Improving Environmental Loading Assessments of *Cry* Protein from GM Plants Based on Experimentation in Cotton

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Abstract: Transgenic Bt-cotton expresses insecticidal *Cry* proteins *via Cry* genes, introduced from the soil bacterium *Bacillus thuringiensis* (Bt). Data on levels of *Cry* proteins expressed in transgenic Bt-crops is important for assessing levels of environmental exposure. During investigations into the rhizosphere impacts of *Cry* proteins from GM cotton we found that *Cry1Ac* expression throughout the season was significantly lower in roots of glasshouse-reared cotton plants (average of 0.03 μg/g in the roots) compared to the same Bt-cotton varieties grown in the field (0.14 μg/g in the roots). We subsequently undertook a whole plant field assessment of both *Cry1Ac* and *Cry2Ab* expression in stem, leaves, root, and flowers, squares and bolls of Australian cotton varieties Sicot 289 Bollgard®II (289B) and Sicot 289 Bollgard®II Roundup Ready (289BR) over an entire season. Significant differences in the expression levels of *Cry1Ac* versus *Cry2Ab* occurred in the whole plant throughout the season. *Cry1Ac* levels remained relatively constant at an average of 6.1 μg/g whilst *Cry2Ab* levels averaged 29.0 μg/g, but decreased over time. Analysis of whole plant expression levels, plant stand densities, and aspects of crop management, estimated that levels of *Cry1Ac* and *Cry2Ab* deposited in the soil at the end of the season were 0.26 and 0.16 μg/g soil, respectively. The undertaken experiments highlight that assessments of environmental loading of proteins from GM plants would be improved with the use of field grown plants, whole plant assessments, increased knowledge on fate and persistence of GM proteins in the soil, and refinement of current ELISA methodologies.

Keywords: Cotton, *Cry1Ac*, *Cry2Ab*, Bollgard®II, expression, *Bacillus thuringiensis*, field grown, *Helicoverpa armigera*, ELISA.

INTRODUCTION

Reliable estimates of transgenic protein expression in genetically modified (GM) crops can provide a valuable tool in assisting with understanding the development and management of resistance, non-target implications, economic performance and environmental loading of the transgenic proteins [1-3]. GM cotton, *Gossypium hirsutum*, expressing the insecticidal *Cry1Ac* gene, from the soil bacterium *Bacillus thuringiensis* (Bt), was introduced commercially in Australia in 1996 [4]. The main target for this GM product was control of *Helicoverpa armigera*, which demonstrated ability to develop resistance to *Cry1Ac* under a laboratory imposed selection regime [5]. These observations, in addition to a reported decline in *Cry1Ac* expression in cotton over a season [6, 7], suggested that the risk of *H. armigera* evolving resistance to a single *Cry* protein Bt crop in Australia was high.

BollgardII® varieties that expressed both *Cry1Ac* and *Cry2Ab* [8] were introduced in the 2003/04 season. Capped limits for BollgardII® crops were initially imposed at 40%, but were removed in 2004/05 on the provision that a resistance management plan (RMP) was followed [9, 10]. Under good RPM practises, conditions for potential resistance development is considered to be retarded, providing that *Cry* expression remains at levels high enough to exert insecticidal control [11-13]. However, there have been concerns that over expression of the GM material could have implications for non-target organisms and increased environmental loading of these proteins. Numerous evaluations have indicated that there is no risk to the health of non-target organisms [14-17], but these studies were conducted without simultaneous measurement of *Cry* expression levels in plants.

Bt plants may have the potential to influence the soil in which they are growing through the release of the Bt proteins in root exudates or from sloughed or decaying plant material [18, 19]. Past work on whole plants suggests that rearing plants in a glasshouse environment can significantly impact on *Cry* expression [19] and aboveground expression levels may not truly reflect expression in roots. We set out to investigate *Cry1Ac* expression in cotton roots and to evaluate Bt expression levels in glasshouse versus field grown plant roots. Results from these studies indicated that *Cry1Ac* levels in below ground material from plants reared in the glass-

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house significantly underestimated expression levels in field grown plant populations. Subsequently, the focus of our work became the measurement of expression of both *Cry* proteins in above and below-ground field grown cotton.

We present information on relative variation in the expression of *Cry1Ac* and *Cry2Ab* in commercially available field-grown Australian cotton varieties throughout a growing season. Analysis of the crop stand and biomass, as well as management options used in preparation of soil for subsequent plantings, were used to conduct an assessment of the loading of soils with transgenic proteins at the end of the growing season.

The results of our experimentation are discussed with a view to offering suggestions on how methods of *Cry* protein assessment in tissues of GM plants could be improved in order to provide better estimations of environmental loading.

MATERIAL AND METHODS

Preliminary Assessment of Glasshouse and Field Levels of Cry1Ac in Cotton Roots

We used a quantitative ELISA method for the assessment of the *Cry* proteins to facilitate the throughput of high sample numbers and present a standardised level of expression comparison. In order to achieve this, plant material was sampled, processed and stored in such a way as to limit degradation of the *Cry* proteins [20] using available facilities and resources.

