

Cotton Research and Development Corporation

FINAL REPORT

“Monitoring efficacy and quantification of *Bacillus thuringiensis* (Bt) toxins in transgenic cotton varieties.”

CRDC123C

October 2000 to June 2001

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Australian Cotton Research Institute
Narrabri (02-6799 1556)

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January, August & Final Reports

REPORTS

Part 1 - Summary Details

Please use your TAB key to complete part 1 & 2.

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Final Reports

Ensure you append the Cover Sheet to this report, and forward 3 copies to Cotton Research & Development Corporation.

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What was the **background** of the project?

The focus of much of the research in the Australian Cotton Industry is to reduce the industry's dependence on synthetic insecticides. There has therefore been a strong push by the industry towards the successful deployment of transgenic cotton crops and the implementation and adoption of a true Integrated Pest Management (IPM) program. The use of transgenic cotton variety has become the key element in future IPM strategies for the industry.

In the last three seasons, it is clear that the expression of the Bt protein (CryIAC), is not consistent throughout the season (Fit, 1998). Research has shown that the expression of the Bt protein begins to decline early in the crop growth cycle. Environmental conditions that effect normal crop development can also further reduce the expression of the Bt protein. As a result significant survival of *Heliothis* can be observed in post flowering transgenic cotton crops. This reduced efficacy has limited the potential for the current Ingard[®] technology to reduce insecticide use.

Two-gene varieties are likely to be available commercially in 2003/2004 season or earlier. The performance of this technology needs to be examined to assess the risk of reduced efficacy in the latter half of the season. Two-gene Bt cotton will still rely on the expression CryIAC. In the development of a suitable resistance management strategy it will be important to understand when the two Bt proteins CryIAC and CryIIA, are working together and when one protein dominates.

The commercially available Enviroligix kit has been promoted as a tool to assess the level of expression of CryIAC and CryIIA. Assessment of CryIIA can be related to CryIAC. If this kit can accurately assess Bt protein expression it will provide a useful tool in the management of transgenic cotton.

The proposed project endeavours to quantify the Enviroligix kit for use in Australia. The project will validate the kit by assessing the expression of CryIAC and CryIIA in various genetic constructs and monitor their efficacy by bioassay with laboratory susceptible insect strains.

The results obtained after completion of the project will assist grower/consultants to optimise their insecticide sprays for *Helicoverpa* and in the long term will contribute the profitability and sustainability of the industry.

What were the **Project Objectives** and to what extent were these achieved?

1. Assessment of the concentration of Bt proteins (CryI_{Ac} and CryII_A) in transgenic cotton varieties throughout the cotton growing season.

Replicated field trial in grower's field was carried out during the 2000/2001 season. Fully opened new leaves were collected for Bt content analysis. The data obtained from duplicated samples indicates that Envirologix kit can produce repeatable and consistent results.

Certain varieties do consistently express higher Bt concentrations than others through the season.

Trend showed by regression test of Bt protein expression during the growing season indicates different slopes for different varieties. Some varieties reduced their expression faster than others as the crop aged.

As far as the comparison of Bt protein express among cotton varieties is concern. Envirologix kit is suitable as a simple tool. The reduction in Bt detection in aged crop due to the inability to extract Bt protein in older tissue might be an issue for researcher who aims to detect total concentration of Bt protein.

2. Monitoring the field efficacy of Bt proteins in young leaves collected from the field by bioassay.

Susceptible, field collected strain of *Helioerpa armigera* was used for bioassay to determine the efficacy of transgenic cotton.

The simple technique using the self-airlocked Falcon petridishes with field collected newly opened leaves and one-day-old, fed neonates were carried out in the laboratory. Insect mortality assessed at five days after exposure was the criterion to determine the field efficacy of the cotton varieties. Result of the bioassay reveals two points that require cautions when using bioassay result to determine field efficacy of transgenic proteins.

- a. Field efficacy is relative as related to level of susceptibility of the tested strain to a given Bt toxin. The lower the susceptibility of the tested strain would result in lower mortality from a given level of Bt protein expression as compared to mortality of another strain, which has higher susceptibility.
- b. Many factors affecting the field efficacy of transgenic cotton. These factors include environmental conditions, plant ages and crop management practices. Beside, results of bioassay may be easily affected by drift; thus data might be rendered unusable if the trial is affected by spray drift from adjacent field. The interpretation of the result of the bioassay therefore should be treated with caution.

3. To correlate the Bt protein concentration and field efficacy.

Means of results obtained from Elisa analysis and bioassay with four replicates were used for correlation and regression analysis.

