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ORIGINAL ARTICLE

Antioxidative responses and expression of insecticidal proteins in Bt cotton under high irradiance

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Effect of high irradiance (HI) on the activity of antioxidant enzymes, rate of lipid peroxidation and hydrogen peroxide accumulation were investigated in non Bt and Bt cotton. The accumulation of malondialdehyde (MDA) and H_2O_2 was higher in Bt cotton. Sustained cultivation of Bt cotton requires stable transgene expression under HI stress. In the present study, Bt toxin proteins (*Cry*1Ac and *Cry*2Ab), which are essential for the control of lepidopteron pests, were found to be reduced in Bt cotton under HI stress.

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Key words: antioxidant enzymes / Bt cotton / high irradiance / insecticidal protein expression

Cotton, a cash crop, plays important role in Indian economy. However, with vast area of cotton cultivation, India ranks only third, next to China and USA in yield. One of the reasons behind the reduction in production might be due to infestation of insect pests. Bollworms (tissue borers) are the most destructive, requiring major efforts to save the crop from them. Among the bollworms, *Helicoverpa armigera* is the most dominant pest. Single *H. armigera* larvae can damage many squares and bolls.

To overcome this problem, Bt cotton with *cry* gene, from *Bacillus thuringiensis*, was introduced. For the Bt cotton to be sustainable, it is important that the toxin protein be expressed in adequate quantities to afford protection against insect pests. It is well known that environmental stresses, such as extreme levels of light, temperature, drought, salinity, or nutrient deficiency, reduce the agricultural production and quality of many crops (Boyer, 1982). The expression of transgenes is also reported to be significantly affected by severe environmental factors in genetically modified crops

including Bt transgenic maize by water stress (Traore et al., 2000), transgenic petunia that contains the gene encoding a dihydroflavonol reductase by high light intensity and temperature (Meyer et al., 1992) and transgenic Bt cotton that carries a toxin gene encoding crystal protein by elevated CO_2 (Coviella et al., 2002).

Light is required for photosynthesis, yet plants need protection from light. High light stress affects several metabolic processes in plants (Long et al., 1994; Baker, 1996). The objective of this research was to compare the physiological responses of non Bt and Bt cotton under high irradiance (HI) stress and their capability to recover from HI stress. In addition, Bt protein content was also detected, in order to better understand the efficacy of Bt cotton under HI stress.

MATERIAL AND METHODS

Plants

Bt cotton (RCH 2), which contains the toxin gene, *Cry*1Ac and *Cry*2Ab from *Bacillus thuringiensis* var. *kursitaki* Berliner, and its isogenic parent line, non-Bt cotton (RCH 2) (purchased from Rasi seeds, Tamilnadu, India), were planted in plastic pots with five seeds per pot. They were grown under controlled lab conditions at 20-25 ^{o}C, 16 hr photoperiod / 8 hr dark period. The young and fully expanded terminal leaves of 20 days old cotton plants (20 days after sowing or DAS) were used for antioxidant studies.

Illumination of leaves

Detached non-Bt and Bt cotton leaves were placed in a controlled environment chamber equipped with a 24 V/250 W metal-halide lamp. The upper leaf surface was exposed to a photosynthetic photon flux density (PPFD) of 1900 µmol m^{-2} s^{-1} for 60 min. Air temperature was 20 ^{o}C and relative humidity was 65 %. After this period, some leaves exposed to HI were returned to normal condition by adapting recovery for 60 min and the samples were analyzed as above.

Measurement of electrolyte leakage

Electrolyte leakage which is used to assess membrane permeability was determined according to Lutts et al. (1996). Leaf discs (1 cm in diameter) from two randomly chosen plants per replicate were taken from the middle portion of youngest fully developed leaf and then were placed in individual stoppered vials containing 20 ml of distilled water after three washes with distilled water to remove surface contamination. After incubating the samples at room temperature on a shaker (150 rpm) for 24 h, the electrical conductivity (EC1) was determined. The same samples were then placed in an autoclave at 121 ^{o}C for 20 min and a second reading (EC2) was determined after cooling the solution to room temperature. The electrolyte leakage was calculated as EC1/EC2 and expressed as percent.

$$\% \text{ injury} = [(\%L_{(t)} - \%L_{(c)})/(100 - \%L_{(c)})] \times 100,$$

where $\% L_{(t)}$ and $\% L_{(c)}$ are percentage ion leakage data for the treatments and control samples, respectively (Arora et al. 1992).

