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Full Length Research Paper

Impact of Physiological Senescence on Cry1Ac Toxin Expression and Leaf Chemistry in Transgenic Cotton

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Quantification of Cry 1Ac protein in two field-grown Bt (*Bacillus thuringiensis*) cotton hybrids (MECH- 184, RCH-2) was performed in relation to tissue maturity. Leaves of upper, middle and lower canopies and in bolls and bracts attached to the plant were chosen. Similar measurements were also made in fully mature tagged leaves attached to plants in the upper canopy and excised leaf discs. The leaf discs were incubated in the dark following the ELISA method with commercially available kits. Cry 1Ac levels declined with tissue maturity and senescence in both attached and excised plant parts of the Bt cotton hybrids examined. With advancing maturity in leaves attached to the plants till 21 days, steady increase in the levels of chlorophyll and decrease in the amounts of total protein were observed. Moreover, with increasing maturity, the concentration of reducing sugars rise in contrast to decline in total soluble amino acids. On the other hand, in case of the excised leaf discs, while total soluble amino acids exhibited an increasing trend, chlorophyll, total protein and reducing sugar contents decreased gradually.

Key words: Bt cotton, senescence, ELISA, Cry 1Ac insecticidal protein, amino acids, reducing sugars, chlorophyll.

INTRODUCTION

The transgenic Bt cotton is rapidly dominating the world agriculture (Ismael et al., 2002). With the genetically modified (GM) crops grown on 90 million ha globally in 2005 (James, 2005). Among these, transgenic cotton expressing insecticidal proteins from *Bacillus thuringiensis* (Bt) is one of the most adopted GM crops in the world (Dong et al., 2005). Bt cotton is considerably effective in controlling lepidopteran pests owing to the presence of Cry genes such as Cry 1Ac, Cry 1Ac + Cry 2Ab or Cry 1Ac + Cry 1F. Moreover, they are beneficial to the grower and the environment as they reduce chemical insecticides and preserving population of beneficial arthropods. However, poor performance of the transgenic traits during boll period and variable performance between different regions has been reported (Olsen and Daly, 2000). The loss of efficacy is associated with a reduction in insecticidal proteins and production of toxin. Those are in turn influenced by plant age, reproductive stage and/or by a variety of environmental factors (Wu et al., 1997). Defoliation is induced by crop senescence which is characterized by loss of chlorophyll and ribonucleic acids (RNA), breakdown of proteins and complex forms of carbohydrates, decrease in inorganic ion levels and resulting in stimulation or inhibition of enzymes due to changes in hormone levels.

A field investigation indicated that the reduction of the insect-resistant efficacy for Bt cotton was due to senescence of the plant cells (Benedict et al., 1993). Hence to ensure that resistance management strategies designed for use with transgenic cotton is successful, the assessment of the insecticidal protein expression by senescence becomes important. It is indicated that insecticidal protein content in Bt cotton is variable with plant age, plant structure and environmental stresses. Variability in Bt cotton efficacy against target insect pests is mainly attributed to the changes in Bt protein content. Still physiological changes associated with the