Sampling of Glasshouse Material

During July 2003, seven seeds each of cotton varieties Sicot 189 (189), Sicot 289-Bollgard®II (289B) and Sicot 289-Bollgard®II Roundup Ready (289BR) were planted in 30 cm diameter pots, containing 3 kg of a self mulching grey vertisol (53% Clay, 0.75% organic carbon, pH 9.1). Sicot 189 was included as a control, as it was the recurrent parent of the tested transgenic varieties. The pots were arranged in the glasshouse in a replicated random block design and a dripper watering system was used to irrigate the cotton daily to ensure that all pots received sufficient water so that moisture was not limiting. Root material was destructively sampled from pots at 42, 84 and 112 days after planting (DAP). Once recovered the roots were dehydrated in a Phoenix 200 (DEC International Inc, USA) at 55°C for four days, ground in a Type 843 coffee mill (Moulinex, France), and stored at -20°C until analysed.

Sampling of Field Material

During the 2003/04 season, four replicated blocks, each containing 189, 289B and 289BR in 8 m by four row plots, were established at the Myall Vale research centre in a soil with 55% Clay, 0.50% organic carbon, pH 8.6. For comparison with the glasshouse material, individual 289B and 289BR plants were dug up with a fork from each replicated plot at 45 and 130 DAP. Roots were recovered, dehydrated and ground, using the methods described for glasshouse plants.

Sample Preparation and Quantitative ELISA of Glasshouse and Field Roots

Glasshouse and field root samples were analysed on EnviroLogix (USA) *Cry1Ab*/*Cry1Ac* QuantiPlate kits, using the manufacturer's standards and according to their instructions. Tissue extraction of the *Cry1Ac* protein was carried out on a weighted 0.1 g sub sample of each milled root. This was extracted with 1 ml of 1x EEB in a microfuge tube by vortexing for 5 seconds before being imbibed for 4 h at 21°C, prior to centrifugation at 5000 rcf for 5 minutes. A 100 µL sample of the supernatant was used for quantitative ELISA. Absorbance was read in a Benchmark Microplate Reader (Bio Rad, Australia) at 450 nm, recorded using Microplate Manager 5.1 (Bio Rad, Australia) and transferred to Excel (Microsoft, USA) for analysis.

Evaluation of Seasonal Levels of Cry1Ac and Cry2Ab in Field Grown Cotton

Sampling of Field Material

To compare below and above ground expression of proteins, four replicated blocks, each containing 189, 289B and 289BR in 8 m by eight row plots, were established in 2004/05. For *Cry* expression analysis, plants were dug up with a fork throughout the season at 34, 54, 97, 137 and 193 days after planting (DAP), which was equivalent to 307, 536, 1081, 1678 and 2327 day degrees.

Plants were returned to the laboratory within an hour of recovery, partitioned into; leaves and petioles (L), stems and branches (S), roots (R), and if present a pooled group of flowers, squares and bolls (FSB). Fresh weight of each of these partitions was recorded. Stem and root material was cut into 2 cm lengths and bolls, when present, were cut open to facilitate rapid and thorough drying. Drying was carried out for four days in a forced air Hurricane dehydrator (Wessburg and Tulander Pty. Ltd., Australia) running at 45°C. Dry weights were recorded and the tissues ground in a 1A Benchmill (Rock Labs, New Zealand) for 40 sec. A subsample of the ground material was transferred to a 2 ml microfuge tube, which was stored at -20°C until analysed.

Quantitative ELISA of Field Plant Tissue

In 2004/05, *Cry1Ac* and *Cry2Ab* coated plates and ELISA reagents were sourced from Strategic Diagnostic Inc. (SDI, USA) and were used according to the manufacturer's instructions for quantitative analysis. For the *Cry1Ac* plates, a spore and crystal suspension of *Cry1Ac* from bacterial strain HD73 (Genesearch, Australia) was used as a standard. For the *Cry2Ab* plates, a ground lyophilised Corn Powder, containing only *Cry2Ab* (Monsanto Australia), was used.

The HD73 (Genesearch, Australia) spore and crystal suspension was supplied as a 62 mg *Cry1Ac*/ml. A 1 in 10 dilution of HD73 was prepared in distilled water and stored at -20°C until required for use, when it was thawed and further diluted to provide standards with a calculated range of 7 to 620 PPB.

From 1 g of the lyophilised Corn Powder, 6.014 mg of *Cry2Ab* was extracted (Monsanto Australia) into 1 ml of ELISA extraction buffer (EEB; EnviroLogix, USA) assuming 100% extraction efficiency and subsequently diluted further in EEB to prepare standards in the range of 0.3 to 300 PPB.

