Int.J.Curr.Microbiol.App.Sci (2020) *9*(7): 1239-1253

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 9 Number 7 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.907.143

Physiological Responses of *Bt* cotton under Drought Stress

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Keywords

Bt cotton, Drought Stress, Physiological Responses

Article Info

Accepted:
11 June 2020
Available Online:
10 July 2020

A B S T R A C T

The present study was conducted to evaluate *Bt* cotton hybrid namely "BG 6488 II" under PEG induced drought stress. Different physiological and biochemical attributes were observed to see whether the plants which were exposed to water deficit stress were acclimatized to the drought stress by altering their metabolism at the later stages of the plant life cycle. Water deficit condition was induced after 60 days of sowing at the reproductive stage by applying 20%, 40% and 60% solution of PEG 6000 and observations were recorded 3 and 6 days after treatment (DAT). A significant decrease in physiological parameters like quantum yield of photosystem (PS) II and relative water content was observed under water deficit conditions after 3 DAT. Membrane injury, MDA content and H_2O_2 content increased significantly with increasing stress conditions. But to alleviate the deleterious effect of drought stress the antioxidant enzymes like catalase and peroxidase were upregulated and osmolytes like proline, total soluble sugar and total soluble protein content also increased with increased PEG 6000 concentration. On the other hand, plants after 6 DAT shows some acclimation to drought by increase in quantum yield of PS II, total chlorophyll content& relative water content and also shows less increase in MI, MDA content and H_2O_2 level. Similarly, catalase and peroxidase level further increased other osmolytes like proline, total soluble sugar and total soluble protein content was also increased more than 3 DAT. Taken together our findings suggest that although the plants were affected negatively by drought stress initially but they also got acclimatized to the new stress environment to sustain their life by alterations in their integrative metabolism.

Introduction

Drought stress is one of the most important factor affecting global agriculture, approximately 40% of the world's land area is under drought stress (Zhang *et al.*, 2014a). It causes decrease in chlorophyll content (Ommen *et al.*, 1999) which results in the reduction of photosynthesis (Moussa 2006). Water deficit stress also induces increased production of reactive oxygen species (ROS) like superoxide radical and hydrogen peroxide (Mittler 2002). These ROS damage the membranes causing lipid peroxidation (Smirnoff 1993). Proline accumulation has been reported to play adaptive roles in plant stress tolerance (Zhang *et al.*, 2014b). Plants protect cellular and subcellular systems from the cytotoxic free radicals with anti-oxidative enzymes such as peroxidase, and catalase

(Alscher *et al.,* 2002). Water deficit is one of the major yields limiting factor for cotton crop (Anjum *et al.,* 2012). Cotton is highly sensitive to water deficit stress during flowering and boll development (Turner *et al.,* 1986), however few studies has been done regarding effects of water-deficit stress on the physiology and metabolism of cotton plants.

Plants have evolved different mechanisms to evade water deficit stress by alteration in their metabolism such as production of compatible osmolytes such as proline, free sugar and antioxidant enzyme system. Acclimation to drought stress is the result of a series of integrated events, ranging from stress signal perception and transduction to the regulation of gene expression and metabolic changes. Upon exposure to drought stress, plants exhibit a wide range of responses at the whole-plant, cellular and metabolite levels (Chaves *et al.,* 2009).

The development of drought-tolerant crops has been hindered by the lack of knowledge of more precise physiological and biochemical parameters that reflect the genetic potential for improving productivity under water deficit conditions (Massacci *et al.,* 2008).So, the present study was taken up to investigate the effect of water deficit stress on *Bt* cotton hybrid namely "BG 6488 II" and to access whether the stressed plants get acclimated to the stress environment for protection of cotton plants against water deficit stress with respect to different physiological and biochemical attributes.

Materials and Methods

Fresh cotton hybrid seeds of BG 6488 II (*Bt* hybrid) were raised in earthen pots filled with 5kg sand dune under screen house conditions. The plants were supplied with nutrient solution (Hoagland & Arnon 1950) at regular intervals. Water stress was given by applying 20%, 40% and 60% solution of PEG 6000 treatment in soil of each pot at reproductive stage (60 DAS). Treatments are:

T_1:- Control plants
T_2:- PEG-6000 (20%)
T_3:- PEG-6000 (40%)
T_4:- PEG-6000 (60%)

Leaf samples were collected uniformly from the plants (penultimate leaf from the top) and analysed at 3 and 6 days after the treatments had been given. Experiment was conducted using two factorial CRD (Completely randomized design) with three replications for each treatment. Statistical analysis was done using OPSTAT programme for all the observations.