Correlation was established only between the bioassay parameters and Bt protein concentrations for three varieties among the five tested varieties. Two possible reasons might contribute to the non-significant relationship.

- a. Tested insect strain was not completely susceptible for CryI_{Ac}
- b. There was significant reduction of Bt concentration in detached leaf over the period of bioassay (due to the fact that CryI_{Ac} is unstable).

In practice, result of pest inspection, especially the number of larvae per metre, might correlate better to Bt protein concentration than that from laboratory bioassay.

What **methodology** was used, and a justification for the use of this methodology?

Bt protein analysis and bioassay.

The Envirologix commercial test kit applied the technique of “Sandwich” Enzyme Linked Immunosorbent Assay (Elisa) were used for protein analysis. This assay utilises colour development, where colour production is proportional to Bt protein concentration in the sample.

The methodology is described in Appendix A.

The Envirologix kit was used as a useful tool for quantifying Bt proteins in transgenic cotton. Although the correlation between Bt protein concentrations and insect mortality was not significant for all the tested varieties, the kit would still be an effective tool to determine the relative level of Bt expression for pest management.

The unknown factor causing the non-significant relationship is the level of susceptibility of the tested field strain of *H. armigera*. The result, however, timely revealed the fact that the *H. armigera* field population has already significant shifted its susceptibility as compared to five years ago when transgenic cotton was first introduced to Australia.

It's also important to realise that in situation where field tested population is not susceptible to the control agent (in this case, it is Bt protein), the field assessment would always be more reliable than laboratory bioassay.

1. ELISA Analysis for Bt protein in transgenic cotton varieties throughout the season

Method:

Six cotton varieties including four one-gene varieties, one two-genes stacked variety and one conventional variety were used in field replicated trial in Oakville, Narrabri in collaboration with Delta Pine International. Four replicates were arranged in a randomised complete block design.

Newly opened first leaves at node 0 was collected weekly, ten leaves per replicate. From each leaf, a leaf sample was collected and weighed. The concentration of Bt's protein(s) was expressed in ppm based on fresh weight. Each sample was analysed twice and the mean was used for statistical analysis. (Analysis was carried out at Grain Technology Service, Narrabri).

Results

There was consistent difference between the Bt's content (CryIAC) of different varieties. The content of CryIAC in Nucot 37 was consistently higher than that of 50 B and V16i throughout the season, indicates that there is the difference in the ability of Bt protein synthesis by the different varieties. (Table 1, Fig1)

Expression of the second gene (CryIIA) was much higher than that of the first gene (CryIAC), the ratio of CryIIA concentration/CryIAC concentration ranged from 12.5 to 46 folds. The expression of CryIIA did not decline with crop age as compared to that of the CryIAC. Therefore the ratio of CryIIA/CryIAC increased towards post-flowering and boll forming stages. (Fig 1, 2, 3 and 4)

Figure 1: Cry1A Contents during growing season (00-01)

