The value of foliar applications of BT for the management of *Helicoverpa spps* (Lepidoptera: Noctuidae) in transgenic cotton varieties.

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Abstract:
Field trial examining the use of *Bacillus thuringiensis* (Bt) foliar sprays on transgenic and conventional cotton varieties showed various levels of *Helicoverpa* control and impacts on beneficial insects. Quantification of Bt protein concentration at various growth stages of transgenic cotton varieties and bioassay with field strain of *H. armigera* revealed great variation of concentration and efficacy.

Introduction:
The introduction of transgenic cotton containing *Bacillus thuringiensis* (Bt) insecticidal proteins offers the possibility to substantially reduce the number of insecticide application for *Helicoverpa* control. However, experience in Australia and in the USA has shown that efficacy of varieties expressing the CryIAc protein is not consistent throughout the growing season and highly variable (Fitt el al,1998 ;Adamczyk el al, 2001). Bt protein expression can be varied from variety to variety and from one plant structure to another (Greenplate, 1991; Gore el al, 2001) and affected by various agronomic and geographical factors. Evaluation of variety which best suits a specific graphical regime and growing conditions would be required for the success of the deployment of such variety. Recently available commercialised kit for quantification of Bt endotoxin would be useful tool for the cotton industry to optimise *Helicoverpa* management.

Insect resistance management guideline has been derived based on the high–dose strategy for *Helicoverpa* control. Any deviation from the effective dose for significant control would lead to resistance in intrinsically tolerant insect such as the cotton bollworm, *Helicoverpa armigera.* The use of non–Bt refuge forms an important component of the Insect Resistance Management Strategy (IRMS) which bases on the temporal mating between moth populations for the refuge and Bt cotton to delay resistance development. (Lieu et al, 1999).Therefore, if current Bt varieties express different levels of CryIAc endotoxin then various reproductive isolates of intrinsically tolerant populations might occur. The occurrence of many isolated populations renders the input of a predictive model oversimplified and therefore undermining the prediction value of the model and in turn adversely affecting the outcome of IRMS which bases on the prediction of the model .

Although few studies have shown that tolerant Lepidopteran pests (ie. bollworms and armyworms) have a large range of susceptibility to Bt endotoxin (Adamczyck et al, 1998; Luttrell et al, 1999), few has tried to relate the efficacy in a commercial crop to the Bt toxin content as well as closely examining the progress of Bt expression throughout the growing season.

The current paper reports the concentration of Bt endotoxin CryIAc in plant parts at different crop ages and the efficacy expressed on young larvae of the cotton bollworm throughout the growing season. The report also addresses the effect of the Bt protein–expression on field insect populations with and without the additional bio–pesticide application.

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Material and methods:

1. **Field trial and foliar application of Bt products (1999–2000 season).**

   i. **Field trial:**
   Conventional cotton variety V2 and transgenic Ingard variety V2I were planted in 8 rows x 20 m plots and replicated four times in a 1.2 ha block at the Australian Cotton Research Institute, Narrabri, NSW. V2 and V2I varieties were planted side by side, tall sorghum was grown in between plots with different treatments to avoid drift. Bt foliar formulation, Costar® (Abbott laboratories) and MVP® (Cyanamid) and Nuclear Polyhydrosis Virus product (NPV-Gemstar®) were applied weekly starting four weeks after planting using the ground rig. Costar® and MVP® were applied at 500 ml/ha in 100 litre of water per ha. Bt proteins components of the Bt formulations are presented in Table 1. Number of *Helivcoverpa spps* larvae and beneficial insects per metre was monitored weekly by visual count.

   ii. Laboratory bioassay:
   The first newly opened leaf was collected from the field and placed with one day old fed neonate in a 50 mm Falcon® petridish. Leaves were collected from random plants in replicated plots, fifty leaves from each treatment. Insect from susceptible strain LB was used for bioassay. The bioassay was conducted at 25°c and 70 % RH. Mortality and development was assessed at 5 days after introduction. The bioassay was carried out weekly starting from 35 days after planting to 133 days (with two weeks interval between 112 and 126 days). Larval development index (LDI) was calculated from larval survival and development according to a scale of 0, 1, 2, 2.5 and 3 was given to larvae that was dead (0), first instar (1), early 2nd instar (2), late 2nd instar (2.5) and 3rd instar (3) ,respectively. Due to the lack of separated facility for bioassay of Gemstar®, only insect bioassay for Bt products was carried out. (Without separated facility, virus disease from Gemstar® sprayed leaves could cause destruction of many important laboratory insect colonies).