For the ELISA assay, ground dehydrated plant material was removed from the freezer and weighed into 0.1 g amounts in 1.5 ml microfuge tubes to which 1 ml of EEB

was added, mixed using a Retsch MM300 (Retsch GmbH & Co., Germany) for 30 seconds, imbibed for 2 h at 21°C before being centrifuged at 13200 rcf for 5 minutes. For *Cry2Ab* analysis, 50 µL of the supernatant was transferred to the ELISA plates for quantitative analysis. For *Cry1Ac* analysis an additional 1 in 10 dilution in EEB was required to bring the tissue *Cry1Ac* content within the detectable range, with 100 µL of this diluted solution transferred to the ELISA plate.

After completion of all field ELISA assays, absorbance was read in a Benchmark Microplate Reader (Bio Rad, Australia) at 655 nm and handled as previously described.

Environmental Loading of Cry Proteins

For the end of season environment loading estimates the 193 DAP samples' quantitative *Cry* data and partitioned plant dry weight measurements were used to calculate partitioned tissue expression and total *Cry* protein levels for individual plants. Stand counts, of plants per metre within the sampled crop population, were taken over 118 individual metres of the crop. This data; stand count, plant biomass and expression levels were used to generate a weight of *Cry* gene expression per metre of crop. Post picking the crop was slashed and root pulled, prior to incorporation into the top 15 cm of the soil. This data was used to estimate the *Cry* protein levels incorporated back into the soil system with the slashed crop.

Statistical Analysis

Sample results were compiled, tabulated and statistically analysed using ANOVA with GenStat version 8 (VSN International Limited, UK). The variance ratio was considered to indicate a significant difference between factors when $P < 0.05$. Normality of the data was assessed from fitted plots of the residuals and none of the data required transformation.

Glasshouse assessment with ANOVA was initially undertaken with a combination of the measured factors; plant varietal traits (conventional, B and BR), environment (glasshouse or field), time of sampling, and root partition (lateral or tap). Subset and individual factors where subsequently assessed with ANOVA.

In the whole plant field assessments the factors were; plant varietal traits (conventional, B and BR), plant partition (L, S, R and FSB) and time of sampling. These factors were initially interrogated in combination and then in subsets and as individuals. The sampled varieties position within the planting design of the field was added as a blocking structure to address the random effects of field location.

Regression curves, when mentioned in the text, were fitted to the data using SigmaPlot version 9 (SYSTAT Software Inc., USA).

Results

Preliminary Assessment of Glasshouse and Field Levels of Cry1Ac in Cotton Roots

Cry1Ac expression in roots of glasshouse plants declined from 0.069 to 0.003 µg/g over the 56 days, whilst *Cry1Ac* in field roots increased from 0.050 to 0.279 µg/g over the 85 days between the fist and last sampling (Fig. **1**). Additionally, under glasshouse conditions levels of *Cry1Ac* expression in roots of 289B were significantly lower than in 289 BR ($P < 0.001$, n=24) throughout the experiment.

Expression of *Cry1Ac* did not differ between lateral and tap roots recovered from glasshouse plants, with mean values of 0.147 and 0.162 µg/g, respectively.

In field grown plants, 289BR had significantly higher levels of *Cry1Ac* than 289B ($P = 0.036$, n=12) in assessed root material.

There were observable differences in the physiology of the roots of glasshouse versus field sampled plants. For example, glasshouse tap roots were thin and the pot soil was heavily proliferated by lateral roots, where as in field samples the tap root was thick with less prolific lateral roots recovered.

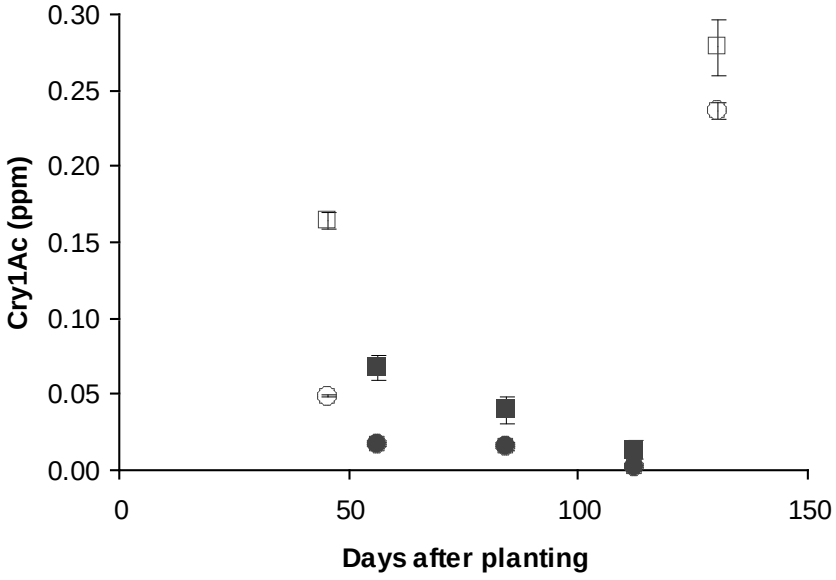

Fig. (1). Comparison of mean *Cry1Ac* levels from roots of field grown (open) and glasshouse (closed) Sicot 289B (circles) and Sicot 289BR (squares). Error bars represent the standard error of the means (n=12).

