera* than does Cry1Ac, with Cry1Ac contributing a consistent 40–45% mortality for concentrations above 3 μg/g. For *H. armigera*, there was no evidence of either synergism or antagonism between Cry1Ac and Cry2Ab proteins ($P > 0.05$) but this aspect was not testable for *H. punctigera* because mortality was mostly close to 100%.

1. Introduction

Bollgard II® cotton, *Gossypium hirsutum*, expresses the Cry1Ac and Cry2Ab δ-endotoxins derived from *Bacillus thuringiensis* variety *kurstaki* (Bt) (Greenplate et al., 2003) and provides highly specific insecticidal activity against a wide range of caterpillar pests of cotton worldwide (Dong and Li, 2007; Fitt, 2003; Greenplate et al., 2003). Bollgard II cotton has the advantage over Bollgard (Ingard in Australia) of expressing two different Cry δ-endotoxins that bind to different larval midgut binding sites; plant efficacy is less likely to

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break down because of cross-resistance in pest species (Luo et al., 2007; Tabashnik et al., 2009), especially as the Domain II amino-acid sequence similarity in Bollgard II's Cry1Ac and Cry2Ab proteins is very low (Carrière et al., 2015). While some level of cross-resistance between the Cry1Ac and Cry2Ab proteins may have been present in one-third of the experiments evaluated in the meta-analysis of Brévault et al. (2013), all of the colonies of *Helicoverpa armigera* and *Helicoverpa punctigera* – the targets of the present study – that were considered by Brévault et al. (2013) (7 and 2 colonies, respectively) showed no evidence of Cry1Ac/Cry2Ab cross-resistance. Additionally, the commercial deployment of Bollgard II has allowed the management of a series of pests not effectively controlled by the Cry1Ac protein alone in Bollgard/Ingard, e.g. *H. armigera*, *Spodoptera* spp., *Pseudoplusia includens* (Adamczyk et al., 2008; Downes and Mahon, 2012; Stewart et al., 2001).

Many studies have demonstrated reductions in field populations of multiple pest species, but without concomitant data on the content of the Cry proteins in the plants, e.g. Chitkowski et al. (2003) and Jackson et al. (2004), while others present data on both season-long field efficacy and seasonal expression profiles of Cry proteins, e.g. Adamczyk et al. (2001a). Additionally, high mortality has been commonly demonstrated in laboratory bioassays using field-grown Bollgard II plant material, but again without concomitant data on Cry protein content (Chitkowski et al., 2003; Llewellyn et al., 2007; Mahon and Olsen, 2009; Stewart et al., 2001). However, Adamczyk et al. (2001b) correlated the Cry1Ac content of two Bollgard varieties with the larval survival of *Helicoverpa zea* and larval development of *Spodoptera frugiperda* throughout the growing season, with the variety with the higher Cry1Ac content producing the highest mortality. Kranthi et al. (2005) used eight Bollgard cultivars to correlate *H. armigera* survival with Cry1Ac content through the season, concluding that survival levels increased where Cry1Ac toxin content fell below 1.8 μg/g Fitt et al. (1998) found a consistent, and sometimes dramatic, decline in bioassay mortality as the growing season progressed on Ingard cotton and a clear relationship between mortality and Cry1Ac protein content as a percentage of total protein content. While these authors fitted a simple linear regression to the data, arguably a sigmoidal dose–response function would have fitted better and made more biological sense given that the mortality response to these toxicants takes a non-linear form (Finney, 1978).