2. **Quantification of Bt proteins concentration in plant structures and bioassay (2000–2001 season).**

   i. Experimental field trial and laboratory bioassay:
   Field trial was planted in Oakville, Narrabri in October, 2000. Conventional, and one-gene transgenic cotton varieties were planted in randomised plots (4 rows x 20 m for each variety) and replicated four times. The whole trial was located in area that was unlikely affected by chemical drift from commercial production.

   From each plot, ten terminal, newly opened leaves were collected for bioassay and Bt protein analysis. For quantification of Bt concentration analysis, 3mm leaf dishes were sampled from ten terminal leaves for each replicate and the rest of the leaves used for bioassay.

   H. armigera strain collected from cotton growing regions in southern Queensland in early October, 2000 was used for bioassay. Terminal leaves

sampled from each plot were used for bioassay using one day old, fed neonate in a Falcon® petridish. Mortality at five days after exposure was criterion to determine the efficacy. Among the four one-gene transgenic varieties, Nucot 37 was the best variety, hence it was chosen for intensive testing.

Quantitative analysis of Bt proteins from plant parts:

Envirologix® Kit (Envirologix Inc, Portland, USA) was used for analysis of CryIAc in transgenic cotton variety. This "Sandwich" Enzyme Linked Immunosorbent Assay (ELISA) utilises colour development steps where colour production is proportional to the protein concentration in the sample. Quantification of endotoxin is determined spectrophoto-metrically (Benchmark®, Bio–Rad, Hercules, CA).
Plant parts are collected from ten plants for each variety and each replicate. Terminal leaves and top squares (flower buds) are sampled from the top one third of the plant, middle leaves and middle squares are from the middle part of the plant (lower than the 3rd node) and lower leaves and lower squares from the lowest one third of the plant. A sample of square is consisted of ten thin cross sections from ten squares collected from ten plants in a replicate. Squares of 1cm in diameter or smaller are used for samples. Fresh weights of all plant structures were collected and the resulting concentrations of CryIAc are expressed in part per million of fresh weight.

Results and discussions:

1. **Effect of foliar spray on transgenic and conventional cotton varieties (1999/2000 season):**
   Tables 2 and 3 show the mortality and larval development index (LDI) of susceptible strain *H. armigera* (LB strain) as bioassayed at different crop growth stages.

   With regards to mortality, the effect of foliar application of Costar® and MVP® on conventional variety V2 is significant at pre–flowering stage (33 DAS). However, no effect was found on Ingard cotton at the same stage. In conventional crop, at flowering stage (77 DAS) the difference in insect mortality between untreated control and the treated plot was less as compared to earlier stage (33 DAS). This reduced mortality in treated plot may be due to less coverage as the canopy at flowering is thicker, but also may be due to higher rate of breakdown of Bt protein during December/January due to UV effect . As the crop grow older (at 105 and 133 DAS), the mortality of the untreated plots increased as the plant nutrients of older crop is less suitable for insect survival. At maturing stage 133 (DAS) there was no significant difference between treated and untreated plot indicating that mortality primarily due to unsuitable plant material as the crop grows old.
   In transgenic cotton, there was no significant difference in insect mortality between untreated and treated plot. The result indicated that the top-up application at pre–flowering stage using Bt products in Ingard cotton is not effective. Moreover unnecessary selection pressure is imposed further for CryIAc.

Apart from efficiacy, results on larval development index (LDI) helps to recognise the differences in level of efficacy between Bt formulations. For example, in using mortality as a criterion to determine the differences between untreated control and Costar® or MVPII® at pre–flowering stage for V2I (33 DAS, Table 2), there was very minimal difference revealed. However, as LDI was used the significant difference was readily recognised (33 DAS, Table 3). This LDI would be considered as effective parameter for differentiation of efficacy from various formulations. Figures 1 and 2 present the mortality of first instar larvae of susceptible laboratory strain (LB) in various treatments of conventional and Ingard cotton varieties.