Evaluation of Seasonal Levels of Cry1Ac and Cry2Ab in Field Grown Cotton

Repeated assessment of HD73 as a *Cry1Ac* standard gave reproducible results over the range of 3 to 60 PPB, which followed a linear relationship ($y=617x^{1.01}$, $R^2=0.91$, n=20). At higher concentrations, e.g. 100 and 600 PPB, increased variability in *Cry1Ac* detection occurred. Aliquots of the 1 in 10 dilution of HD73 were not affected by at least three cycles of freeze thawing (data not shown).

Ground corn powder (Monsanto, Australia), previously used in published ELISA and bioassay studies [21, 22], was found to produce a reliable standard over the range of 0.3 to 70 PPB ($y= -12.7x^2 + 62.0x + 0.5$, $R^2 = 0.99$, n=15). However, it was found to be susceptible to freeze/thawing, thus aliquots and dilutions were prepared fresh when required. Substitution of EEB with water in the preparation of *Cry2Ab* standards resulted in ELISA plate detection over too narrow an absorbance range at 655nm to be useful (data not shown).

During the 2004/05 season, analysis of the partitioned plants revealed that there was always a significant difference in recovered dry weights between the segregated plant tissues (in all cases $P<0.001$, data not shown) and that at certain sampling points these differences also existed between 289B and 289BR ($P=0.036$ and 0.002 for comparisons between varieties at 3rd and 5th sampling, respectively, and $P<0.001$ in both cases for partition comparisons). These differences did not significantly affect final lint yield of 2929 and 3022 kg/ha for the 289B and 289BR plots, respectively. Analysis of the partitioned tissues over the entire season showed a significant difference in *Cry* expression. *Cry1Ac* was significantly higher ($P=0.012$) in the roots than in the leaves, but similar to stem and FSB whilst, in contrast, *Cry2Ab* expression was significantly less in the leaves and FSB ($P<0.001$), but similar in roots and stem.

ELISA results from 0.1 g of dehydrated plant material were adjusted for actual plant partition weights and used to calculate *Cry* expression means for 289B and 289BR over the 2004/05 season (Fig. **2**). This data indicated both expression pattern and level differences between *Cry1Ac* and *Cry2Ab* within the assessed plant tissues. *Cry1Ac* levels in both cultivars generally increased toward the end of the season, whilst levels of *Cry2Ab* decreased after 97 DAP (Fig. **3**). Expression levels in leaves and fruit flowers/squares/bolls (Fig. **4**) followed a similar trend with equivalent levels of expression to those of the whole plant.