Physiological observations of leaves

Indices of stress

Total chlorophyll content

Leaves were washed, blotted dry then cut into discs and dipped in test tubes containing dimethyl sulfoxide (DMSO) overnight as described by Hiscox & Israelstam (1979). The extracted chlorophyll in DMSO was estimated by recording its absorbance at 663 and 645 nm.

Quantum Yield

Quantum Yield was recorded in intact plants using CIP chlorophyll fluorometer (OS-30p, Opti-Science, Inc., Hudson, USA). The fully expended leaf was first acclimated to dark for minimum two minutes by fixing clip on it. Initial (F_0) and maximum (F_m) fluorescence were recorded and variable fluorescence (F_v) was derived by substracting F_0 from F_m. Quantum yield/photochemical efficiency which is F_v/F_m, was then calculated.

Membrane injury (MI) and Relative water content (RWC)

Membrane injury was analyzed based on method of Sullivan & Ross (1979). One hundred mg of leaf tissue was taken separately in a test tube containing 10 ml of de-ionized water and, incubated for 24 hrs at 4ºC. The conductance of decanted liquid at 25ºC, designated as EC_a (Before boiling). Then the samples were in a water bath (100ºC) for 10 minutes, the electrical conductivity of the solution was designated as EC_b (After boiling).

Electrolyte leakage (%) = $(EC_a/EC_b)\times100$

The RWC (%) was calculated by using the formula given by Weatherly (1950).

RWC (%) = ((Fresh weight – Dry weight) / (Turgid weight – Dry weight))×100

Lipid peroxidation

Level of lipid peroxidation was measured by thiobarbituric acid (TBA) reaction with minor modifications of the method of Heath & Packer (1968). The OD of final reaction mixture was read at 532 nm and the value for non- specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Osmolytes

Proline content

The proline content of cell free extract was estimated by the method of Bates *et al.*, (1973).Three hundred mg of leaf tissue was homogenized in 3ml of 3% sulphosalicylic acid and centrifuged at 12,000g for 10 min. Two ml each of extract, acid-ninhdrin and glacial acetic acid was taken in a test tube. Test tubes were kept in water bath for 1 hour at 100°C and the reaction terminated in an ice bath. After this 4 ml toluene was added and mixed vigorously. Then its optical density was measured at 520 nm using toluene as blank. The proline concentration was determined from a standard curve using L-Proline.

Total soluble protein content

The protein content of cell free extract was estimated by the method of Bradford (1976). Three hundred mg sample of leaf was homogenized by using 5 ml of potassium phosphate buffer (50mM) containing 5mM polyvinyl pyrrolidone (pH 7.0). The homogenate was centrifuged at 10,000 rpm for 15 min at 4ºC. One hundred µl of extract was mixed with 5 ml of Bradford reagent, shaken well and its absorbance was recorded at 595 nm against blank. Standard curve of bovine serum albumin was used for calculation.

Total soluble sugar content (TSC)

Total soluble carbohydrates were determined with the method of Yemm & Willis (1954). Three hundred mg fresh leaf was homogenized separately in 80% ethanol. The homogenate was refluxed thrice with 80% ethanol. The supernatant from different extractions were pooled and volume made to 5 ml with 80% ethanol. An aliquot of 0.2 ml was evaporated to dryness in a test tube in a boiling water bath. On cooling the residue left in the tube was dissolved in 1 ml of distilled water and mixed with 4.0 ml of the anthrone reagent.

The mixture was heated in a water bath for 10 minutes. After cooling, absorbance was recorded at 620 nm using Spectrophotometer. Standard curve was prepared using D-Glucose.

Oxidative Metabolism

H_2O_2 content

H_2O_2 content was estimated by the method of Sinha (1972). Five hundred mg of leaf tissue was homogenized in 6 ml of chilled 0.8 N $HClO_4$ and centrifuged at 10,000 rpm for 30 minutes. Supernatant was decanted and neutralized with 5 M K_2CO_3. The supernatant obtained was carefully decanted and the corresponding volume of each preparation was recorded.

