Season-long Monitoring of Transgenic Cotton Plants - Development of an Assay for the Quantification of *Bacillus thuringiensis* Insecticidal Crystal Protein

Dr Helen E. Holt, CSIRO Division of Entomology

Background

This project was designed to support the deployment and continued use of transgenic (Ingard®) cotton in the Australian cotton industry. To maximise the efficacy and useful lifespan of transgenic cotton we need to monitor the production of the toxin from *Bacillus thuringiensis* (Bt) in cotton plants throughout the growing season. Transgenic cotton contains a gene which encodes for the production of an insecticidal crystal protein (referred to as the "toxin") which is highly toxic to lepidopteran species including *Helicoverpa*, a major pest in the Australian cotton industry. These plants have been shown to successfully produce the toxin, but field studies indicated that the efficacy of plants was reduced later in the season (Fitt *et al.*, 1994). The cause of this reduced efficacy was not understood, although it is possible that production of the toxin is influenced by plant age or reproductive stage, and/or by a variety of environmental factors. Also, to ensure that resistance management strategies designed for use with transgenic cotton are successful, we need to assess the exposure of insects to the toxin.

It is also important to consider how information on toxin levels in plants can be used. Currently we do not know the nature of the relationship(s) between toxin level and plant efficacy, and these relationships may be complex. Data concerning toxin levels in plants probably will be most valuable when used in conjunction with field efficacy, bioassay and plant physiological data. Using this combination of data we can develop an accurate picture of plant performance and the importance of a number of influences on efficacy.

Project Aims

This project continues earlier work developing extraction methods for the toxin from a variety of cotton plant tissues and detection methods for the toxin in plant extracts. Both projects have involved the development of key components including:

1. Extraction methods for good quality protein from cotton tissues, including the Bt toxin.
2. Techniques to detect the toxin in extracts from a variety of cotton tissues - "yes/no" or "positive/negative" methods.
3. Quantitative methods to determine whether we can measure changes in Bt toxin levels over time or under differing conditions.
4. Refinement of the quantitative methods, including the extraction method, to give more repeatable measurement of the Bt toxin.

Results to Date

The development of extraction methods and initial detection of Bt toxin in extracts has been discussed in an earlier paper (Holt, 1996) and will not be discussed here except to outline refinements and more recent results from this work. Briefly, early work showed that our extraction method gave sufficient yields of good quality protein, including the Bt toxin, which we could detect in cotton tissues of all ages and developmental stages.

More recent work has focussed mainly on the development and testing of an assay which was capable of testing a large number of samples from field and glasshouse-grown cotton and producing reliable and reproducible results. This method has been based on an assay used by Dr Danny Llewellyn (CSIRO Plant Industry, Canberra) which has previously been used only to provide a positive/negative answer concerning whether Bt toxin is present in young cotton tissue. This assay, known as an Enzyme-linked Immunosorbent Assay or ELISA, can be adapted using a set of standard amounts of Bt toxin which allow us to construct a standard curve. Tissue extracts can then be compared to the standards and toxin levels determined. This assay relies on the recognition of Bt toxin in an extract by an antibody which recognises only the toxin. Our antibody, obtained from Dr Danny Llewellyn, was first tested in a Western blot which allowed us to check that the antibody bound only to the toxin. The results indicated that the antibody has good specificity for the toxin. The antibody was then tested to determine whether it showed similar specificity in the ELISA, and results indicated that it did. The ELISA system operates as below:

1. Bind specific antibody to wells of an assay plate and allow it to bind to the plate well.
2. Wash to remove any antibody which is not bound.
3. Add blank and standards to coated plate wells as required.
4. Add a known amount of extract to remaining coated wells. Up to 42 samples can be tested on a single plate.
5. Incubate plates to allow the toxin in standards and extracts to bind to the antibody bound to plate wells.
6. Wash plates to remove any extract which is not bound to the antibody.
7. Add a second antibody, also specific for the toxin. This antibody has an enzyme linked to it and will bind only where the toxin is bound to the first antibody.
8. Incubate the plates to allow the second antibody to bind to the toxin.
9. Wash plates to remove any unbound second antibody.
10. Add a substrate with which the enzyme on the second antibody reacts. When the reaction occurs colour is produced. The intensity of the colour is relative to the amount of enzyme and thus the amount of toxin, present in the sample.
11. The intensity of the colour reaction is measured in a plate reader and the amount of toxin present is determined by comparison of the samples to the standards.