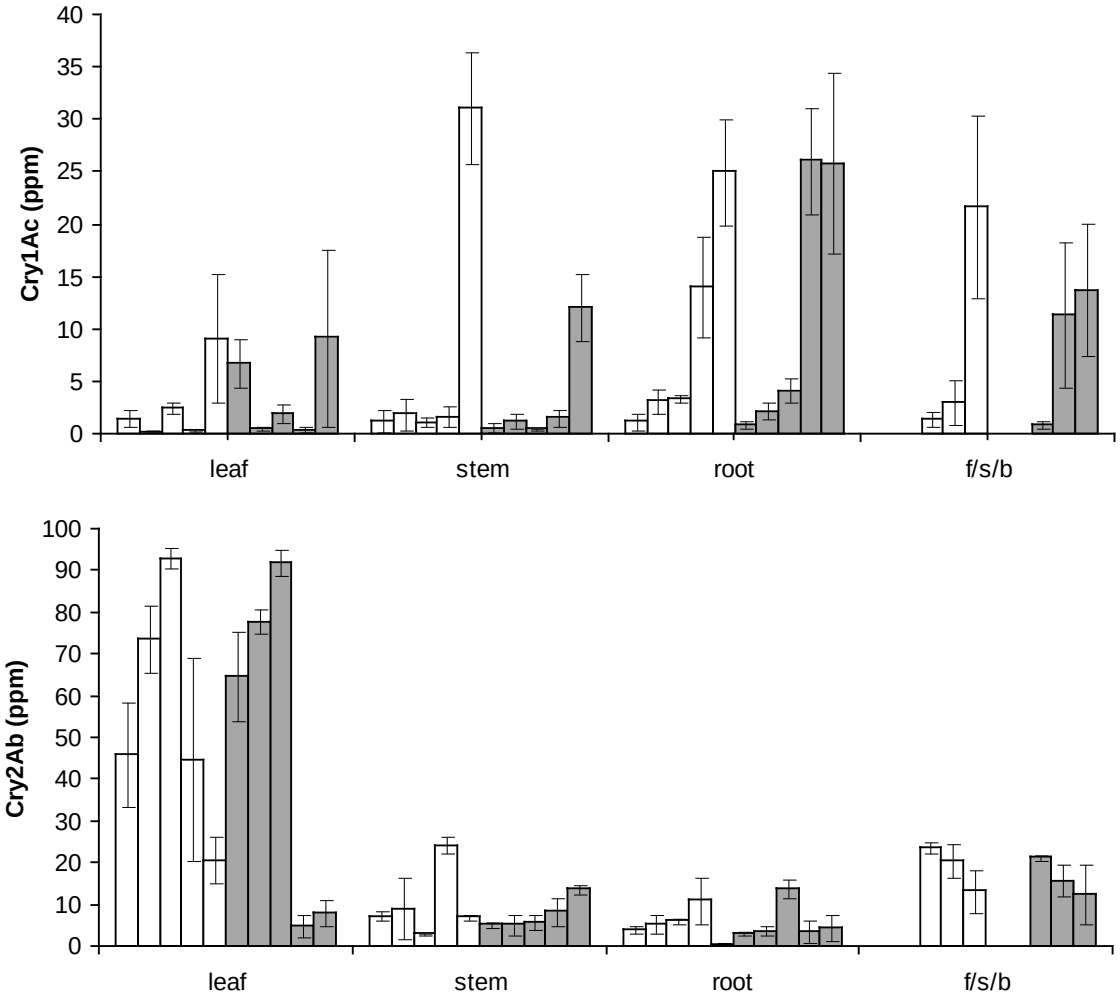

Fig. (2). PPM (μg/g) measurements of *Cry1Ac* and *Cry2Ab* in leaves, stem, root, and flowers/squares/bolls (f/s/b) of 289B (white) and 289BR (grey). Each tissue was measured at 5 times over the 2004/05 season at 34, 54, 97, 137 and 193 days (indicated as 1 to 5 on axis, respectively) after planting. The error bar represents the standard error of the means, based on a minimum of four partitioned plants.

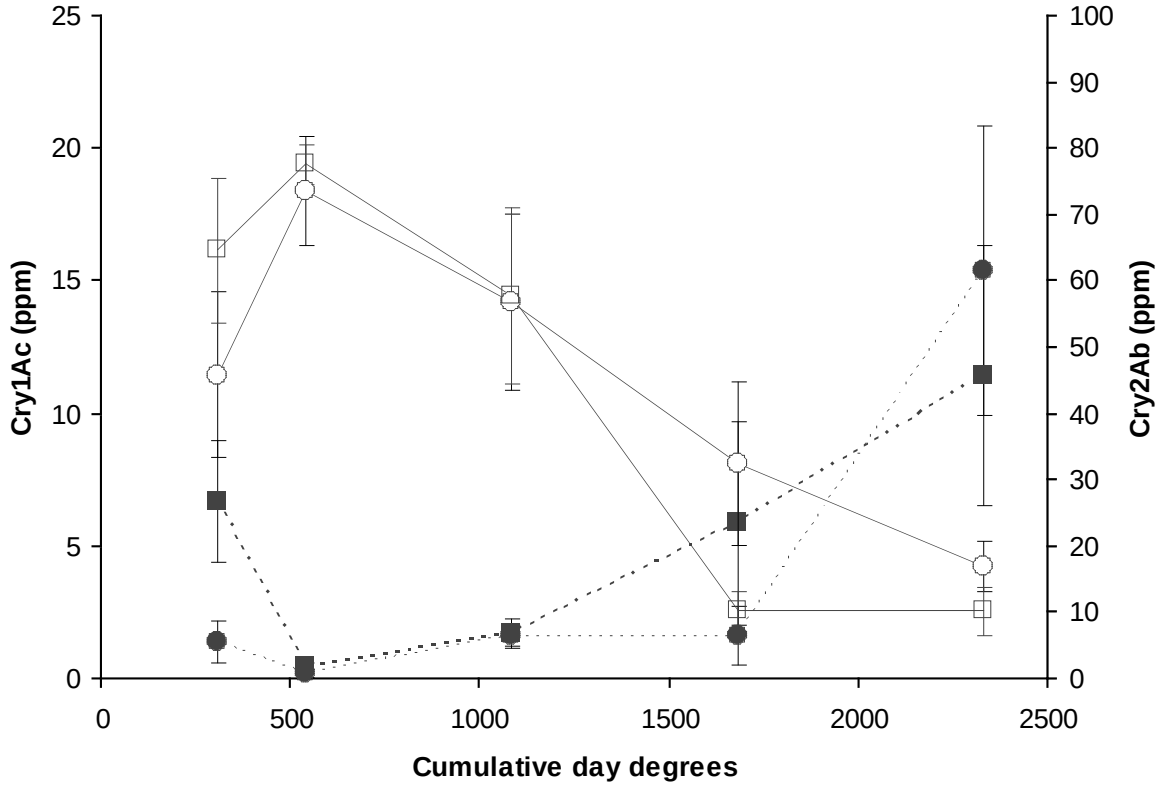

Fig. (3). Seasonal variation in the expression of *Cry1Ac* (closed) and *Cry2Ab* (open) in whole plants as analysed by ELISA of dehydrated field grown Sicot 289B (circles) and 289BR (squares). Means of analyses of 4 partitioned plants (representing 12 to 16 samples) at each point are presented. Error bars represent the standard error of the means. Time throughout the season is expressed as cumulative day degrees.