This supernatant was used for H_2O_2 estimation. Two hundred µl of extract was made to 1 ml with 0.1M phosphate buffer (pH 7.5). 2 ml of 5% potassium dichromate and glacial acetic acid (1:3 v/v) was added to it. The mixture was then heated in boiling water bath for 10 minutes and cooled. Its absorbance was read at 570 nm against reagent blank which was without sample extract.

Catalase (CAT) and Peroxidase (POX) activity

The catalase activity was estimated according to the procedure described by Aebi (1984). One gram of tissue taken and macerated in a chilled pestle & mortar in presence of 3.0 ml of cold extraction buffer (potassium phosphate) containing 0.1 mM EDTA, 1% (w/v) PVP, 0.5% triton X-100 and 20% glycerol (pH 7.8). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. Reaction was initiated with the addition of H_2O_2 and reading was taken at 240 nm for 2 minutes. The enzyme activity was calculated using the extinction coefficient value of 39.4 M^{-1} cm^{-1} for H_2O_2.

The procedure of Siegel & Siegel (1969) was followed for estimating peroxidase activity. The extraction of enzyme was same as done in CAT. Reaction was started with the addition of H_2O_2 and absorbance taken at 470 nm for 2 min. The activity was calculated using the extinction coefficient value of 26.6 mM^{-1} cm^{-1} for guaicol.

Results and Discussion

Pigment

On the 3rd day after treatment it was observed that total chlorophyll content decreased significantly with 20%, 40 and 60% PEG treatment (Figure 1). The total chlorophyll content decreased by 12.65, 24.10 and 29.10% at 20%, 40 and 60% PEG respectively with respect to control plants (No PEG). On the other hand at 6 DAT total chlorophyll content got relatively less decreased by 7.29 and 22.50% at 20% and 40% PEG respectively but it got decreased more at 60% PEG by 30.85% from control plants (Figure 1).

Quantum yield (Fv/Fm) and Relative water content (RWC)

The Fv/Fm value is also regarded as quantum yield or photochemical efficiency of PS II. Data represented in Figure – 2 showed that quantum yield decreased significantly with increasing level of PEG treatment on the 3rd day after treatment.

There was 12.92, 18.84 and 22.88% decrease in quantum yield at 20, 40 and 60% of PEG treatment respectively with comparison to control plants at 3 DAT. But at 6 DAT the percentage decrease of Fv/Fm got reduced to 6.55 and 13.89% at 20% and 40% PEG treatment respectively. The percent decrease at 60% PEG at 6 DAT was higher than 3 DAT i.e. 31.45%.

The effect of water deficit was clearly observed by the RWC of leaf which got

reduced significantly with the increase in severity of drought stress (Figure-3). At 3 DAT, RWC of leaves got reduced by 10.29, 16.24 and 25.94%at 20, 40 and 60% of PEG treatment respectively with comparison to control plants. But at 6 DAT the RWC values got increased and hence the percent reduction got decreased at 20 and 40% of PEG treatment and not at 60% PEG. So, at 6 DAT there was 9.34, 15.14 and 31.71% decrease in RWC at 20, 40 and 60% of PEG treatment respectively.

Membrane Injury (MI), H_2O_2 level and MDA content

Due to the drought stress membrane injury, H_2O_2 level and MDA content increased significantly from the control plants. MI level increased by 1.9, 3 and 3.7-foldat 20, 40 and 60% of PEG treatment respectively with comparison to control plants at 3 DAT. At 6 DAT the increase in MI was 1.7, 2.7 and 4-fold at 20, 40 and 60% of PEG treatment respectively (Figure – 4).

MDA and H_2O_2 contents reflect the extent of cellular damage. As the drought stress intensity increased the level of H_2O_2 content also increased at both 3 and 6 DAT (Figure – 5). The H_2O_2content increased by 36.1, 54.1 and 104.1% at 20, 40 and 60% of PEG treatment respectively at 3 DAT. But at 6 DAT the percent increase got increased to 51.1, 66.2 at 20 and 40% PEG respectively.