Samples were then checked in the ELISA to determine whether this antibody recognised toxin in extracts from all tissues of transgenic plants and did not cross-react with any compounds in the corresponding conventional cotton tissue extracts. Figure 1, below shows that the toxin in transgenic extracts was successfully detected without cross-reactivity.

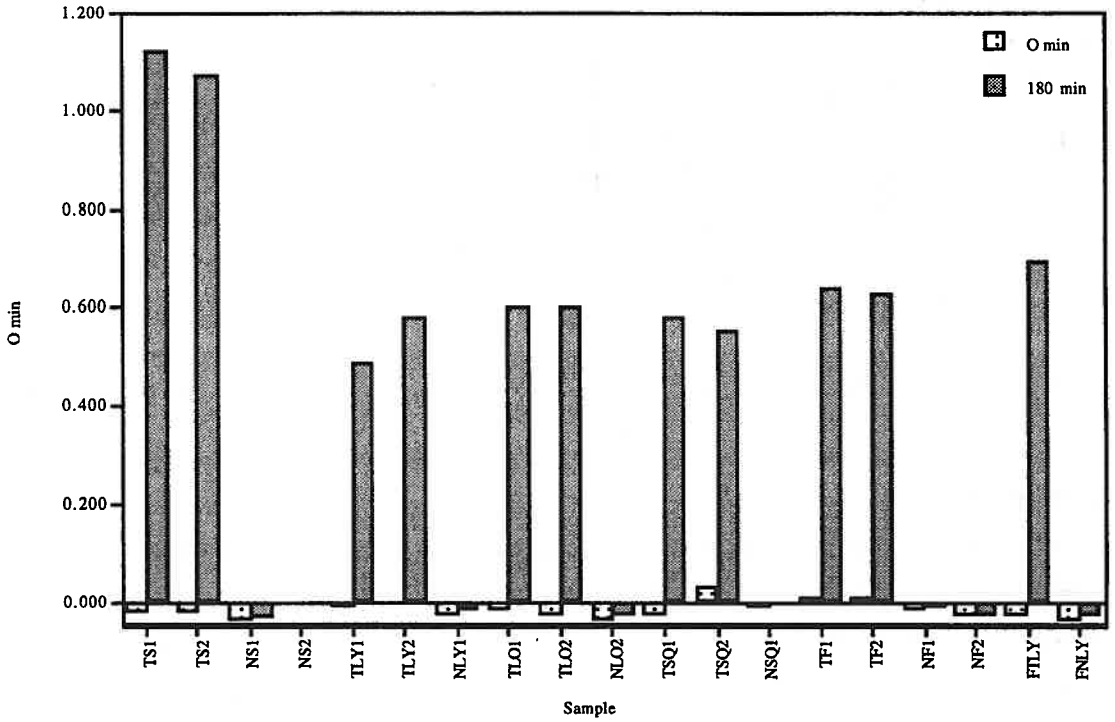

Figure 1 : Initial testing of cotton extracts using ELISA. Samples with the "T" prefix are transgenic, samples with an "N" prefix are non-transgenic. Samples with "F" prefix are field samples. "S" samples are seeds, "LY" samples are young leaves, "LO" samples are older leaves, "SQ" samples are square samples and "F" samples are flowers. The two samples at the far right of this figure are field leaf samples, transgenic and non-transgenic respectively. Zero and 180min ELISA optical density (OD) readings are shown.