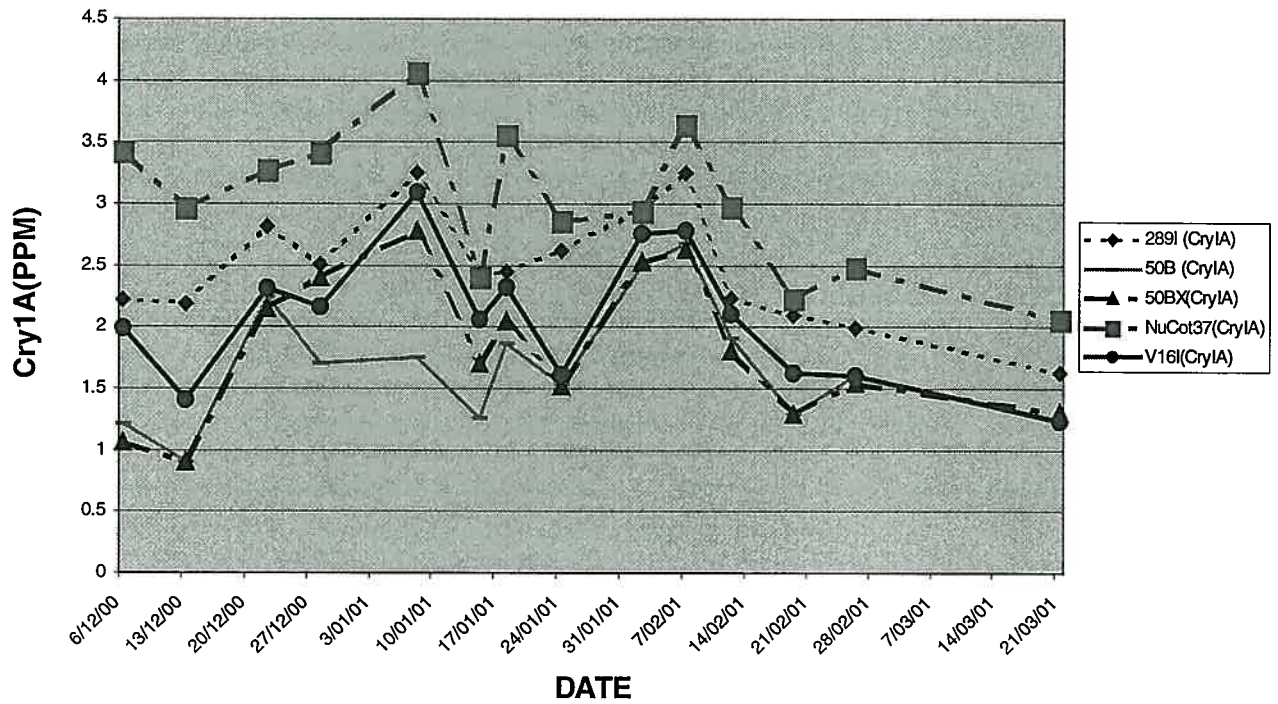


Figure 2: Cry2A contents and trend line in cotton variety 50BX during growing season (00-01)

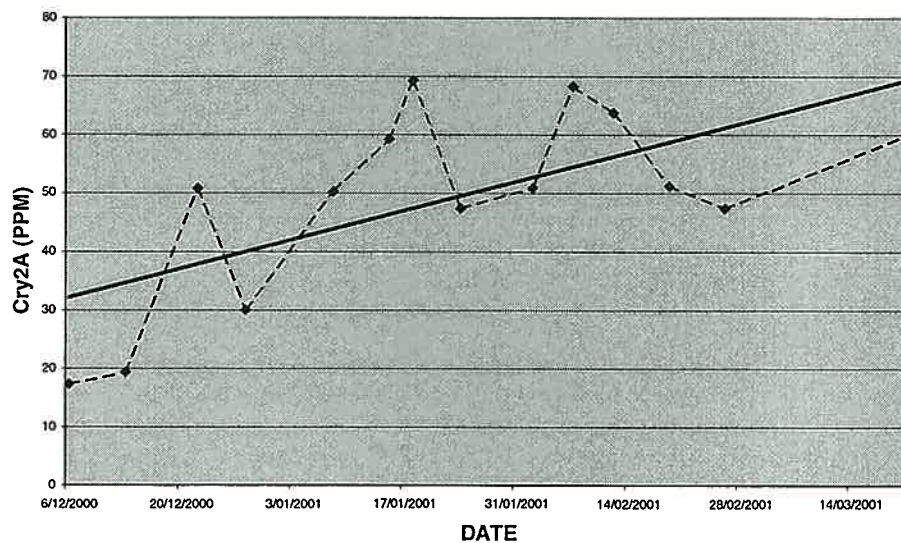


Figure 3: Trend lines for Cry1A protein content in transgenic cotton variety during growing season (00-01)