The H_2O_2content under 60% PEG at 6 DAT was increased by 82.5% from the control plants. Similarly, the malondialdehyde (MDA) content increased by 0.9, 1.6 and 2.3-fold at 20, 40 and 60% of PEG treatment respectively at 3 DAT (Figure – 6). At 6 DAT the increase in MDA content was 0.4, 0.8 and 1.4-fold at 20, 40 and 60% of PEG treatment respectively which is lesser than the values at 3 DAT.

Osmolytes

Proline, Total soluble sugar and Total soluble protein

Drought triggered the accumulation of osmolytes such as proline, sugar and soluble proteins to combat the water deficit condition. Proline content increased by 1.48, 2.32 and 3.38-fold at 20, 40 and 60% of PEG treatment respectively with comparison to no PEG treated plants at 3 DAT. At 6 DAT the proline content gets stabilized a little but still the level got increased by 1.22, 2.26 and an abnormal 9 fold at 20, 40 and 60% of PEG treatment respectively from their control plants (Figure – 7).

Total soluble sugar also got increased by 25.92, 35.33and 62.36% at 3 DAT and at 6 DAT it got increased by 43.96, 48.19 and 60.06%with 20, 40 and 60% PEG treatment respectively from their control plants (Figure – 8). Similarly the soluble protein content was increased by 12.63& 21.58% at 3 DAT and 12.24 & 20.92% at 6 DAT under 20 and 40% PEG treatment respectively from their respective control plants (Figure – 9). But the protein content decreased by 7.89 % and 20.41 % under 60% PEG treatment at 3 and 6 DAT respectively.

Antioxidant Enzyme system

Catalase (CAT) and Peroxidase (POX)

The antioxidant enzymes activities significantly increased in response to drought induction under 20, 40 and 60% PEG treatment and 40% PEG treatment showed highest catalase activity. Catalase activity increased by 12.66, 28.89 & 5.98% at 3 DAT and 13.67, 32.98 & 6.59% at 6 DAT under 20, 40 and 60% PEG treatment respectively from the control plants (Figure – 10). Similarly peroxidase activity increased by

16.67, 47.62 & 26.19 at 3 DAT and 19.57, 43.48 & 17.39% at 6 DAT under 20, 40 and 60% PEG treatment respectively from the control plants (Figure – 11).

Fig.1

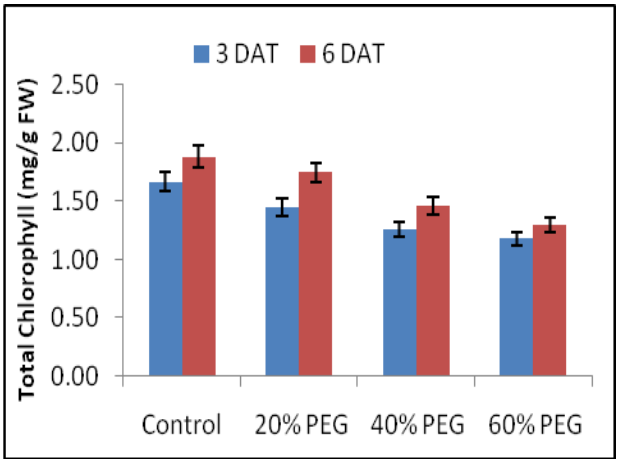


Fig.2

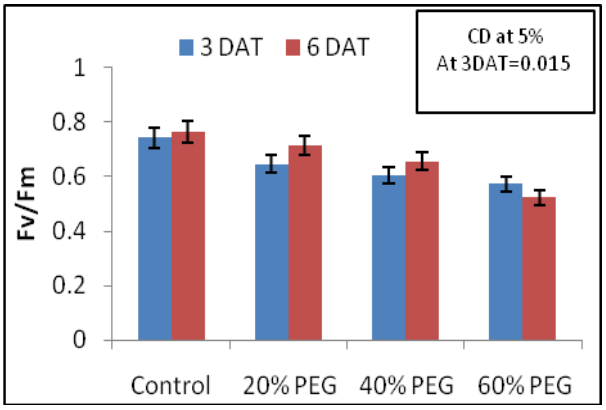
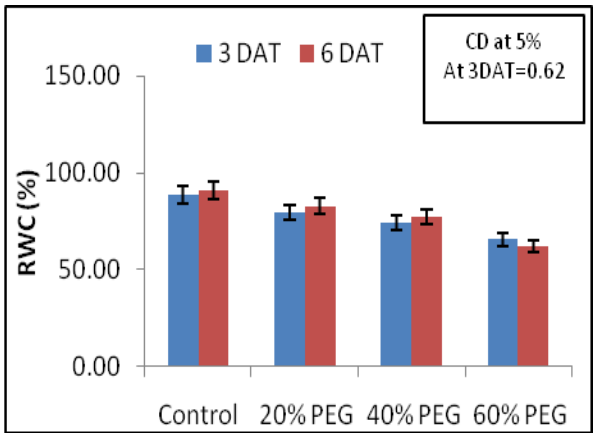