There are few equivalent data for Bollgard II cotton that have concomitant insect-bioassay data and Cry1Ac and Cry2Ab protein content but Greenplate et al. (2003) did demonstrate that the effects of the two proteins on larval mortality were additive for *Helicoverpa virescens* and *H. zea* and that Cry2Ab was the main contributor to total insecticidal activity. They evaluated synergism/antagonism effects on mortality using the formula of Salma et al. (1984) (from Finney (1964)), using seasonal-average mortalities, but did not directly quantify the joint relationship between Cry1Ac and Cry2Ab protein levels and mortality. Consequently, the equation of Salma et al. (1984), as used by Greenplate et al. (2003), is not relevant to the data analysed here because it is based on combining mortalities from the Cry1Ac and Cry2Ab proteins rather than quantifying the combined relationship between the concentrations of the two Cry proteins and the resultant larval mortality. There are data for *H. armigera* but they are based on artificial-diet studies (Ibargutxi et al., 2008; Wei et al., 2015). Ibargutxi et al. (2008) reported synergism between the two Cry proteins that are in Bollgard II at Cry1Ac: Cry2Ab ratios of 1:1 and 1:4. Wei et al. (2015) tested a series of combinations of Cry1Ac and Cry2Ab each up to 4.76 μg/cm^2 with a susceptible *H. armigera* strain. They found evidence of antagonism and synergism in four and two of 36 combinations, respectively, with all Cry1Ac concentrations showing either antagonism or synergism being 0.18 μg/cm^2, or lower. Thirty of the 36 combinations showed neither antagonism nor synergism. The combinations showing synergism tended to be combinations where the Cry2Ab concentration was ~3 times lower than for Cry1Ac, while the antagonism examples tended to occur where Cry2Ab concentration was 27–238 times higher than the Cry1Ac concentration. Both these studies were conducted on artificial diet and spanned Cry1Ac: Cry2Ab ratios substantially different from those known to occur in Bollgard II plants in the field (Knight et al., 2013).

We report here data for *H. armigera* and *H. punctigera* using field-grown leaf material from multiple sites in eastern Australia with Cry-protein content measured on the same cohorts of leaves as was used for the laboratory bioassays. These data extend the season-long expression profiles reported by Knight et al. (2013) by providing direct estimates of efficacy associated with the Cry protein content, as well as data on the relative importance of the Cry1Ac and Cry2Ab proteins in producing *H. armigera* and *H. punctigera* mortality in Bollgard II cotton. As such, these data provide the first estimates of the conjoint action of the Cry1Ac and Cry2Ab proteins in Bollgard II plant tissue against *H. armigera* and *H. punctigera* and provide understanding of the different responses of the two *Helicoverpa* species to these two Cry proteins.

2. Materials and methods

2.1. Study overview

The laboratory bioassay experiments were conducted at the Monsanto Research Facility in Toowoomba, Australia during the 2009/10 cotton growing season using leaf material collected in two cotton-growing areas of Queensland, Australia; St George and Emerald from Bollgard II cultivar 71BRF. At St George, data were collected from three farms, while at Emerald samples were taken from two fields on one farm; Field 1 had a history of larvae being present while Field 2 did not. The Emerald and St George fields were planted in the week commencing 5 October 2009. Collections of first unfurled leaves were taken fortnightly from early squaring (1 square per row-metre), weekly through the flowering period and fortnightly again until first defoliation. At each sampling date, 20 first unfurled leaves were collected within 2 rows on either side of the sampling point for the field position i.e. head ditch, middle of the field or the tail drain. Once collected, the tissue was placed into pre-labelled zip lock plastic bags and placed directly into a cooler with an ice brick. For the Emerald experiment, the tissue was sent to the Monsanto Research Facility in Toowoomba, Queensland, Australia by overnight courier. For the St George trials, the material was transported to the Monsanto Research facility in a car fridge with a temperature of approximately 5 °C. Leaf material from the same collections was frozen for Cry-protein quantification, as described by Knight et al. (2013). At all field sites, fixed points for sampling throughout the season were established at the first sampling date, referred to as field positions in this paper. These field positions were at least 30 m from the head ditch and the tail drain, and in the middle of the field. Laboratory bioassays were conducted with leaves from St George on each of 10 sampling dates, while bioassays were conducted with leaves from Emerald on four dates during the season (Fig. 1).

The laboratory bioassays were conducted at 25 °C, with a 14:10 (L:D) photoperiod using 32-well larval-rearing trays with 5-mL of 2% agar and 0.1% sorbic acid per well. Field-collected leaves were washed in 10% bleach solution and allowed to dry before an entire leaf was placed in a well. Ten leaves were set up per field position per sampling date using 30 of the 32 wells. One neonate of either