Oxidative damage

Lipid peroxidation was measured in terms of MDA content, as described by Davenport et al. (2003). Fresh leaves (0.2 g) were homogenized with 2.0 ml of 5 % (w/v) trichloroacetic acid (TCA) in an ice bath, and centrifuged at 10,000 x *g* for 10 min at 4 °C. 2.0 ml supernatant mixed with 2.0 ml of 0.67 % (w/v) thiobarbituric acid was incubated in boiling water for 30 min, then cooled and centrifuged. The absorbance of reaction supernatant was assayed at 450, 532, and 600 nm. The MDA content was calculated based on the following formula,

$$\text{MDA } (\mu\text{mol g}^{-1}) = [6.45 (A_{532} - A_{600}) - 0.56A_{450}] \times Vt/W,$$

where Vt = 0.002l; W = 0.2 g.

H_2O_2 content was determined according to Velikova et al. (2000). Fresh leaves (0.5 g) with 5.0

ml of 0.1 % trichloroaceticacid (TCA) were homogenized in an icebath. The homogenate was centrifuged at 10,000 x g for 10 min and 0.5 ml supernatant was mixed with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1.0 ml of 1 M KI. The absorbance of the mixture was read at 390 nm. The content of H_2O_2 was calibrated based on the standard curve.

Enzyme extraction and protein determination

Fresh leaves (0.3 g) were homogenized with 3.0 ml ice-cold extracting buffer in an ice bath. The homogenized slurry was centrifuged at 10,000 x g for 15 min at 4 °C and the supernatant was collected. The extracting buffer was 0.05 M potassium phosphate buffer (pH 7.0) containing 1 % (w/v) PVP. Protein concentration in the homogenate was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme assays

The SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (Beauchamp and Fridovich 1971). One unit of the SOD activity was defined as the amount of the enzyme required to cause 50 % inhibition of the reduction of nitro blue tetrazolium (NBT), monitored at 560 nm.

Peroxidase (POD) activity was assayed by the method of Kumar and Khan (1982). Assay mixture of POD contained 2 ml of 0.1 M phosphate buffer (pH 6.8), 1 ml of 0.01 M pyrogallol, 1 ml of 0.005 M H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. One unit is defined as the change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

Polyphenol oxidase (PPO) activity was assayed by the method of Kumar and Khan (1982). Assay mixture for PPO contained 2 ml of 0.1 M phosphate buffer (pH 6.0), 1 ml of 0.1 M catechol and 0.5 ml of enzyme extract. This was incubated for 5 min at 25 °C, after which the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The absorbancy of the purpurogallin formed was read at 495 nm. To the blank, 2.5 N H_2SO_4 was added at zero time of the same assay mixture. PPO activity was expressed in U mg^{-1} protein (U = change in 0.1 absorbance min^{-1} mg^{-1} protein).

Glutathione reductase (GR) activity was assayed at 25 °C in a 3 ml reaction volume containing 1.5 ml potassium phosphate buffer (0.1 M, pH 7.0), 150 µl GSSG (20 mM), 200 µl enzyme extract, 1 ml distilled water and 150 µl NADPH (2 mM, dissolved in Tris HCl buffer pH 7.0). GR activity was measured according to Carlberg and Mannervik (1985) by following the oxidation of NADPH spectrophotometrically at 340 nm.

Proline and ascorbic acid determination

Proline content was measured according to the method of Bates (1973). 0.5 g sample of fresh leaves was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and filtered through Whatman #2 paper. Then, 2 ml of filtrate was mixed with 2 ml of acid- ninhydrin and 2 ml of glacial acetic acid and heated at 100 °C for 1 h. The reaction was terminated in an ice bath; then 4 ml of toluene was added to the mixture and contents of tubes were stirred for 15 to 20 s. The chromophore was aspirated from the aqueous phase, and the absorbance was read at 520 nm.

Ascorbic acid content was assayed as described by Omaye et al. (1979). The extract was prepared by grinding 1 g of fresh material with 5 ml of 10 % TCA, centrifuged at 3,500 rpm for 20 min, re-

extracted twice and supernatant made up to 10 ml and used for assay. To 0.5 ml of extract, 1 ml of 6 mM 2,4-dinitrophenylhydrazine-thiourea-$CuSO_4$ (DTC) reagent was added, incubated at 37 ºC for 3 hr and 0.75 ml of ice-cold 65 % H_2SO_4 was added, allowed to stand at 30 ºC for 30 min and the resulting colour was read at 520 nm. The AA content was determined using a standard curve prepared with ascorbic acid.