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production of secondary compounds in plant tissues may also play an important role. As a part of total protein, the insecticidal protein in plant tissues changes its level through inhibited synthesis, degradation or translocation to developing plant parts particularly under environment stresses (Dong et al., 2006). The synthesis of the Bt protein and its cycle in plant was controlled by several key enzymes such as NR, GPT, GOT, protease and peptidase (Steward, 1965). Control efficacy of Bt cotton is dependent upon the expression of Cry genes through synthesis of insecticidal protein in Bt cotton (Gutierrez et al., 2006). The temporal, spatial and environmental variation of efficacy may lead to insufficient control of targeted pests and evolution of resistance to Bt cotton. In recent years, it is becoming increasingly evident that several natural and induced defense mechanisms operate in host plants against different diseases. One of such defense mechanism is the presence of certain compounds inhibitory to the pathogen. Sometimes, the host plant is induced to synthesize these compounds on infection. In view of present day of Bt cotton, it was thought to analyse the biochemical in Bt cotton and accordingly non-Bt cotton (Hosagoudar et al., 2008). They also reported that the leaf insecticidal protein content of Bt cotton has close correlation with amino acids, chlorophyll and reducing sugars. This indicates that the biochemical aspects of the Bt cotton and the Bt protein content were reduced due to excised and intact senescence. This research aims to: (i) monitor Cry 1Ac protein in upper, middle, lower canopies, bolls and bracts at different ages of the plant, (ii) assess Cry 1Ac levels in relation to chlorophyll, total protein content and soluble amino acids in tagged intact leaves at different ages of their maturity and (iii) time course determination of Cry 1Ac levels in relation to chlorophyll, total protein content and soluble amino acids in detached dark-incubated leaves.

MATERIALS AND METHODS

Plant material and experimental design

The experiment was conducted under field conditions at International Institute of Biotechnology and Toxicology, Paddapai, India (Temperature maintained during season 32°C) during 2005 - 2006 in cotton growing season. Two Bt transgenic varieties (Medium in maturity, *Gossypium hirsutum L)* such as Mahyco cotton hybrid cotton (MECH)-184 (Maharashtra Hybrid seeds company Ltd, Jalna, India) and Rasi cotton hybrid (RCH)-2 (RASI seeds Pvt Ltd, Attur, Tamil Nadu) were grown. The plot size was 40 sq.m laid in Complete Randomized Block Design with four treatments replicated thrice. A spacing of 90 x 60 cm for irrigation was maintained and the seeds were dibbled. The organic manure at 10 to 12.5 tons per hectare was incorporated into soil for 3 to 4 weeks before sowing. The basal dose of 40:60:40 kg of N: P: K per hectare was applied at the time of sowing. Then the first and second top dressing was done, 30 days after sowing (DAS) and 60 DAS @ 40:0:20 kg of N: P: K per hectare. The irrigation was done at the critical stages of crop growth that is, germination, seedling growth, flowering and boll formation. The Bt varieties MECH-184 and RCH-2 flowered at 38 and 45 DAS.

Sample preparation

After square initiation, leaf samples were collected from different parts on the upper, middle, lower canopy, bolls and bracts of the plant on day 50, 70, 110 and 130; flowers were collected on day 38 and 45 from the squares. All the samples were frozen in liquid nitrogen, lyophilized and stored in -20°C for the quantification of the Cry 1Ac protein content.

Intact senescence

Phenotypically similar leaves and bolls were marked in the initial budding stage of the Bt cotton plant. The leaf samples were collected on day 0, 7, 14, 21 and 28. The samples were lyophilized on the same day and stored in -20°C for the estimation of Cry 1Ac protein. Ethanol extraction was done from leaves and bolls for the estimation of chlorophyll, reducing sugars, amino acids and total proteins.

Excised senescence

Phenotyphically similar leaves were collected during initial budding stage and the leaf discs were immersed in petri plates containing 10 ml of sterile water and incubated in dark. The leaf discs were taken from the petri plate on day 0, 2, 4, 6, 8 and 10, lyophilized and stored in -20°C for the estimation of Cry 1Ac protein. Ethanol extraction of the leaves was done for the estimation of chlorophyll, reducing sugars, amino acids and total proteins.