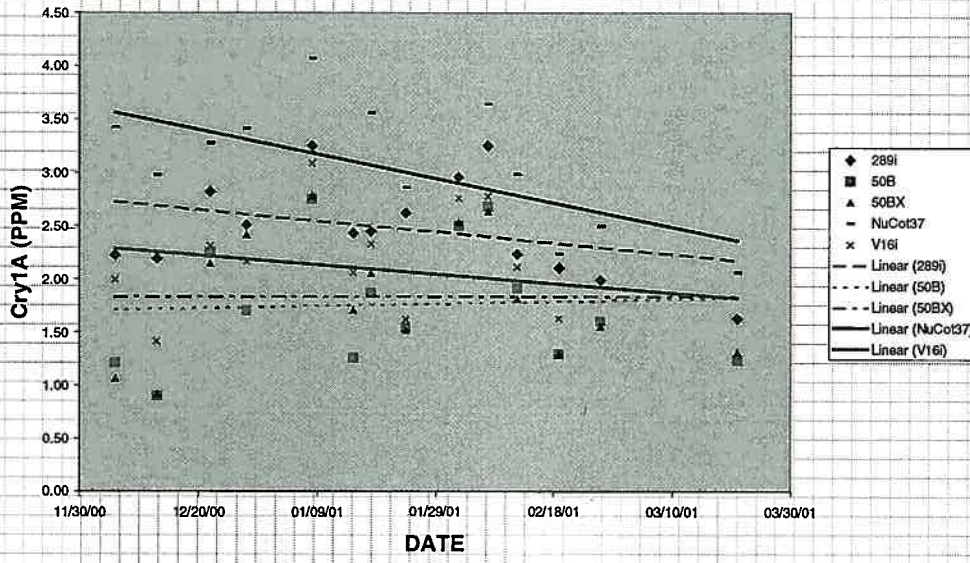
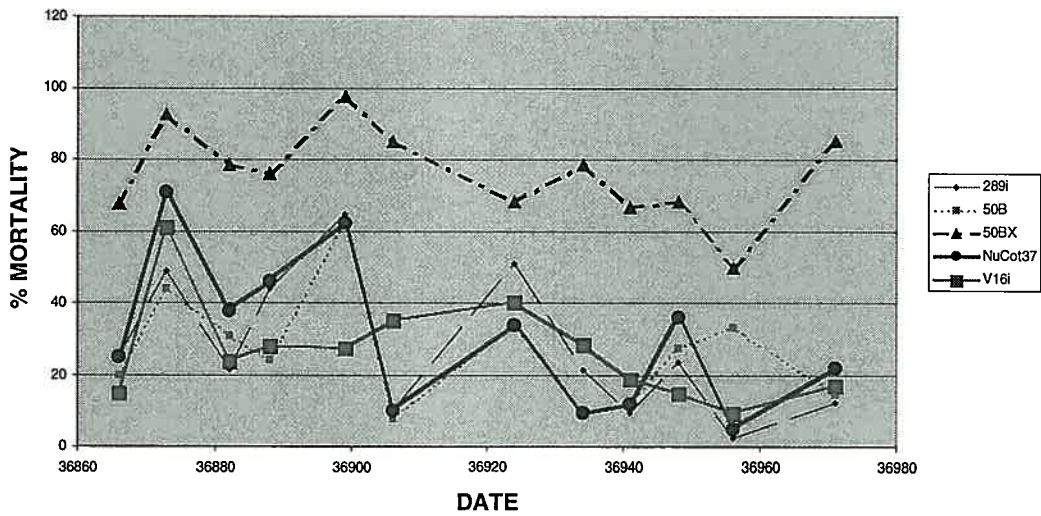


Figure 4: Percent mortality of *H. armigera* larvae tested on leaves of transgenic cotton variety during growing season (00-01)



Some varieties had high expression of CryIAC at early parts of the season, however, the expression declined faster than other varieties starting at flowering stage (Fig 3) In all varieties CryIAC expression was highest at squaring stage (Table 1). In different varieties, the reduction of expression of CryIAC occurs at various stages ie. Nucot 37 at 116 day after feeding (DAS) as compared to others: 289i, 50B and 50 BX at 95 DAS. (Table 1, Fig 1, 2 and 3).

Conclusions

More research is required to establish the relationship of the expression of the two genes. Results indicates the advantageous aspects from high expression of CryIIA as compared to those of CryIAC and also the steady expression of CryIIA throughout the season giving the better performance of the two-gene stacked transgenic variety. In one-gene transgenic varieties, their expression of CryIAC protein gradually declined to low level at about 95 to 115 DAS, thus supplementary control measure is required in the pest management program.

2. Bioassay to assess the expression of Bt proteins (CryIAC and CryIIA) in transgenic cotton varieties throughout the cotton growing season.

Methods

The insects: through the collaboration with Dr Neil Forrester, field strain was collected from Southern Queensland areas in early part of the cotton season. The objective of using field susceptible strain instead of laboratory susceptible strain can not be over emphasised, as the result of the bioassay will reflect the realistic situation of crop- pest interaction under field conditions.

The technique : simple method of bioassay using the Falcon petridish with one small leaf and one neonate per dish was carried out. The neonate was one day old fed and introduced onto the leaf using the wet hair- brush. Falcon petridish was self- locked and air tight to prevent drying of the leaf. Ten petridishes were used for each variety and each replicate. Assessment was made at five days after introduction of the neonate onto the leaf.

To establish the Larval Developmental Index (LDI), the assessment includes number of insect dead (Index = 0), and number of first, second, late second, third and fourth instar (Index = 1, 2, 2.5, 3 and 4 respectively.) LDI was calculated as followed:

$$LDI = (\sum x i)/n$$

x = Number of larvae at given stage (instar)at assessment time .

i = Index applied for a given stage

n= Total number of tested larvae .

Results

The percentage mortality of *H. armigera* larvae on variety (50 BX) was much higher than that on the single-gene varieties. The highest insect mortality among the single-gene varieties was 70.7% and the lowest insect mortality in the first 100DAS was 9.5%. The results indicate that the tested field strain was not completely susceptible to CryIAC toxin.