Fig.3



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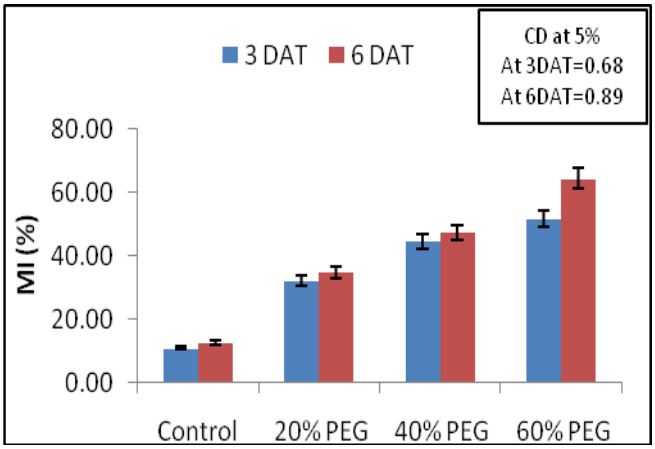
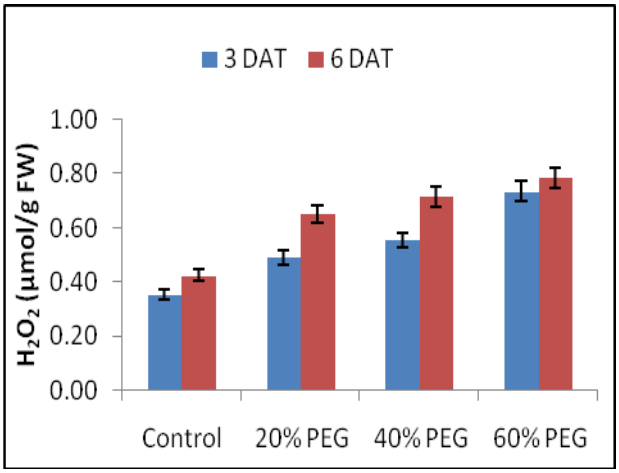


Fig.5



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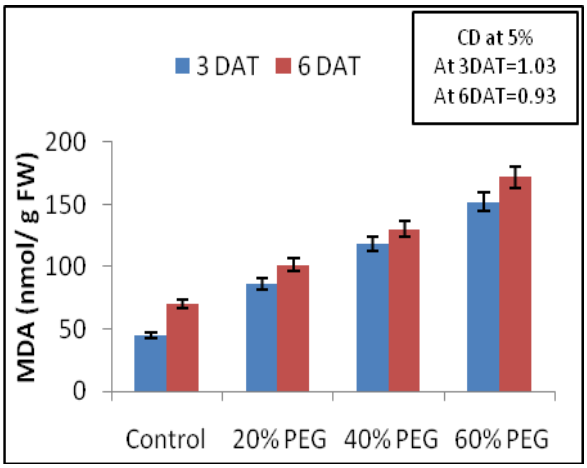