Cry 1Ac protein concentration assay

The Cry 1Ac protein in cotton leaf extracts was determined through immunological analysis by means of the ELISA (Shan et al., 2007). Leaf tissue extract of about 0.5 mg was prepared by homogenizing the lyophilized tissue in 500 µl of the ice cold 1 X sample extraction buffer (The complete recipe contains the following ingredients (in wt%): phosphate-buffered saline containing 0.05% Tween 20 and 1% polyvinyl pyrrolidone and the extract was then treated with trypsin). The lyophilized tissue was macerated at 3000 rpm using mortar driven pestle for 30 s, chilled on ice for 30 s and macerated again for 30 s then centrifuged at 8000 rpm for 15 min. Then the supernatant was collected for the quantification of Cry 1Ac. The antibodies against the Cry 1Ac, that is, goat anti-Cry 1Ac *(Ab2)* was added to each well. Buffer blank, standards, positive and negative controls were added to each well and incubated at 37°C for 1.5 h in a humid environment. AP–Conjugated Affinipure Donkey Anti-Goat IgG (Cat.No.705-0550147, Jackson immunological Research Laboratories Inc., USA) was added next and incubated at 37°C for 45 min in humid environment. Finally, the buffered enzyme substrate pNPP was added and the enzyme reaction was carried in dark at room temperature for 30 min. The absorbance was measured at 405 nm.

Assay of chlorophyll, amino acids, reducing sugars and total proteins

The extraction of the plant tissues in alcohol were prepared by using the following procedure. Four leaf discs of the same size were plunged into hot 80% ethanol (10 ml) and maintained to boil for 10 min at 60°C. Then the tissue was ground in a mortar with pestle and filtered through the Whatman No. 41 filter paper. The

Table 1. Lethal dose of Cry 1Ac level in the leaves of different canopies and in bolls and bracts of two Bt cotton cultivars MECH-184 and RCH-2. Values in µg/g are mean ± SD of three observations

DAS*	Upper canopy		Middle canopy		Lower canopy		Bolls		Bracts	
	MECH-184	RCH-2	MECH-184	RCH-2	MECH-184	RCH-2	MECH-184	RCH-2	MECH-184	RCH-2
50	2.67±0.7^{a}	3.77±0.4^{a}	2.52±0.8^{a}	3.52±0.6^{a}	2.42±0.9^{a}	3.21±1.8^{a}	2.26±0.5^{a}	2.24±0.1^{a}	1.50±0.1^{a}	2.25±1.9^{a}
70	1.62±0.5^{b}	2.77±0.5^{b}	1.28±0.2^{b}	2.24±0.2^{b}	1.16±0.4^{b}	2.40±0.5^{a}	1.26±0.2a^{b}	1.17±1.2^{b}	1.21±0.5^{a}	1.16±1.8^{bc}
90	1.20±0.2^{c}	1.16±1.6^{c}	1.14±0.1^{b}	1.22±0.5^{c}	1.01±0.9^{b}	0.94±0.3^{b}	1.04±0.9^{ab}	1.07±0.2^{b}	1.02±1.5^{a}	0.11±1.1^{c}
110	0.94±0.1^{c}	1.06±1.2^{c}	0.74±0.3^{b}	0.96±0.8^{c}	0.88±0.2^{b}	0.57±0.0^{b}	0.99±0.5^{ab}	0.98±0.8^{b}	0.94±0.8^{a}	0.01±1.5^{c}
130	0.21±0.1^{b}	0.71±0.6^{d}	0.34±0.01^{b}	0.01±0.5^{c}	0.25±0.01^{b}	0.06±0.1^{b}	0.07±0.2^{b}	0.01±1.5^{c}	0.12±0.2^{a}	0.01±0.01^{bc}

*DAS – Days after sowing. Mean values of different superscript letters (a, b, c) are significantly different (P<0.05) as determined by Duncan's multiple range test.

extract was made up to 5 ml for the estimation of chlorophyll, amino acids and reducing sugars. Finally, the residue was used for the estimation of total proteins. The chlorophyll estimation was done on the same day and the absorbance was measured at 665 nm. The amino acid content was determined by Ninhydrin assay (Moore and Stein, 1948), absorbance readings were converted to fresh tissue (µg/g) of amino acid using a standard curve prepared with glycine. The total protein content was determined by Coomassie Blue Dye–binding assay (Bradford, 1976). The absorbance readings were converted using bovine serum albumin (BSA) as standard curve. Reducing sugar was determined by Nelson's method (Nelson, 1944), absorbance readings were converted to fresh tissue (µg/g) of reducing sugar using glucose as standard curve.