The expression of Cry1Ac protein in variety 50BX and 50B was similar throughout the season . However, the insect mortality on 50B declined at 67 DAS while remained

high on 50BX until the end of the season . Furthermore, insect mortality on variety 50BX during early parts of the season was significantly higher than that on variety 50B . These differences indicate that in 50BX, the second gene conferring CryIIA protein plays a major role in causing death to *H. armigera* larvae.

Similarly to the trends of Bt regression, insect mortality declined with crop age.

NuCot 37 had higher rate of declining among the single gene varieties (Fig 5).

The Larval Development Index (LDI) of *H. armigera* tested on the two–gene stacked varieties was significantly lower than that of the single–gene varieties. (Table 3). LDI of larvae tested on all transgenic varieties was significantly smaller than that of larvae tested on conventional variety (Pearl Table 2, 3, 4 and 5, Fig 4 and 5)

Table 4: Relationship between *B. thuringiensis* protein content and percent mortality of *H. armigera* on various cotton varieties.

| Variety | Toxin | Regression Equation ¹ | R ² |
|-----------------------|--------|----------------------------------|----------------|
| 289i ³ | CryIA | Y = 22.4X -22.9 | .476ns |
| 50B | CryIA | Y = 8.13X +13.8 | .0371n |
| 50BX | CryIA | Y=7.91X +62.9 | .0927ns |
| Nucot 37 ³ | CryIA | Y = 18.6X - 20.9 | .3880ns |
| V16i | CryIA | Y = 5.77X +15.4 | .0065ns |
| 50Bx | CryIIA | Y = .104X +71.4 | .0148ns |
| All Varieties | CryIA | Y = 3.86X +29.7 | .0144ns |

1. X = Bt content in ppm and Y = % mortality (linear regression analysis-SigmaStat 2.03)
2. ns = non significant at 5% level (SigmaStat 2.03)
3. Correlation coefficients for 289I and NuCot37 are significant (NuCot37:.622-P=.0285 and 289I:.648-P=.0203)

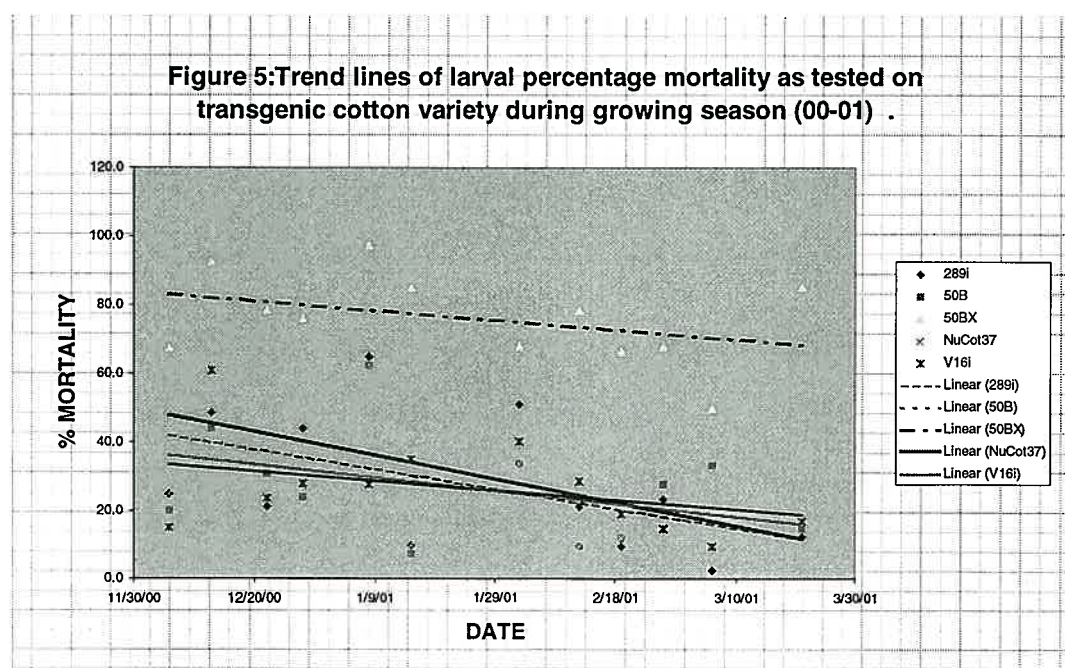


Table 5: Relationship between *B. thuringiensis* protein content and Larval Developmental Index of *H. armigera* on various cotton varieties.