Fig.7

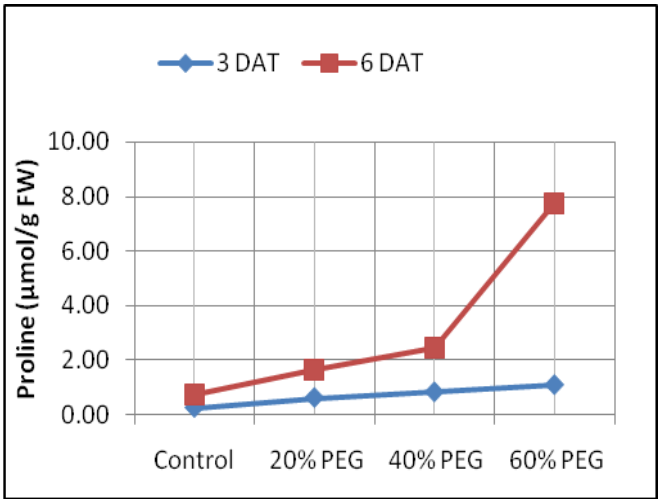


Fig.8

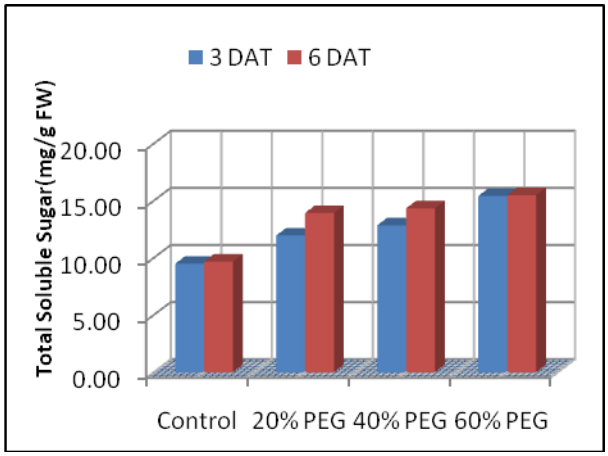


Fig.9

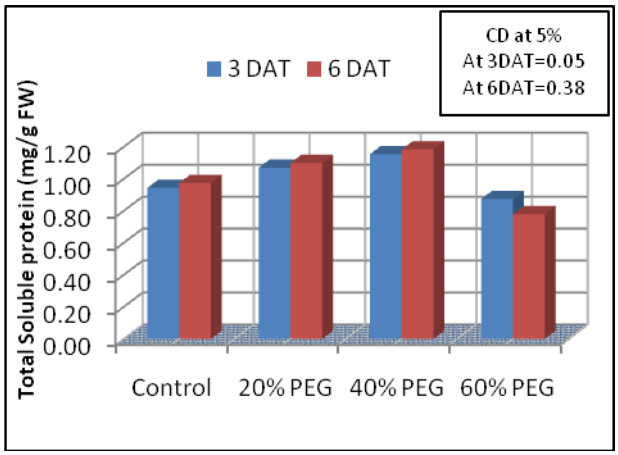


Fig.10

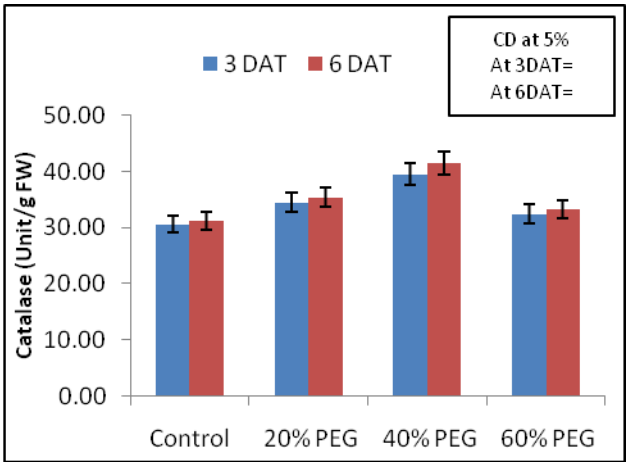
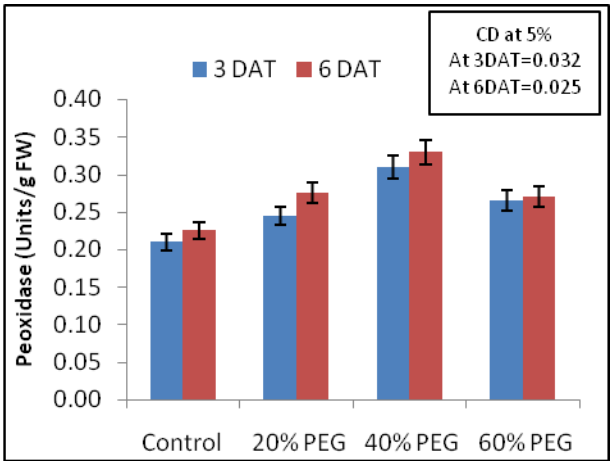


Fig.11



Drought is one of the major yield limiting factors for cotton crop (Anjum *et al.*, 2012). Plant growth is markedly affected by soil water deficiency (Li *et al.*, 2001; Liu *et al.*, 2004). This study was conducted by employing an integrated physiological and biochemical approach for elucidation of the responses to drought stress to cotton and to access the acclimation capability of cotton to the induced drought stress.