Assay of insecticidal Bt protein

The level of *Cry*1Ac and *Cry*2Ab was determined in freeze-dried leaf material by an ELISA, by using a kit from Desigen Diagnostics (Maharashtra Hybrid Seed Company, Jalna, India). The test procedure described by the manufacturers was followed and optical densities of the wells were measured at 405 nm, by using a multiskan EX photometric microplate reader (Thermo Inc., Finland).

Statistical analysis

All data were subjected to ANOVA test and means were compared by the Tukey test using sigma plot 11. Comparison with P value < 0.01 was considered significantly different.

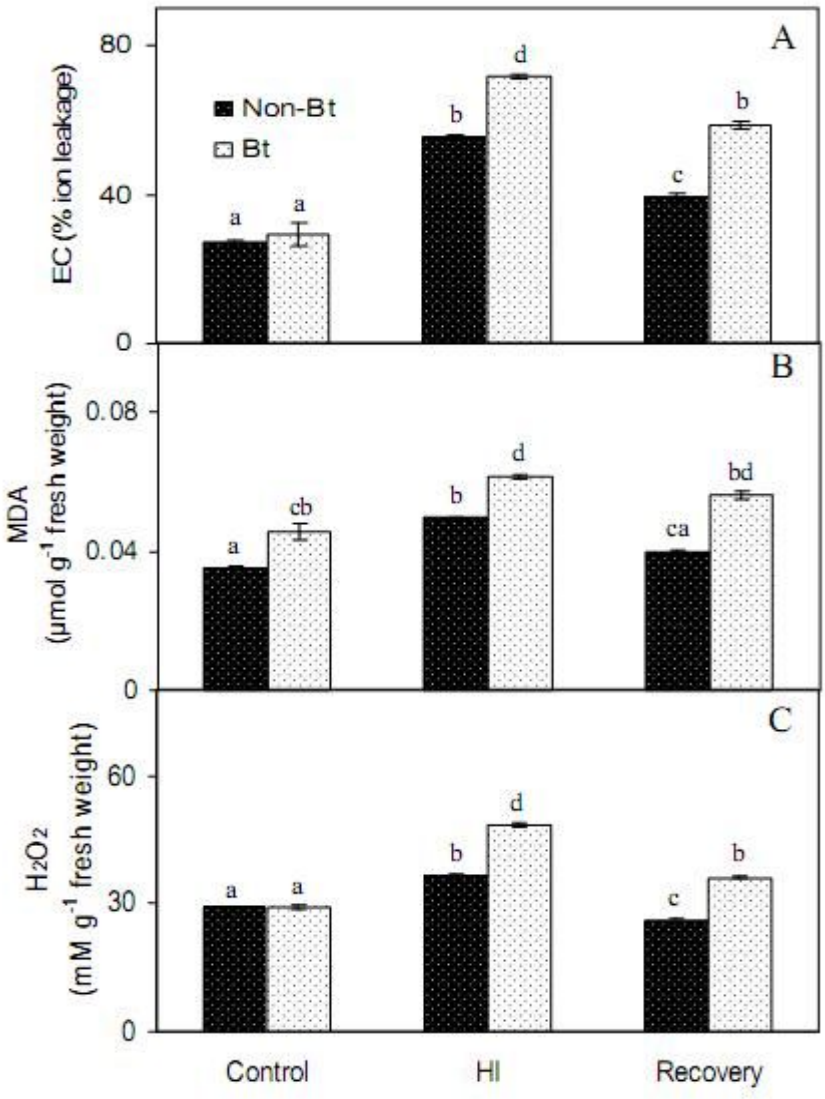

Figure 1. Changes in EC (A), MDA (B) and H_2O_2 (C) content in the leaves of non Bt and Bt cotton under HI stress and recovery. Mean ± SE (n = 5). The different letters indicate significant differences at P <0.01 as determined by Tukey test.