RESULTS

Cry 1Ac levels influenced by the age of Bt cotton hybrids MECH-184 and RCH-2 in different canopies

In upper canopy, the Cry 1Ac levels were initially high in a range 1.20 - 3.77 µg/g during 50 - 90 DAS. The Cry 1Ac level decreases with time in both hybrids. The decline started relatively early in the season in Bollgard MECH-184 and RCH-2 with a range of 0.21 - 1.06 µg/g during 110 - 130 DAS. In middle canopy, the Cry 1Ac levels were initially high in a range of 1.14 - 3.52 µg/g during 50 - 90 DAS. At 110 - 130 DAS, Bollgard MECH-184 and RCH-2 displayed a gradual decline of Cry 1Ac levels and the decline started relatively early in the season with a range of 0.34 - 0.96 µg/g. The Cry 1Ac expression in the lower canopy leaves initially during 50 - 90 DAS ranged between 1.01 - 3.21 µg/g in both hybrids. At 110 - 130 DAS, in both Bollgard MECH-184 and RCH-2, a gradual decline was observed and the rapid rate of decrease started relatively early in the season with a range of 0.025 - 0.57 µg/g. The Cry 1Ac expression in the bolls and bracts initially during 50 - 90 DAS ranged between 1.04 - 2.24 and 0.02 - 2.25 µg/g in both hybrids. At 110 - 130 DAS, in both Bollgard MECH-184 and RCH-2, a gradual decline was observed and rapid decrease was started relatively early in the season with a range of 0.07 - 0.98 and 0.12 - 0.01 µg/g (Table 1).

Biochemical changes influenced by senescence of intact tissues of Bt cotton hybrids

The contents of the leaf Cry 1Ac proteins, chlorophyll, reducing sugars, amino acids and total proteins were different over the duration of the intact senescence of the Bt cotton. The contents of the Cry 1Ac protein decreased from 0 to 28 days for MECH-184, which was 2.11 - 0.74 µg/g of lyophilized tissue, the content of RCH-2 1.62 - 0.87 µg/g of lyophilized tissue. The chlorophyll content was increased for both MECH-184 and RCH-2 varieties with the range of 1.52 - 2.90 and 1.37 - 2.21 absorbance at 665 nm respectively. The results of the reducing sugar decreased between 0 to 28 days for MECH-184, which was 18.66 - 40.26 µg/g of fresh weight and the content of RCH-2 were 14.56 - 62.67 µg/g of fresh weight. The contents of leaf amino acid decreased from 0 to 28 days for MECH-184, which was 7.94 - 1.03 µg/g of fresh weight, the contents of RCH-2 was 8.77 - 1.66 µg/g of fresh weight. The total protein content in the leaf decreased from 0 to 28 days for MECH-184, which was 13.21 - 3.13 µg/g of fresh weight; the contents of RCH-2 were 15.55 - 5.62 µg/g of fresh weight (Figure 1).

Biochemical changes influenced by senescence of excised leaves of Bt cotton hybrids

The contents of the leaf Cry 1Ac proteins, chlorophyll, reducing sugars, amino acids and