| Variety | Toxin | Regression Equation ¹ | R ² |
|-----------------------|--------|----------------------------------|----------------|
| 289i ³ | CryIA | Y = .429X +2.49 | .218ns |
| 50B | CryIA | Y = -.261X +1.97 | .120ns |
| 50BX ³ | CryIA | Y=-.184X + .607 | .326ns |
| Nucot 37 ³ | CryIA | Y = -.447X +2.65 | .422ns |
| V16i | CryIA | Y = -.247X +2.05 | .041ns |
| 50BX | CryIIA | Y = .00141X +.236 | .011ns |
| All Varieties | CryIA | Y = -.107X +1.49 | .0006ns |

- 1 X = Bt content in ppm and Y = Larval Developmental Index (linear regression analysis-SigmaStat 2.03)
- 2 ns = significant at 5% level (SigmaStat 2.03)
- 3 Correlation coefficients for 289I ,50BX and NuCot37 are significant (289I:-.67-P=.015 , 50BX:-.58-P=.045 and NuCot37:-.66-P=.0186)

Conclusions

The highest percentage mortality of *H. armigera* larvae tested with leaf from two-gene stacked variety (50 BX) compared to that from single-gene varieties indicated the superior performance of the two-gene technology, especially in situation where susceptibility to the first protein (CryIAc) has been shifted.

To retain this outstanding performance on the two-gene technology, care should be taken to maintain the susceptibility in field population to CryIAc at current level.

3. Correlating the Bt expression and field efficacy:

Methods

The means of Bt protein content from 4 replicates were tested for regression and correlation with the corresponding means of the percentage mortality and larval developmental index.

The regression and correlation tests were carried out using Sigma-Stat (2.03).

Results

The regression analysis with Percentage Mortality (PM) and Larval Developmental Index (LDI) as dependent variable and Bt protein(s) content as independent variable which would describe the effect of Bt protein(s) content on resultant bioassay parameters (table 4 and 5).

Correlation analysis differs from regression in that it measures the degree of association between variables without specifying a priori which is the cause and which is the effect.

All regression coefficients (R^2) were not significant, however, there were significant correlation coefficients.

The significant positive correlation coefficients were established between Bt protein content (CryI Δ c) and percent mortality for variety 289i and NuCot 37 (Table 4).

Further, the significant negative correlation coefficient were established between Bt protein content (CryI Δ c) and Larval Developmental Index for variety 289i, 50 BX and NuCot 37 (Table 5).

For other varieties the resulting regression and correlation coefficient was not significant which might be due to the fact that the field strain was not susceptible for the low Bt protein contents in these varieties to take effect. The field strains collected from the South Queensland areas were found later possessing certain degree of resistance. Although these strains were composed of high degree of heterozygotes for resistance, they have shown to have higher survivorship than the corresponding generation of the previous season.

Further research should include field pest inspection report in situation where field population has been resistant to the tested toxin. In that situation, data from field count (specially on larval number) might be better correlated to Bt protein content than data obtained from a laboratory trial. The host-insect interaction reflected in field count data is more completed than from the laboratory test where only on stage of insect and one part of a plant is used for assessment.

Conclusions

Possible correlation coefficient could be obtained in situation when tested insect strain is susceptible to a Bt toxin.

Both larval mortality and larval development index could be considered as acceptable parameter for bioassaying to monitor field efficacy, provided that insect tested strain is susceptible.

Plain English Summary

Insect control accounts for about one third of the variable costs in cotton production and resistance is the major threat to sustainable cotton production in Australia. Transgenic cotton varieties already provide a backbone in current IPM programs for 30 % of the Australian crop. The expression of Cry1Ac by current transgenic varieties has been shown to be variable.

A commercial available ELISA kit represents a useful tool for quantifying Bt proteins in current (single gene) and future (stacked) transgenic cotton varieties . In this study, Enviroligix ELISA test kit was used for *B. thuringiensis* (Bt) protein analysis and laboratory bioassay was carried out to assess the field efficacy of transgenic cotton varieties.

Results indicated that Enviroligix kit is user-friendly and producing consistent results.

Due to the fact that the field strain of *Helicoverpa armigera* was not completely susceptible to the Bt protein toxin (CryIAc) the results of the laboratory bioassay was only partly correlated to the protein content in the field crops. For transgenic varieties that contain sufficiently high amount of Bt protein for the control of *H. armigera*, the correlation coefficient were significant for bioassay both parameters, namely, larval mortality and larval developmental index.

Further research is warrant to evaluate the mortality of the ELISA analysis in field crops grown under various agronomic conditions to determine the field efficiency. The future research would require detail field insect population monitoring.