In conventional cotton, Costar® foliar application produced better control than MVP® except at flowering stage. Mortality on untreated control increased significantly as the crop aged (27 and 25% mortality at 126 and 133 DAS, respectively – Fig 1). In Ingard cotton, control level decreased after post–flowering stage (100 days) indicating that major component of the efficacy is from the transgenic Bt protein, rather than from the top-up effect of the foliar application of Bt formulations. Although there was no significant difference among the treatments in Ingard cotton, Costar® foliar spray shown better control than MVP. As the crop age, at 133 DAS there was still supplementary effect of the low CryIAc concentration that caused additional mortality in Ingard cotton as compared to conventional cotton (as V2 control compared to V2I control, Figures 1 and 2).

Table 4 shows the number of *Helicoverpa* larvae from field count. There was no insect infestation at 33 DAS. At 56 DAS (early squaring stage), there was significant difference between insect count of treated and untreated plots. The differences were more profound in conventional than in Ingard cotton. The differences became less as the crop grew older indicating that the efficacy of Bt foliar application was higher at earlier growth stage than later in the growing season.
Table 5 shows the number of beneficial insects counted at different growth stages and different biopesticide treatments. In general, there was no difference between untreated and treated plots and between conventional and Ingard cotton. The presence of beneficial insects is highly related to the number of *Helicoverpa* larvae. As the crop matured, the number of beneficial insects reduced. At 133 DAS, although there were still medium and large *Helicoverpa* larvae present, no beneficial insects was found.

2. Bt protein expression in various plant structures and at different growth stages:

Table 6 shows the concentration of CryIAC in terminal leaf (in ppm) of Nucot 37 at different growth stages. The concentration of CryIAc significantly reduced at post–flowering and first opened-boll stage.

Greenplate et al (1998) reported the similar trend of CryIAc concentration in the fruiting structures, however, in the fruiting structures the change was more profound (1–3 ug/g and 10–15 ug/g at 120 and 40 days after planting, respectively). This trial was affected by the flood in November, 2000 although

the field was not completely submerged, part of the field was affected by drainage from other fields. The early expression at pre–squaring (35 days after seeding – DAS) and squaring stage (56 DAS) should be higher if the crop was not flooded (Table 6). The results indicated that CryIAc delta–endotoxin level decreases as the crop aged (Adamczyk, 2001, Fitt, 1998, Sads et al, 1998, Greenplate et al, 2000).

Table 7 shows the concentration of the proteins in different plant parts analysed at flowering stage (77 DAS). The concentration of CryIAc was highest in terminal leaf and lower in square and bract. In general, either in leaf or flowering structure, the younger tissue contains higher CryIAc protein than older tissue. Among the top part of the canopy, terminal leaf contains three fold higher concentration of CryIAc than the square.

Our preliminary investigation revealed that young larvae of *H. armigera* forage for suitable plant structure before settling in sustained feeding activity and during the process they were able to feed on tissues with lower concentration of Bt protein. Thus, as squares contains lower concentration of CryIAc than leaf, the ultimate feeding site for the larvae would be inside the square where protection from natural enemies and chemicals is much enhanced.

Table 8 shows the results of the leaf bioassay using first instar larval in Falcon® petridish at various stages of the crop cycle. Due to the fact that the insect strains used in this study was a field collected strain which has been selected for CryIAc resistance under field condition, the larval mortality on Nucot 37 observed in this trial was much lower than that observed on V2I in previous season (Table 2 – V2I untreated control – UTC). The mortality of neonate was very low started at 70 days after seeding (DAS).

Summary:

The results obtained in these trials and in other reports (Adamczyk et al, 2001, Greenplate et al, 2000, Fitt, 1998) indicated that there are significant spatio–temporal variations in concentrations of CryIAc in transgenic cotton varieties. With regards to larval feeding behaviour, the lower concentration of CryIAc expressed by fruiting and flowering structures causes feeding preference on or inside these structures. Thus the larvae are well protected from natural enemies and from supplementary control with synthetic insecticides. However, the implication of the modified feeding behaviour is more profounded as most of the new chemistry insecticides for *Helicoverpa* control in cotton have common mode of action which requires high efficacy through food ingestion. The spatio–temporal variation of CryIAc concentration in Ingard cotton would have implication not only directly on survivorship of larvae, but also on the selection for resistance as the high dose strategy initially applied for the technology does not hold true.

Field trials with supplementary application of foliar Bt formulation indicated that the top-up option was not effective in enhancing the performance of transgenic cotton. Moreover, in term of effective resistance management, products having other modes of action should be used.

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Table 1: Products tested against *Helicoverpa spps* as foliar application.