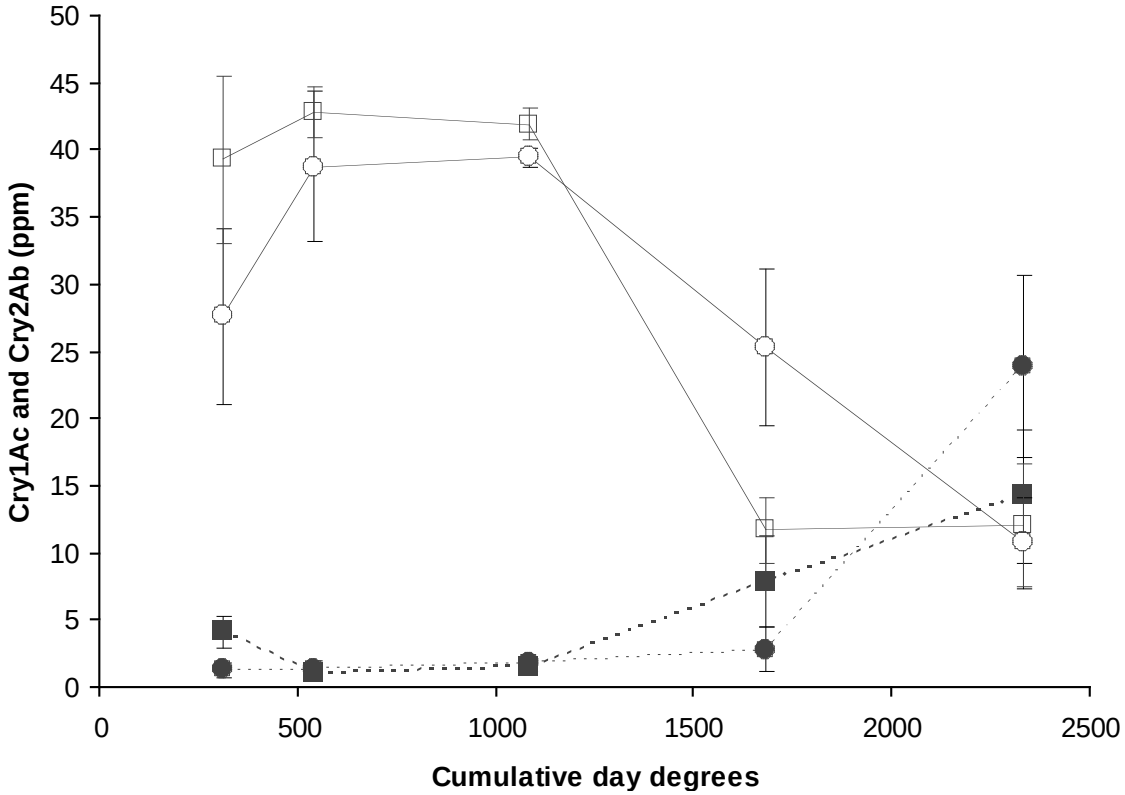

Fig. (4). Seasonal variation in the expression of *Cry1Ac* (closed) and *Cry2Ab* (open) in leaf and flower/square/boll material from field grown Sicot 289B (circles) and 289BR (squares). Means of 4 plants at each sample point are presented with error bars representing the standard error of the means. Time throughout the season is expressed as cumulative day degrees.

Statistical analysis revealed that time of sampling and tissue type both resulted in significant differences in detected *Cry* protein levels (main effect for both tests, $P \leq 0.05$), but that there was no significant difference in expression of *Cry1Ac* or *Cry2Ab* between 289B and 289BR or any interaction between variety, partitioned tissue and sampling time (in all cases $P > 0.10$).

Environmental Loading of Cry Proteins

The mass of *Cry* proteins incorporated into the soil at the end of the season were estimated from the 193 DAP (7 days post defoliation) sampling, which had mean *Cry1Ac* and *Cry2Ab* levels of 19.1 µg/g and 11.5 µg/g, respectively. Plant biomass at this time had a mean of 385.3 g dry weight per plant with a stand count of 8 plants per metre, which represented 8000 plants in a hectare. The top 15 cm of field soil, the depth to which the soil was mechanically worked post slashing of the crop, had an averaged bulk density of 1.49 g/cm^3. This data provided an estimate for the soil mass, into which the plants were incorporated at the end of season of 2.24×10^6 kg. Stands of 12 plants per metre, the industry recommended plant density, were recorded elsewhere on the station and an assessment based on 12 plants per metre was included for comparison (Table **1**).