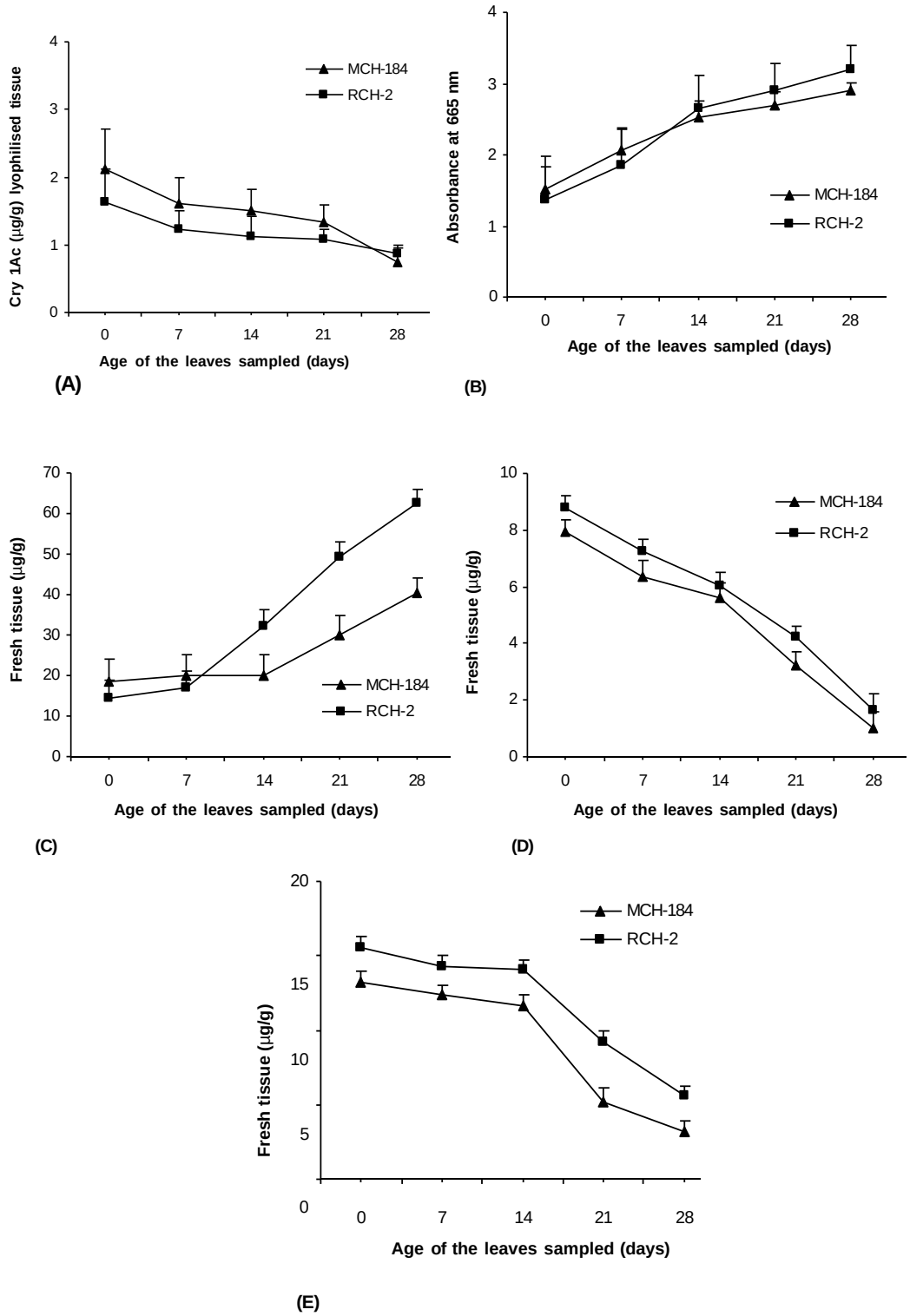

Figure 1. Changes in (A) Cry 1Ac expression, (B) chlorophyll, (C) reducing sugar, (D) amino acids and (E) total proteins of the intact senescence. Symbol MCH-184 and RCH-2 are the name of the two Bt cultivars, vertical bar represent S.E. of the mean (n = 4), when value exceeds the size of the symbol. The value at 0 day was the level of the control.