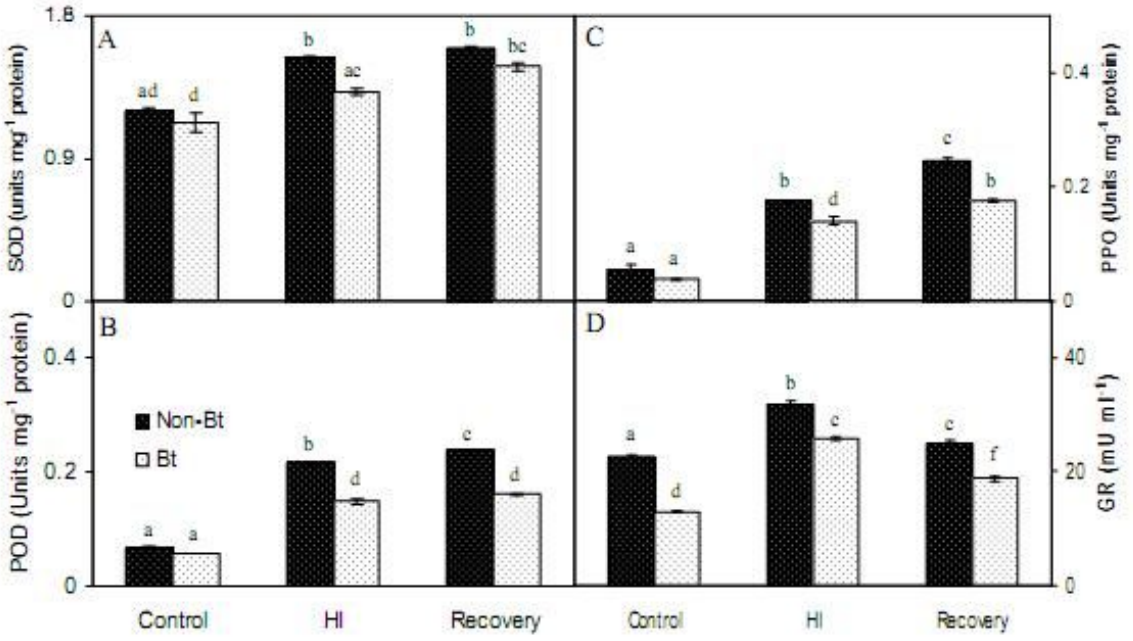

Figure 2: Alterations in the activities of SOD (A), POD (B), PPO (C) and GR (D) in the leaves of non Bt and Bt cotton leaves under HI stress and recovery. Mean ± SE (n = 5). The different letters indicate significant differences at P <0.01 as determined by Tukey test.

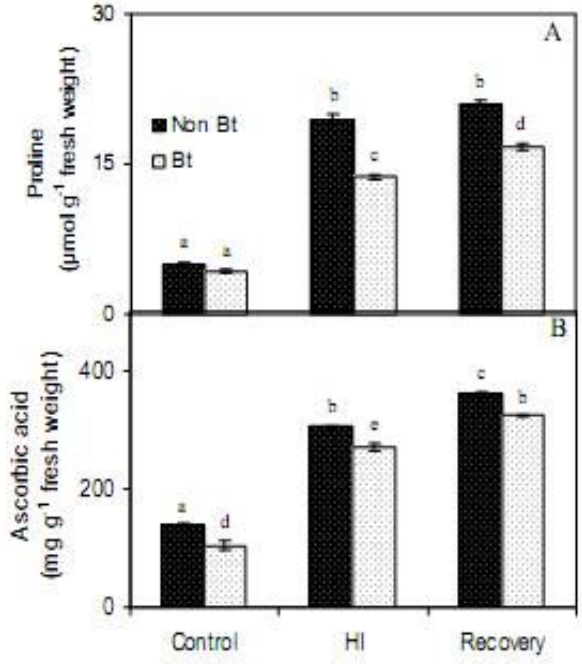

Figure 3: Changes in the contents of Proline (A) and Ascorbic acid (B) in the leaves of non Bt and Bt cotton under HI stress and recovery. Mean ± SE (n = 5). The different letters indicate significant differences at P <0.01 as determined by Tukey test.