Having shown the suitability of the antibody to detect toxin in an ELISA we needed to focus on the development of a standard curve using purified Bt toxin. We showed that we could do this at a range suitable for comparison with the levels in extracts. Field tissue extracts were checked using the ELISA and we were able to detect differing amounts of Bt toxin in the plant extracts across the growing season. This showed us that the assay could detect gross changes in the amount of toxin present in plant extracts, despite some variation in assay results. The preliminary data also suggest that the decline in plant efficacy during the latter part of the season was correlated with a decline in the level of Bt toxin (Figure 2). The toxin level determined by the ELISA can be used to give us a measure of toxin levels in relation to plant dry weight or plant wet weight.

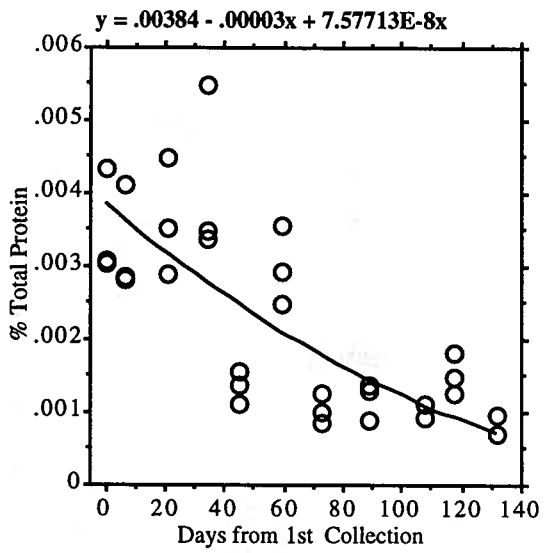

Figure 2 : Changes in Bt toxin across the season 96/97 in Narrabri. Levels of Bt protein as a percentage of total plant protein across the season 96/97. Regression r^2=0.55233

Current Work

Having demonstrated that the ELISA was suitable for detecting differing amounts of Bt in tissue extracts we have determined a number of areas to improve its reliability and accuracy. The first is the re-examination of the extraction method now that we can actually quantify the extracted Bt. This allows us to determine whether all, or most, of the Bt is extracted in the initial extraction process, and whether extraction is uniform for all tissues. This work is currently underway and initial results indicate that our buffer provides better total protein extraction than the only other buffer cited in literature (Benedict *et al.*, 1996) under our extraction conditions. We have run extractions on samples of mixed transgenic and conventional cotton tissues in known ratios which allows us to also assess the efficiency of the extraction for the toxin itself. Results of this work are currently being analysed and look promising. Secondly, we are refining the ELISA itself by standardising the conditions of the assay, especially with respect to the antibody used and the purified toxin standards which are produced and used in the assay. Because the use of the ELISA hinges on the specificity and sensitivity of the antibody used it is vital to ensure that the antibody has both the qualities. It is also vital to ensure a long-term supply of the antibody, so that re-calibration of the assay is unnecessary. This way the results from one season are readily comparable to those from other seasons. The reliability of the standards is being addressed by ensuring the development and use of standard methods for the production and purification of the toxin.

Another important area of interest is determining the relationships which may exist between toxin levels and plant age or reproductive stage, environmental influences and/or efficacy (determined either in the field or by bioassays). This is being determined using field samples collected throughout a full growing season and glasshouse trials in which we can

control environmental factors such as temperature, light, moisture or other influences, to determine what effects these have on toxin levels, efficacy and the relationships between the two. Samples are currently being examined from field and glasshouse trials.

The Future

A major aim of this work is to provide a test which can be used by other laboratory personnel in strategic laboratories. One of the final tasks in this project will therefore be to teach the refined method to people in these laboratories and to ensure that the method is robust when used by a number of people in a range of locations. It is envisaged that this test will be a lab test because of the difficulties in extracting the toxin and the repeatability necessary in the assay which requires laboratory conditions and equipment. It should also be noted that until relationships between toxin levels and the variability of these relationships under differing growing conditions are determined, a measure of toxin level, alone, will not be useful in determining how a crop should be managed. In the longer term it is likely that this assay will provide important data with applications in the long-term management of transgenic crops, in design and refinement of resistance management strategies and in the selection of elite lines of transgenic plants.

References

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