total proteins were different over the duration of the intact senescence of the Bt cotton. The contents of the Cry 1Ac protein decreased from 0 to 28 days for MECH-184, which was 2.64 - 1.15 µg/g of lyophilized tissue and the content of RCH-2 was 2.73 - 0.56 µg/g of lyophilized tissue. The chlorophyll content decreased for both

MECH-184 and RCH-2 with the range of 2.58 - 0.52 and 1.92 - 0.16, absorbance at 665nm respectively. The results of the reducing sugar decreased during 0 to 28 days for MECH-184, which was 40.57 - 14.66 µg/g of fresh weight and the content of RCH-2 was 50.25 - 10.09 µg/g of fresh weight. The contents of the leaf amino acid decreased from 0 to 28 days for MECH-184, which was 2.01 - 6.11 µg/g of fresh weight, the content of RCH-2 was 1.20 - 7.05 µg/g of fresh weight. The total protein content in the leaf decreased from 0 to 28 days for MECH-184, which was 7.21 - 1.89 µg/g of fresh weight; the contents of RCH-2 were 8.97 - 2.90 µg/g of freshweight (Figure 2).

DISCUSSION

Cry 1Ac expression in different durations of cotton growth

Dong and Li (2007) reported that the efficacy of transgenic Bt cotton against target pests varies with plant age, plant part or structure and also due to aging of plants. It is also considerably affected by environmental stresses, such as high temperature, heavy drought, water logging, elevated CO_2 and nitrogen deficiency (Luo et al., 2008). They also reported that the variability in efficacy is mostly attributed to reduction in the amount of endotoxin proteins in plant tissues, but physiological changes accompanied with production of some secondary compounds also play an important role in changes in Bt cotton. Chen et al. (2000) demonstrated that toxin protein content in the fully expanded leaves was significantly higher than those in roots, stems and petioles. During the seedling stage, ovaries at anthesis expressed considerably more toxin protein than pistils and stamens at the flowering stage. Based upon multi-site experiments with 35 transgenic Bt cotton varieties, Greenplate et al. (1999) suggested that differences in field efficacy for less sensitive insect species such as armyworm and cotton bollworm are likely functions of differences in levels of Cry 1Ac as influenced by plant age, field site and variety background. Kranthi et al. (2005) reported that the quantitative levels of Cry 1Ac and the seasonal decline in expression differed significantly among the Bollgard hybrids and also between the different parts of the plant. The Cry 1Ac expression declines progressively over the crop growth with toxin level falling below the critical level of 1.9 µg/g after 110 DAS. The variability in toxin expression and the pest control properties are unlikely to be affected significantly at least, until the crops are 100 - 125 days old (Greenplate et al., 1998).

The Cry 1Ac δ-endotoxin protein content decreased as plant ages during the profiling season–long expression in Bollgard cotton (Adamczyk and Sumerford, 2001). Mahon et al. (2002) commented that salinity stress (200 *mM* NaCl) significantly decreases the insecticidal protein leaves, but it does not affect the control efficacy against Bollworm in terms of Bioassay results. Changes in efficacy are seen to be mediated through modification of the physiological background of the plant rather than changes in the level of Cry 1Ac expression or in the concentration of Bt toxin. The secondary compounds alter the toxicity of Bt proteins against lepidopteran larvae, either negatively or positively (Olsen and Daly, 2000; Zhang and Guo, 2000). Loss of reproductive organ can be induced by myriad of causes and such losses can elicit many morphological and physiological responses including compensatory growth. They also suggested that fruit loss may enhance photosynthetic rate in cotton. In comparison to the control, the leaf insecticidal protein content was reduced for both the hybrids after 50 DAS and suggests a rapid decline in Cry 1Ac expression in the early stage of the different canopies and in bolls and bracts.