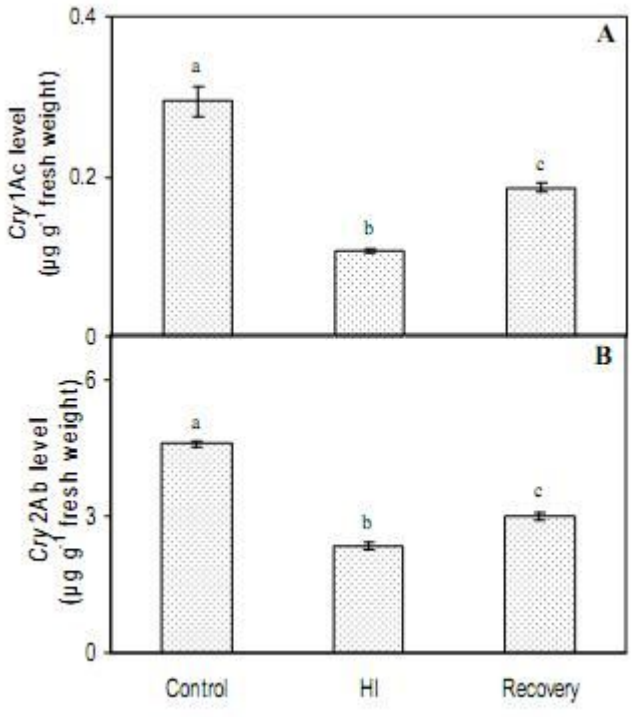

Figure 4: Production of insecticidal Bt toxin proteins *Cry*1Ac (A) and *Cry*2Ab (B) in Bt cotton under HI stress and recovery. Mean ± SE (n = 5). The different letters indicate significant differences at P <0.01 as determined by Tukey test.

RESULTS

Electrolyte leakage

Under HI stress, electrolyte leakage was significantly increased in non Bt cotton as well as in Bt cotton with respect to their control values. But the increment was more in case of Bt cotton. On the contrary, recovered leaves exhibited decreased electrolyte leakage both in non Bt and Bt cotton, but the recovery percentage was higher in non Bt cotton (Fig. 1 A). The percentage injury was higher in Bt cotton (60 %) than in non Bt cotton (39%) under HI stress.

Oxidative damage

MDA and H_2O_2 contents of non Bt and Bt cotton were shown in Fig. 1B and 1C respectively. Relative to controls, both non Bt and Bt cotton showed a significant increase in MDA and H_2O_2 level but there was a significant difference between the non Bt and Bt cotton under HI stress. Under recovery, there was a significant decrease in the content of H_2O_2 in both the cotton varieties, whereas, MDA level was significantly decreased in non Bt cotton but not in Bt cotton. Compared to Bt cotton, non Bt cotton showed maximum recovery from oxidative damage.

Antioxidant enzymes

The SOD activity was significantly increased in non Bt and Bt cotton under HI stress, but the activity was more in non Bt cotton. Similar results were observed in recovered leaves of non Bt and Bt cotton in SOD activity but there was no significant difference between HI stressed and recovered leaves of both the cotton varieties (Fig. 2A). Activities of POD and PPO were significantly increased in non Bt and Bt cotton but the activity was more in non Bt cotton. Under recovery, both non Bt and Bt cotton showed a significant increase in POD and PPO activities, but non Bt cotton maintained much higher

activity than Bt cotton (Figs. 2B and 2C). GR activity was significantly increased in non Bt cotton under HI stress than in Bt cotton. In addition, under recovery there was a significant decrease in GR activity both in non Bt and Bt cotton but the curtailment was higher in Bt cotton (Fig. 2D).

Proline and ascorbic acid

The contents of proline and ascorbic acid in non Bt and Bt cotton were significantly increased under HI stress. Similar results were observed in recovered leaves but the increment was higher in non Bt cotton (Fig. 3A and 3B). There was a significant difference between the non Bt and Bt cotton under HI stress and recovery.

Insecticidal Bt protein

The contents of *Cry*1Ac and *Cry*2Ab were shown in Fig. 4A and 4B. The decrease in both *Cry*1Ac and *Cry*2Ab contents with respect to their control values under HI stress was statistically significant. HI stress produced an immediate inhibiting effect on the expression of Bt proteins. Under recovery, Bt protein contents recovered from the stress and there was a significant difference between the recovery and control values.

DISCUSSION

The contents of electrolyte leakage, MDA, H_2O_2 and the activities of SOD, POD, PPO and GR are the important parameters in determining the physiological responses in plant leaves under stress conditions. Fig. 1 clearly shows that the increment in electrical conductivity was higher in Bt cotton than in non Bt cotton. In addition, percentage injury was also elevated in Bt cotton under HI stress. This allowed relatively quick assessment of the cellular membrane injury in Bt cotton. MDA and H_2O_2 contents were used as indicators for cellular membrane damage to assess the effect of HI stress. The generation of ROS was indicated by an increase in the lipid peroxidation in our study. There was a significant increase of MDA and H_2O_2 content both in non Bt and Bt cotton but the induction was more in Bt cotton. The increase of MDA and H_2O_2 contents in our study are in agreement with the results of other studies (Bailly et al., 1996; Queiroz et al., 1998), which supports our hypothesis that high light increases the breakdown of lipid membrane.