Acknowledgments

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References

- Anon, 1992. Catalogue No. 003, CryIAb/CryAc/CryIIA kit (use, sample extraction and result interpretation) Envirologix Inc. PP4 Portland Maine USA
- Dang. H.T, Gunning. R and Holloway. J (2001) Resistance in Australia *Helicoverpa armigera* to *Bacillus thuringiensis* protein (CryIAc). Paper presented at the Resistance 2001 Conference. Rodhmsted, UK PP 1-7
- Fitt. G.P.,Daly,J.C.,Mare,C.L.and K.Olsen.1998.Changing efficacy of transgenic Bt cotton patterns and consequences .PP189-196. In: M.P.Zalucki, RAI Drew,G.G.White (eds). Pest Management future challenges. Proceedings of the 6th Australian Applied Entomology Conference. .Vol. 1, University of Queensland Press, Brisbane .

Table 1: Content of *B. thuringiensis* proteins (ppm) in first opened leaves during the 00–01 crop season.¹

| Variety | 6/12 | 13/12 | 22/12 | 28/12 | 8/01 | 15/01 | 18/01 | 24/01 | 2/02 | 07/02 | 12/02 | 19/02 | 26/02 | 21/03 |
|---------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 289I (CryIA) | 2.22c | 2.19c | 2.82c | 2.51c | 3.25c | 2.43d | 2.45cd | 2.62b | 2.96c | 3.25c | 2.23c | 2.10c | 1.99b | 1.63b |
| 50B (CryIA) | 1.21a | 0.90a | 2.24a | 1.71a | 2.75a | 1.26a | 1.87a | 1.54a | 2.50a | 2.68a | 1.91a | 1.29a | 1.60a | 1.23a |
| 50BX(CryIA) | 1.06a | 0.91a | 2.15a | 2.41c | 2.78a | 1.71b | 2.05b | 1.52a | 2.53a | 2.63a | 1.81a | 1.29a | 1.55a | 1.31a |
| NuCot37(CryIA) | 3.42d | 2.97d | 3.28d | 3.41d | 4.07d | 2.41d | 3.56d | 2.86c | 2.94c | 3.64d | 2.98d | 2.23d | 2.49c | 2.06c |
| V16I(CryIA) | 1.99b | 1.41b | 2.31b | 2.16b | 3.09b | 2.06c | 2.33c | 1.62a | 2.76b | 2.78b | 2.11b | 1.63b | 1.60a | 1.24a |
| Mean(CryIA) | 1.98 | 1.676 | 2.56 | 2.44 | 2.988 | 1.974 | 2.452 | 2.032 | 2.738 | 2.996 | 2.208 | 1.708 | 1.846 | 1.494 |
| 50BX(CryIIA) | 17.33 | 19.4 | 50.78 | 30.02 | 50.26 | 59.24 | 69.28 | 47.42 | 50.9 | 68.27 | 63.77 | 51.25 | 47.35 | 59.93 |
| DAS ² | 32 | 39 | 48 | 54 | 60 | 67 | 75 | 81 | 90 | 95 | 100 | 107 | 114 | 137 |

1. Means in a column followed by common letter are not significant difference at 5% level (Genstat 4.2)
2. DAS = Day after seeding.

Table 2: Percent mortality of first instar larvae of *H. armigera* at 5 days after feeding on leaves of transgenic and conventional cotton varieties during the 00–01 crop season.¹

| Variety | 6/12 | 13/12 | 22/12 | 28/12 | 8/01 | 15/01 | 2/02 | 12/02/ | 19/02 | 26/02 | 6/03 | 21/03 |
|------------------|-------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|--------|
| 289i | 25.0c | 48.8a | 21.4a | 44.0b | 64.9b | 10a | 51.0c | 21.4b | 9.5a | 23.4b | 2.4a | 12.2a |
| 50B | 20ab | 43.9a | 30.6b | 24.0a | 62.4b | 7.5a | 34.0a | 9.5a | 11.9a | 27.6b | 33.2c | 14a |
| 50BX | 67.5d | 92.6c | 78.4d | 76.0c | 97.3c | 85.0c | 68.0d | 78.4d | 66.5c | 68.0d | 49.9d | 85.3 |
| Nu37 | 25c | 70.7c | 38.0c | 46.0b | 62.4b | 10.0a | 34.0a | 9.5a | 11.9a | 36.1c | 4.8ab | 21.9b |
| V16i | 15a | 60.9b | 23.8a | 28.0a | 27.5a | 35.0b | 40.4b | 28.5c | 19.0b | 14.9a | 9.5b | 17.1ab |
| Mean | 30.0 | 63.4 | 38.4 | 43.6 | 61.7 | 29.5 | 45.5 | 29.5 | 23.8 | 19.5 | 19.9 | 30.2 |
| DAS ² | 32 | 39 | 48 | 54 | 60 | 67 | 90 | 100 | 107 | 114 | 122 | 137 |