Effect of environmental stress on Bt cotton

Boyer (1982) and Martins et al. (2008) explained that environmental stresses, such as extreme levels of light, temperature, water deficit, salinity or nutrient deficiency, reduce the agricultural production and quality of many crops. In transgenic Bt cotton, the Cry gene expression in terms of concentration of toxin proteins is impacted by environmental factors. Reduction in toxin concentration with the elevated CO_2 was found in a Bt transgenic cotton cultivar Ck-12 (Coviella et al., 2002). The elevated CO_2 enhances water-use efficiency and photosynthetic production of the crop (Samarakoon and Gifford, 2004). Chen et al. (2005) commented that the exposure of the Bt transgenic cotton to high temperature (37°C) significantly reduced the Cry 1Ac protein content at boll-setting stage. Under some circumstances, the amount of insecticidal protein in Bt cotton tissues is considerably reduced, but the toxin level does not fall below the critical level, and still maintains a relative high efficacy against the insect pest. They added that exposure of Bt transgenic cotton plants to high temperature resulted in a significant decline in glutamic-pyruvic transaminase (GPT) activity and soluble protein content, suggesting that high temperature may result in the degradation of soluble protein in the leaf, with a resulting decline in the level of the toxin Cry 1A. Otherwise Abel and Adamczyk (2004) reported that low chlorophyll content of leaf tissue does not fully express Cry 1A and further suggested that photosynthesis regulating factors related to mRNA transcription and translation should have effects on Cry 1A production and insect control.

Olsen and Daly (2000) concluded that there is less Bt protein in older plants and it appears that the protein is either less available or less toxic to neonates. The concentration of Cry 1Ac protein, as a proportion of total protein, also declines during the season.