The total chlorophyll content decreased significantly with 20, 40 and 60% PEG treatment (Figure – 1).This decrease in chlorophyll content due to stress environment has been reported by Alyemeni & Al-Quwaiz (2015) & Herbinger *et al.*, (2002).The reduction in chlorophyll content due to drought stress might be ascribed by the fact that chloroplasts are explicit sources of ROS production in plant cells exposed to adverse environments hence therefore; it is conceivable that this organelle could experience oxidative damage when ROS generation overwhelms the antioxidant defense mechanisms, leading to degradation of photosynthetic pigments (Foyer & Shigeoka 2011). But we also observed that the percent decrease of total chlorophyll under 20 and 40% PEG treatment was less at 6 DAT than 3 DAT showing some acclimation to the stress environment which

was not in the case of 60% PEG due to the severity of stress

The dynamic changes in chlorophyll fluorescence are a direct reflection of photosynthesis in crops (Maxwell 2000). Under water deficit condition quantum yield decline significantly from the unstressed plants at both the stages but the reduction was less at 6 DAT than 3 DAT (Figure – 2). Shahenshah & Isoda (2010) had reported that water stress leads to less transpiration causing high leaf temperature which had negative effects on the efficiency of PSII. Decrease in quantum yield due to water stress has been reported by by Massacci *et al.,* (2008) in cotton cultivars, Faraloni *et al.,* (2011) in olive cultivars.

Leaf RWC is one of the best growth indices revealing the stress intensity (Alizade 2002). Relative water content plays a huge role in plant growth and development because cell division and elongation are influenced by leaf water status or cell turgor (Heckenberger *et al.,* 1998). In this investigation it was found that gradual increase in PEG concentration, subsequently decreased the relative water content of leaves significantly (Figure – 3). Under water deficit, cell membrane increases in penetrability and decreases in sustainability (Blokhina *et al.,* 2003).

This decreasing trend of RWC with respect to water stress have also been reported by Siddique *et al.,* (2000) in wheat, Mensah *et al.,* (2006) in sesame, Hayatu *et al.,* (2014) in cowpea and Neto *et al.,* (2017) in sunflower. We also found that the RWC of leaves got increased at 6 DAT and the percent decrease at 20 and 40% PEG treatment was less at 6 DAT than 3 DAT (Figure – 3). This increase in RWC may be attributed to the increase in osmolytes like proline (Figure – 7) and total soluble sugar (Figure – 8).

The degree of cell membrane stability i.e. less membrane injury is related to more membrane stability and vice-versa, is considered to be one of the best physiological indicators of drought stress tolerance. A progressive increase in membrane injury with gradual increase in PEG concentration i.e. 20%, 40% and 60% PEG treatment was observed (Figure – 4). Water-stressed cotton leaves had higher levels of H_2O_2content (Figure – 5) and MDA content (Figure – 6), which might have caused the membrane destruction by ROS-induced oxidative damage there by increasing the electrolyte leakage. This increasing trend of membrane injury in stress conditions is in accordance with Abdel-Kader *et al.,* (2015) in cotton under water stress, Ahmad *et al.,* (2016) in chickpea under salinity, Molaei *et al.,* (2012) in sesame under water stress.

The role of H_2O_2 in stress-induced damage has been recognized, but it is now also generally accepted that H_2O_2 is an integral component of cell signalling cascades (Mittler 2002; Vranova *et al.,* 2002) and an indispensable second messenger. Accumulation of H_2O_2 in stress conditions causes oxidative damage to the cell components but its accumulation also induce its scavenging by enhancing the antioxidant system related genes. Based upon the results (Figure – 5) it is clear that water stress induced by gradual increase in PEG concentration, subsequently increase the H_2O_2 content significantly in both at 3 and 6 DAT but the percent increase was lesser at 6 DAT than at 3 DAT showing some acclimation to drought.