1. Means in a column followed by common letter are not significant difference at 5% level (Genstat 4.2)
2. DAS = Day after seeding

Table 3: Larval Developmental Index (LDI) of *H. armigera* at 5 days after feeding on leaves of transgenic and conventional cotton varieties during the 00–01 crop season.¹

| Variety | 6/12 | 13/12 | 22/12 | 28/12 | 8/01 | 15/01 | 2/02 | 12/02/ | 19/02 | 26/02 | 6/03 | 21/03 |
|-------------------|------|-------|-------|-------|------|-------|-------|--------|-------|-------|------|-------|
| 289i | 1.4b | 1.2c | 1.7a | .9b | .9c | 2.1b | .7b | 1.7c | 1.8b | 1.8d | 1.9c | 1.8bc |
| 50B | 2.0d | 1.3c | 1.5c | 1.6c | .7b | 2.2b | 1.2d | 1.7c | 1.7b | 1.6c | 1.3b | 1.9c |
| 50BX | .4a | .1a | .1a | .1a | 0.0a | .4c | .3a | .4a | .5a | .4a | .7a | .2a |
| Nu37 | 1.3b | .6b | 1.2b | 1.0b | .8bc | 2.2b | 1.1cd | 1.9d | 1.7b | 1.4b | 2.0c | 1.6b |
| V16i | 1.8c | .7b | 1.7d | 1.6c | 1.2d | 2.1b | 1.0c | 1.4b | 1.7b | 2.0e | 2.0c | 1.7bc |
| Mean ² | 1.38 | .78 | 1.24 | 1.04 | .72 | 1.8 | .86 | 1.42 | 1.48 | 1.44 | 1.58 | 1.56 |
| Pearl | 3.95 | 2.90 | 2.95 | 2.76 | 2.03 | 2.9 | 1.81 | 2.36 | 2.4 | 2.47 | 2.48 | 2.54 |
| DAS ³ | 32 | 39 | 48 | 54 | 60 | 67 | 90 | 100 | 107 | 114 | 122 | 137 |

1 Means in a column followed by common letter are not significant difference at 5% level (Genstat 4.2)

2 Mean of LDI on transgenic varieties .

3 DAS = Day after seeding

Appendix A: Methodology

1. Cotton varieties to be assessed:

- a. Delta Pearl (Conventional)
- b. NuCotn 37 (531) (one-gene transgenic)
- c. Sicot 289i(531) (one-gene transgenic)
- d. Siokra V16i (531) (one-gene transgenic)
- e. DP50B (531) (one-gene transgenic)
- f. DP50Bx (985) (two-genes stacked transgenic)

2. Bt Assays

The quantitative analysis using Envirologix test kit was carried out by contractor (Grain Technology Services (GTS) Narrabri). Varieties were planted in four randomised replicated field plots.

Ten newly opened leaves from each replicate; each variety was processed in the laboratory at ACRI. The leaf disc samples were developed at ACRI and supplied to contractor's (GTS) staff in Narrabri. The weight of ten leaf discs was written on the sample packets.

- a. Each ten disc sample was placed in one tube and macerated in 1.5 ml of CryAb Extraction/Dilution buffer, using the ball bearing method in Mini Bead Beater.
- b. Sample dilutions for terminal leaves were 1:51 early season, 1:26 mid season and 1:11 late season.
- c. Two negative control samples were done per sampling occasion and averaged for subtraction from sample and calibration readings.
- d. Two samples were done for each of the three standard CryIAb calibration solutions (0.5 ppb, 2.5 ppb and 5.0 ppb) per sampling occasion and averaged to produce a standard curve.
- e. Results were supplied by Contractor as reading for each plot expressed as micrograms of CryIAC toxin per gram of leaf dish's weight.
- f. The same leaf disc extraction can be used for CryIIA. However, separate negative controls were used.

The Envirologix commercial test kit applies the technique of "Sandwich" Enzyme Linked Immunosorbent Assay (ELISA). This assay utilises colour development, where colour production is proportional to Bt concentration in the sample.

3. Bioassay

Susceptible strains of *Helicoverpa armigera* were used for bioassay to determine the efficacy of transgenic cotton.

Forty leaves from four replicated field plots were sampled fortnightly for each variety. Newly opened leaves were used, One leaf and one young first instar larval were placed in a Falcon petridish. Insect mortality was assessed at five days after introduction.