DISCUSSION

The quantitative *Cry1Ac* ELISA method used in the initial investigation of glasshouse versus field material has been used extensively in other work [23] and found to produce highly repeatable results. Our decision to desiccate and grind plant material was made based on existing facilities, the requirement to process both soft and woody plant tissue, and to facilitate the simultaneous throughput of samples in a 96 well format. A preferred method of tissue preparation and storage prior to extraction would have been freeze drying [20], but was not available to us at the time of the experimentation.

Comparison of the roots from glasshouse and field grown plants showed that expression of *Cry1Ac* varied with plant growth and environment. The glasshouse material experienced a steady decline in expression as plant age increased. In contrast, our field root material measurements showed that expression levels of *Cry1Ac* increased over the season. Several factors may have contributed to these differences, particularly different root architecture. Differences in the physiology of the field and glasshouse root systems could have implications for plant nutrient and water acquisition [24] that could in turn affect *Cry1Ac* expression levels [23, 25]. As well as visual differences in the plant root systems, field grown cotton was exposed to prolonged environmental wet and dry periods, several flood irrigations, diurnal temperature fluxes of approximately 20°C, and repeatedly exposed to insect pest pressures. In contrast, glasshouse plants were watered daily to prevent water stress, experienced diurnal temperature fluxes of only 12°C, and were not exposed to insect pests.

Analysis of the field grown cotton in the subsequent season permitted further evaluation of *Cry1Ac*. The change to the SDI plates facilitated improved analysis of both *Cry* proteins by adding the ability to quantify *Cry2Ab* and removed the use of a *Cry1Ab* calibrator for *Cry1Ac* assessments, which increased detection levels (direct comparison not shown, see Figs. (**1**) and (**2**)). With the change of plates and seasons came a change in the extraction methodology. A ball mill was used to grind the desiccated plant material, in place of the coffee grinder, which provided a much finer and more evenly disrupted material to work on. This change in the extraction methodology was considered to be the major reason behind the increase in *Cry1Ac* detected between the 2003/04 and 2004/05 seasons, although changes in plate manufacturers were also made.

Expression of *Cry1Ac* increased toward the end of the season in most of the tested tissues (Fig. **2**) and when assessed in whole plants (Fig. **3**), which is in contrast to some published literature [26]. Possible explanations for this discrepancy are due to the use of field plants, the environmental conditions of the particular season, and season length, which in Australia is around 180 days, but in the United States of America is nearer to 150 days. Our measured increase in *Cry1Ac* expression occurred at 137 DAP (Figs **3** and **4**), which would correspond to very late season sampling in America, when fruit loads are largely determined, as opposed to a period of continued crop development within the longer Australian season.

In contrast to *Cry1Ac*, in most of the tested tissues *Cry2Ab* peaked early in the season and then declined after 1000 day degrees (Figs. **2-4**). This pattern of expression of *Cry2Ab* was similar to that reported by Adamczyk *et al.* (2001) working with DP50 Bollgard® II [26]. Reasons for this decline in *Cry2Ab* expression are currently unknown and could be due to either one or a combination of a number of factors. Differences in transformation events and promoters

Table 1. The *Cry1Ac* and *Cry2Ab* Levels from Field Grown Cotton Obtained in this Study Compared to Values Available from Previously Reported Studies (Sims and Ream 1997; U.S. EPA. 2002). FW = Fresh Weight, DW = Dry Weight

	Sample Preparation	Tissue Type	Plants	ug/g Expressed		Plant Mass		g/ha		ug/g Soil	
			Per ha	*Cry1Ac*	*Cry2Ab*			*Cry1Ac*	*Cry2Ab*	*Cry1Ac*	*Cry2Ab*
8 Plants/m	Dehydrated and ground	Partitioned/whole	80000	19.14	11.5	385.3 g	DW	590.0	354.5	0.263	0.158
12 Plants/m	Dehydrated and ground	Partitioned/whole	120000	19.14	11.5	385.3 g	DW	885.0	531.7	0.395	0.237
BRAD	*Not reported*	*Not reported*	149400					3.56		0.0016	
EPA	*Not reported*	*Not reported*						1.44			
Sims & Ream	Fresh leaf	Leaf	149400	34		238 g	FW	1174		1.6	
Head *et al.*	Dry cotton plant	*Not reported*	149400	20		250 g	DW	747		0.65	

could affect transgenic protein stability [25], however, all commercial cotton varieties derived their transgenic material from specific and stable insertion events MON531 [27] for *Cry1Ac* and MON15985 [10] for the *Cry2Ab*. This similarity in the inserted transgenic material suggests that variation in expression levels of *Cry* proteins is more likely to reflect differences between the parentage of tested lines, plant stress and/or the environmental conditions of the season [28]. Transcriptional regulation and temperature differences have been reported to effect *Cry1Ac* expression [7, 29] and could affect *Cry2Ab*. Whilst purified *Cry2Ab* has been reported as temperature stable in water [12], we observed instability of the Corn powder *Cry2Ab* standard when freeze/thawed. Onset of higher summer temperatures could also increase expression of heat shock proteins to the detriment of other proteins [28]. Whatever the reason for the decrease in *Cry2Ab* expression, it was common to both the assessed 289B and 289BR cultivars tested [6, 23, 30].