The accumulation of H_2O_2 under water stress condition can be due to the superoxide production by the photosynthetic electron transport chain (via the Mehler reaction) which is accelerated by drought (Noctor *et al.,* 2002). Similar increasing trend in H_2O_2 accumulation in stress conditions has been

reported by Zhao *et al.,* (2008) in reeds under water stress, Deeba *et al.,* (2012) in cotton cultivars under water stress.

Increase in ROS causes the peroxidation of lipids which are the major constituent of the bilayer membrane of cells and organelles. The concentration of MDA reflects the degree of peroxidation of lipid in the cell membrane under stress conditions (Zhang *et al.,* 2005). Based on result (Figure – 6) it was observed that the MDA content increased significantly with the gradual increase in PEG concentration at both the stages. Increased MDA is a characteristic feature of oxidative membrane damage that has been reported as a common response to stress conditions by Cechin *et al.,* (2015) in sunflower under water stress, Hai-hua *et al.,* (2002) in wheat under salinity, Deeba *et al.,* (2012) in cotton under water stress.

Plants produce higher levels of osmolytes such as proline, soluble sugars and soluble proteins in the cytosol and other organelles to overcome the negative impacts of osmotic stress (Abdel Latef & Miransari, 2014). Proline has the ability to scavenge ROS and shield the cell from the oxidative damage (Ahmad *et al.,* 2010; Khan *et al.,* 2010; Jogaiah *et al.,* 2013). We observed that the proline content increased progressively with the increasing concentration of PEG at both stages (Figure – 7). The high accumulation of proline was attributed to the increased biosynthesis of proline due to the enhanced expression of proline biosynthetic genes (Zhang *et al.,* 2008). Increase in proline content due to stress conditions were also reported by Hai-hua *et al.,* (2002) in wheat under salinity, Alyemeni & Al-Quwaiz (2015) in *Vigna radiate* under water stress. Based upon the results it was observed that on all the sampling stages total soluble sugar and total soluble protein content increased significantly with increase in PEG concentration (Figure – 8 & 9). Due to the increase in these osmolytes the RWC of leaves were also higher (Figure – 3). Increased accumulation of total soluble sugars in response to saline stress was reported by Liu *et al.,* (2016), Mafakheri *et al.,* (2011), Shinde & Deokule (2015).

The avoidance of ROS production during drought stress is also an important strategy that enables plants to cope with water shortage without extensive damage (de Carvalho 2008). In environmental stress conditions such as drought, high activities of CAT and POX enzymes are important for plants to tolerate stresses. There was significantly higher CAT and POX activity upon exposure to drought stress in all the sampling stages (Figure – 10 & 11). We also observed that the osmolytes and antioxidant enzymes content was more pronounced at 6 DAT than the initial drought stage. This might be due to the fact that plants adapted more to the water deficit condition after certain time period than the initial drought induction phase. The upsurge in CAT and POX activity is might be due to the up regulation of CAT genes (Luna *et al.,* 2004). The increase in CAT and POX activity under stress conditions is well supported by Sudhakar *et al.,* (2001), Mohammad & Mahdiyeh (2013), Hernandez *et al.,* (2000), Acar *et al.,* (2001), Shinde & Deokule (2015), Zhang *et al.,* (2014b), Alyemeni & Al-Quwaiz (2015).

In summary our study provides further information about physiological & biochemical responses in the leaves of a *Bt* cotton hybrid "BG 6488 II", in response to drought stress which might be helpful proving basis for development of promising approach for drought stress management. This study also demonstrates that this hybrid was able to acclimatise to the drought environment under less to moderate drought condition but was unable to do so under severe drought.

Acknowledgement

The authors would like to extend their sincere appreciation to their Institution and acknowledge the Chaudhary Charan Singh Haryana Agricultural University for all the support.

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How to cite this article:

Biswabiplab Singh, Sunita Sheokand and Anita Kumari, Sarita. 2020. Physiological Responses of *Bt* cotton under Drought Stress. *Int.J.Curr.Microbiol.App.Sci.* 9(07): 1239-1253.
doi: https://doi.org/10.20546/ijcmas.2020.907.143