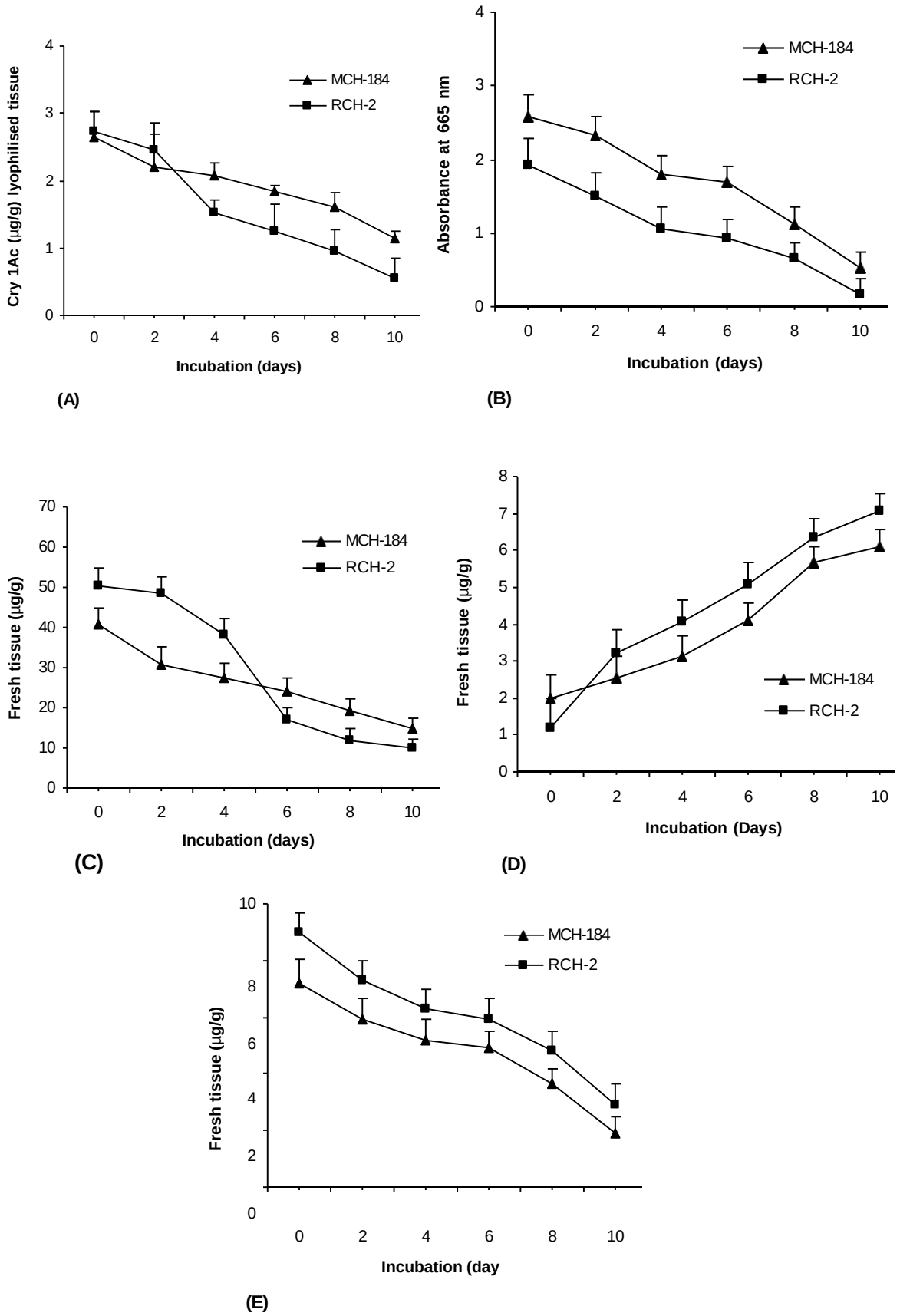

Figure 2. Time course study on the levels of (A) Cry 1Ac expression, (B) chlorophyll, (C) reducing sugar, (D) amino acids and (E) total protein content in excised leaf discs of two Bt cotton hybrids treated on water and incubated in dark. Symbol MCH-184 and RCH-2 are the name of the two Bt cultivars, vertical bar represent S.E. of the mean (n = 4), when value exceeds the size of the symbol. The value at 0 day was the level of the control.

Brown and Oosterhuis (2003) stated that chaperone, a plant growth regulator appears to increase the protein concentration and the efficiency of endotoxin expression even at high temperature stress. Pettigrew and Adamczyk (2006) mentioned that reduced levels of toxin protein in cotton leaves planted early were presumably caused by the remobilization of the leaf to support the larger developing boll load as compared with late planted cotton. There are many physiological changes in plants either as the plant ages or under environmental stresses, which may contribute to the variation in endotoxicity in Bt cotton. Studies on non transgenic cotton indicate that there is a change in the level of secondary compounds, such as phenolics and terpenoids as plants mature.

The results of the senescence induced changes in intact leaves of Bt cotton hybrid MECH-184 and RCH-2 showed that the Cry 1 Ac insecticidal protein, total proteins and reducing sugar decrease due to the aging of plant and the chlorophyll and amino acids increase. Amino acid content increases due to the breakdown of the proteins (Figure 1). In excised leaves of Bt cotton hybrids, that is, MECH-184 and RCH-2, it was observed that the Cry 1Ac, chlorophyll, reducing sugar and total protein content decreases, and the amino acid increases due to aging of plant. This can attributed to the reduction of total protein content under artificially induced senescence incubated in dark. The variation of Cry 1Ac insecticidal protein and other biochemical constituents is not only due to the transformation of the Cry 1Ac protein in the cotton hybrids but also due to the environmental conditions as well as the ageing of the plant. Therefore, it is recommended to spray 5% NSKE (Neem seed kernel extract), HaNPV (*Helicoverpa armigera* nuclear polyhedrosis virus) and farmers should mentally prepare to spray the insecticides such as thiodicarp, quinolphos, chlorpyriphos, navaluron etc., during weekly intervals of fruiting phase of crop as normally bollworm is susceptible to these insecticides (Kranthi et al., 2002). The companies should evaluate their hybrids critically for the highest levels of expression of toxin in late season.

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