Cry protein expression data can facilitate the assessment of environmental loading of the Bt proteins from the transgenic plants [2, 18, 31] and provide information on seasonal expression levels of relevance in continued evolution of insecticidal resistance [9, 32]. Previous assessments of the cotton plant contribution of *Cry* proteins to the soil post season have been made [33-35], however, these analyses differed from ours on a number of aspects; (i) the reports dealt solely with *Cry1Ac*, (ii) *Cry* protein levels were not assessed for the whole plant, but predominantly from leaf material, (iii) plant population measures were estimated, and (iv) field incorporation was not based on actual management strategies.

In our study, we estimated an end of season incorporation of 0.26 µg/g soil *Cry1Ac* and 0.16 µg/g soil for *Cry2Ab*, similar to findings of previously published studies [12, 33, 35, 36] (Sims and Ream 1997; U.S. EPA. 2002), but this record presents the first estimate of both *Cry1Ac* and *Cry2Ab* that we are aware of. There are, however, constraints and sources of error even within our estimates. We assumed equal extraction efficiency of both *Cry* proteins from all plant tissues, but in reality the composition of different tissues and their age is likely to impact on both the readiness by which the proteins are extracted and the content of other substances that could inhibit the ELISA [37-39]. Tissue extraction is clearly important [20], but in the absence of access to freeze drying facilities we opted for a method that permitted extraction of all plant tissues, whether soft or woody. During our experiments we obtained experimental evidence that desiccating and grinding leaf tissues caused a 2.5 increase in the levels of detected *Cry1Ac*, but reduced levels of *Cry2Ab* to 0.4, when compared to fresh leaf tissue extractions. Field loading of *Cry* protein through root exudation or plant senescence and degradation was not included in our assessment as there is currently insufficient understanding of the levels of deposition, persistence and degradation of transgenic *Cry* proteins within soil from these processes [12, 34]. The amount of root material recovered averaged only 0.093 of the recovered plant dry weight biomass, which although similar to other published data [40] did not represent complete root system recovery. Our expression measurements are for 8 plants per m plant stand and evidence exists that Bt expression is density dependant [18] therefore our estimations for 12 plants per m plant stand are estimations only.

Much of the work on the expression levels of Bt proteins in BollgardII® cotton has been assessed or inferred from experiments involving (i) transgenic donor material that is rarely grown commercially [13], (ii) plants reared in glasshouse conditions [38], and (iii) field trials of non-commercial varieties often containing *Cry* constructs that were not commercially released [35, 41, 42]. This study highlights that physiological and environmental differences between glasshouse and field grown cotton have an effect on the expression levels of transgenic *Cry* proteins.

CONCLUSION

Analysis of *Cry1Ac* protein in glasshouse and field grown cotton suggested that environmental factors contributed to both plant physiology and expression levels of transgenic proteins within roots. Field analysis of whole plants indicated that *Cry1Ac* and *Cry2Ab*, the two transgenic proteins found in Bollgard®II cotton, differ in their expression profiles over the growing season. The ELISA methods used provided reproducible estimates of both proteins from specific standards at levels ranging from 0 to 60 PPB and 0 to 70 PPB for *Cry1Ac* and *Cry2Ab*, respectively, allowing estimates of the amount of *Cry* protein incorporated back into the soil at the end of the season to be made. These estimates were comparable with previous reports and present an improvement to currently available data due to the assessment of both *Cry* proteins in all tissues of field grown cotton. The reported values for protein loads are, however, still estimates.

Analysis of the various plant tissues revealed significant differences in expression levels between above and below ground tissues, but that overall plant trends were similar. Differences between partitioned tissues' level of expression, of both *Cry1Ac* and *Cry2Ab*, indicated that there is a requirement to analyse entire plants to improve environmental loading predictions. Our results would imply that assessments based solely on leaf material would under estimate *Cry1Ac* expression and overestimate *Cry2Ab* expression (Fig. **2**). Whilst ELISA is currently the preferred way to interrogate specific protein expression from a range of tissues, there is room to improve the system in terms of standard selection and tissue preparation methodology, and the provision and use of suitable and accurate standards. The outlined discrepancies between results obtained in this study and those presented in others, investigating expression levels in transgenic plants, highlights the need for standardised and reproducible field procedures, and the assessment of transgenic cultivars on a case by case and season by season basis.

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