Components	Genes Present	Product	
		Costar®	MVPII®
Genes	CryIAa	+	–
	CryIAb	+	–
	CryIAc	+	+
	Cry2Aa	+	–
	Cry2Ab	+	–
Spore		+	–

Table 2: Mortality of first instar *H. armigera* at 5 days after exposure to cotton leaf with different bio–pesticide application (1999–2000 Season).

Treatment	Days After Seeding				
	33	77	105	133	Average
Conventional					
V2+Costar®	86ab[1]	56c	62a	61a	66
V2+MVPII®	76b	58b	48b	47ab	57
V2 UTC	4c	9d	15c	25b	13
Ingard					
V2I+Costar®	96a	92a	57ab	60a	76
V2I+MVPII®	92a	84a	55ab	51ab	70
V2I UTC	92a	79ab	47b	40ab	64

1/ Means of four replicates, each composed of 50 insects. Means in a column followed by common letter one not significantly different at 5% level (New Duncan Multiple Range Test - NDMRT).

Table 3: Larval Development Index of *H. armigera* larvae at 5 days after exposure to cotton leaf with different bio–pesticide application (1999-2000 season).

Treatment	Days After Seeding				
	33	77	105	133	Average
Conventional					
V2+Costar®	.12cd[1]	1.82a	1.93a	.10b	.99
V2+MVPII®	.24c	1.95a	1.27b	.23ab	.92
V2 UTC	2.2a	1.86a	1.80a	.28a	1.53
Ingard					
V2I+Costar®	.02d	1.37ab	1.70a	.05b	.78
V2I+MVPII®	.25c	.89c	1.28b	.26a	.67
V2I UTC	1.04b	1.12b	1.61ab	.18ab	.99

1/ Means of four replicates, means in a column followed by common letter are not significantly different at 5% level (NDMRT)

Table 4: Number of *Helicoverpa spps* larvae per metre at different crop growth stages(1999-2000 season).

Treatment	Days After Seeding				
	56	77	105	119	133
Conventional					
V2+Gemstar®	1.00b[1]	8.00a	4.70ab	6.30a	8.40a
V2+Costar®	0.00c	2.50ab	4.50ab	4.30b	5.80ab
V2+MVPII®	1.00b	2.50ab	5.00ab	7.00a	7.00a
V2 UTC	3.30a	3.00a	5.30ab	3.80ab	7.00a
Ingard					
V2I+Gemstar®	1.00b	2.00a	3.30b	6.00ab	3.60b
V2I+Costar®	1.00b	2.00b	4.80ab	3.00c	3.50b
V2I+MVPII®	1.00b	2.00b	6.30a	4.50b	5.00ab
V2I UTC	2.50a	3.50a	4.30b	5.80ab	4.00b

1/ Means of four replicates, means in a column followed by common letter are not significantly different at 5% level (NDMRT)

Table 5: Numbers of beneficial insects per metre at different crop growth stages(1999-2000 season)..

Treatment	Days After Seeding			
	56	77	105	119
Conventional				
V2+Gemstar®	4.8ab[1]	4.3b	6.0a	1.7cd
V2+Costar®	5.3ab	6.0ab	7.8a	2.0bcd
V2+MVPII®	4.0b	4.5b	5.8a	3.0ab
V2 UTC	5.0ab	5.0ab	6.0a	2.5abc
Ingard				
V2I+Gemstar®	5.0ab	6.8a	6.0a	2.5abc
V2I+Costar®	4.3b	5.3ab	5.3a	2.8abc
V2I+MVPII®	6.0a	5.3ab	7.3a	3.5a
V2I UTC	4.0b	7.0a	7.0a	1.8cd

1/ Means of four replicates, means in a column followed by common letter are not significantly different at 5% level (NDMRT)

Table 6: Concentration of CryIAc (ppm) in terminal leaves of Nucot 37 and Delta 50 BX at different growth stages. (2000/01 season)[1]

Growth Stage (DAS)[2]	Nucot 37 (CryIAc)
Pre–squaring (35)	3.42ab[3]
Squaring (56)	3.29abc
Flowering (77)	3.53a
Post–flowering (102)	2.98cd
First opened boll (133)	2.06e

1/ Concentration in part per million, analysed with Envirologix Kit.
2/ DAS = Days after seeding
3/ Mean of four replicates, means in a column followed by common letter are not significant different at 5% level (Turkey test)

Table 7: Concentration of CryIAc (ppm) in different parts of cotton plant at flowering stage (77 DAS) (2000-01 season).[1]

Plant Part	Nucot 37 (CryIAc)
Terminal Leaf	3.53a[2]
Middle leaf	3.30a
Lower leaf	1.87ab
Top square	1.34ab
Middle square	.83ab
Top bract	1.82ab
Middle bract	1.33b

1/ ppm = part per million based on fresh weight, DAS = day after seeding .
2/ Means of four replicates , means in a column followed by common letter are not significantly different at 5% level (Turkey test).