A significant increase in SOD, POD, PPO and GR in leaves under HI stress might have been resulted due to the formation of ROS. This enables the plants to protect themselves against the oxidative stress (Scalet et al., 1995). Our results are in good agreement with those of Khan et al. (2009) who observed a similar result in mustard under salt stress. Bray et al. (2000) and Sheen and Calvert (1969) also specified that superoxide dismutase is a major scavenger of superoxide and its enzymatic action results in the formation of H_2O_2 and O_2. POD and PPO decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. Glutathione reductase is also involved in scavenging the products of oxidative stress such as hydrogen peroxide (Gamble and Burke, 1984; Bowler et al., 1992) and thus helps in ameliorating the adverse effects of oxidative injury. Under stress, GR is responsible for maintaining the reduced form of glutathione pool (Foyer et al., 1997).

The level of antioxidant enzymes are higher in tolerant than in sensitive species under various environmental stresses (Demiral and Turkan, 2005). Accordingly, we found that the antioxidant enzymes were more in case of non Bt cotton under HI stress. This suggests that non Bt cotton is more tolerant towards HI stress than Bt cotton.

In the present investigation, the diverse response of SOD, POD, PPO and GR on HI stressed plants suggests that oxidative stress may be the influential component of environmental stresses. HI stress led to a significant increase of SOD, POD, PPO and GR

activities. But under recovery period, except GR activity, the activities of the SOD, POD and PPO were slightly increased. In contrast MDA, H_2O_2 content and electrolyte leakage were curtailed under recovery. This shows that the increasing enzyme activities lead to the decrement of oxidative damage. But the reduction was more in non Bt cotton compared to Bt cotton. The lesser degree of oxidative damage, as indicated by low electrolyte leakage, MDA, H_2O_2 and the higher activities of SOD, POD, PPO and GR in HI stressed non Bt cotton indicates that non Bt cotton has a higher capacity for scavenging ROS generated by HI stress than Bt cotton.

Proline metabolism is a typical mechanism of the biochemical adaptation in living organisms subjected to stress conditions (Delauney and Verma, 1993). In addition, like glutathione, ascorbic acid is also essentially required in scavenging of H_2O_2 by ascorbate-glutathione cycle and for elimination of ROS (Conklin, 2001). In our present study, proline and ascorbic acid contents were significantly increased under HI stress and recovery, both in non Bt and Bt cotton but the production was more in non Bt cotton. Similar results have been observed by Yao et al. (2007) in dragon spruce under UV-B stress. Increased proline in the stressed plants may be an adaptation to overcome the stress conditions. Proline accumulated under stressed conditions supplies energy for growth and survival and thereby helps the plant to tolerate stress (Jaleel et al., 2008). Proline could function as a hydroxyl radical scavenger to prevent membrane damage and protein denaturation (Ain-Lhout et al., 2001). Our result suggests that scavenging system forms the first line of defense against ROS.

In addition to the physiological changes in Bt cotton, we also found marked decline in the contents of *Cry*1Ac and *Cry*2Ab toxin proteins under HI stress. Similar reduction in toxin concentration was found in Bt transgenic cotton under elevated CO_2 (Coviella et al.,, 2002; Chen et al., 2005) and drought stress (Parimala and Muthuchelian, 2010). Mahon et al. (2002) also specified that the amount of Bt toxin present in the stressed and control plants was comparable. Decline in endotoxin proteins in cotton tissues might be due to the deletion of necessary defense genes during integration of foreign genes or it might also be due to the methylation of promoter.

CONCLUSION

Our results confirmed that ROS metabolism is crucial for the better environmental adaptation of cotton plants. It also indicated that the relative HI tolerance of non Bt cotton may be due to enhanced production of antioxidative enzymes and lower accumulation of MDA and H_2O_2. In addition, the Bt toxin proteins which are key for the control of insect pests were also reduced sharply in Bt cotton. Environmental factors that affects gene expression and protein synthesis or degradation is also likely to influence Bt toxin expression. This changeability in toxin protein contents under stress condition may lead to the evolution of resistance of pests against Bt cotton. So, the biotechnologists should be careful during integration and selection of the promoter. However, additional investigations such as the study on the influence of secondary compounds and insect bioassay are required to ascertain that the Bt cotton performance is not compromised by HI stress.

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