Table 8: Percent mortality of *H. armigera* larvae at 5 days after exposure to leaves of transgenic cotton. (2000–00 season).

Variety	Days After Seeding				
	35	56	70	102	133
Nucot 37	25.0b[1]	46b	10b	9.5b	22b

1/ Means of four replicates , means in a column followed by common letter are not significantly different at 5% level (Turkey test).

Fig.1: Percent mortality of *H. armigera* in conventional cotton with biopesticides (1999/00 season).

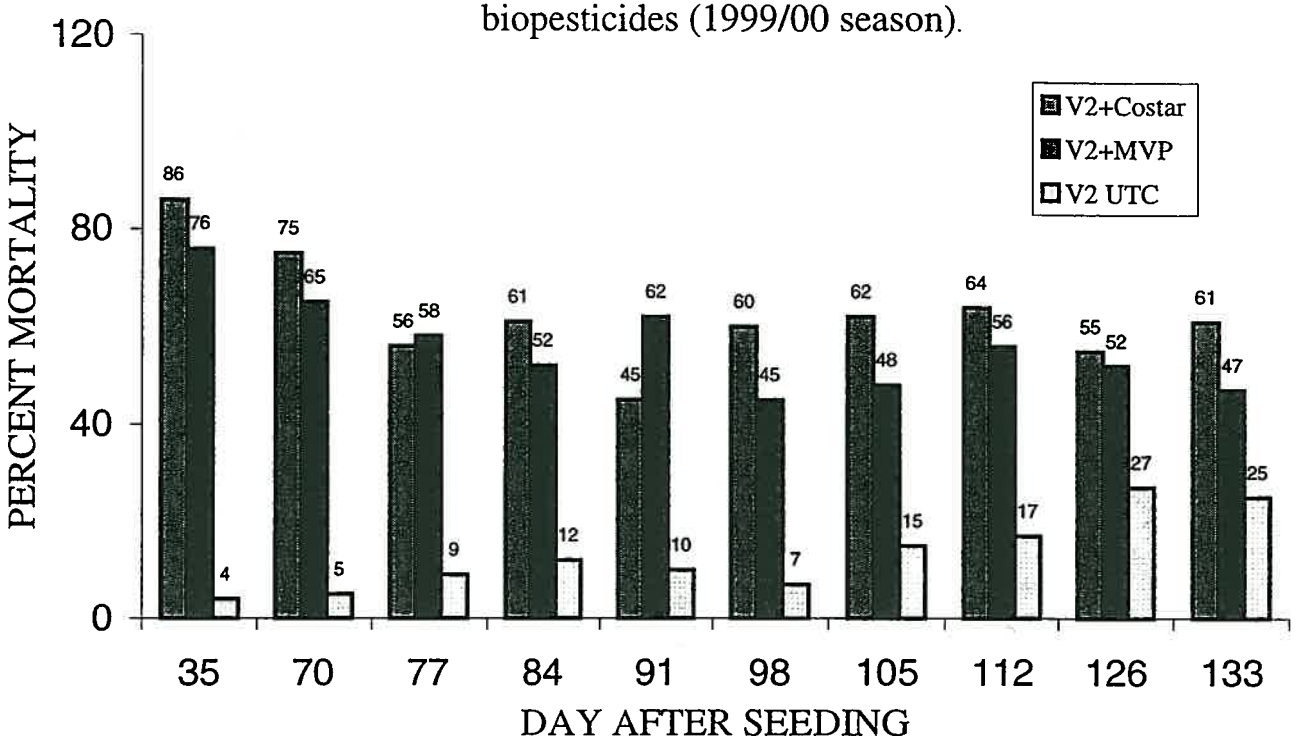

Fig.2 : Percent mortality of *H. armigera* in Ingard cotton with biopesticides(1999/00 season) .