

Full Length Research Paper

Diurnal gradual heat stress affects antioxidant enzymes, proline accumulation and some physiological components in cotton (*Gossypium hirsutum* L.)

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Even though high temperatures significantly reduce both vegetative growth and yield in cotton, very little is known about the effects of heat stress on cotton antioxidant system. Thus, the effects of gradual heat stress on cotton growth in controlled conditions were investigated in the present study. At squaring stage, cotton plants were subjected to two different temperatures, 38 and 45°C to determine the influence of heat stress on the plants. The results of the present study showed that heat stress did not significantly alter the levels of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) in the leaves, whereas there was a remarkable decline in proline quantity of the leaves of plants subjected to 45°C heat stress. As for the amount of total chlorophyll content, a slight increase at plants treated with 38°C temperature was observed. Furthermore, the activities of some enzymes such as superoxide dismutase (SOD), which were associated with heat stress response in other plants was also investigated. For example, there was decline in the activity of SOD in the plants exposed to high temperatures. On the contrary, catalase (CAT) activity increased at 45°C; peroxidase (POX) activity increased at 38°C and ascorbate peroxidase (APX) activity increased at 38 and 45°C. The results from this study suggest a potential role for CAT, POX and APX in the reduction of elevated levels of H₂O₂ in cotton plants grown under heat stress condition. To sum up, it could be concluded that, diurnal gradual heat stress caused a low oxidative injury in cotton.

Key words: Antioxidant enzymes, cotton, heat stress, lipid peroxidation, proline.

INTRODUCTION

Both plant growth and yield are adversely affected by abiotic stresses such as high or low temperatures, drought, salinity and high-light intensity. Among abiotic stresses, heat stress influences photosynthesis, cellular and subcellular membrane components, protein content in cell and antioxidant enzyme activity; thereby significantly limiting crop production (Georgieva, 1999). Heat stress also induces oxidative stress in plants caused by the generation and the accumulation of super-oxides (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH⁻), which are commonly known as active oxygen

species (AOS) or reactive oxygen species (ROS) (Breusegem et al., 2001). Plants get rid of these harmful ROS products by converting them to less reactive chemicals. For example, superoxide dismutase (SOD) converts free O₂⁻ radicals to H₂O₂ and O₂. Catalase (CAT), ascorbate peroxidase (APX) and non-specific peroxidase (POX), in turn, scavenge the hyped level of H₂O₂ to non-toxic levels by catabolizing it to water and oxygen (Mittler, 2002). One of the most common responses of many plant species exposed to different abiotic stresses is the accumulation of compatible organic solutes such as proline, glycine betaine and choline O-sulfate (Rhodes and Hanson, 1993; Ashraf and Fooland, 2007). Proline accumulation has been observed in many plant species treated with a variety of abiotic stresses. From these observations, it has been suggested that proline may play

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a role in the protection of plants by acting as a cellular osmotic regulator between cytoplasm and vacuole and by detoxifying ROS, thereby protecting membrane integrity and stabilizing antioxidant enzymes (Bandurska, 1993; Bohnert and Jensen, 1996). However, it has also been suggested that over accumulation of proline could be toxic to plant cells (Rizhsky et al., 2004).

Although the daily average temperature for optimal growth conditions of cotton is 27 - 29°C (Reddy et al., 2004), cotton is often grown in hot and semi-arid regions of the world, where the daily temperatures occasionally could soar up to 48 - 50°C. High temperatures reduce the vegetative growth and boll production in cotton (Ashraf et al., 1994; Rahman et al., 2004). With the increase of every 1°C in daily maximum temperatures, the amount of cotton fiber harvested per hectare is reduced by 110 kg/ha, hence highlighting the significant and negative correlation between heat stress and cotton fiber yields (Singh et al., 2007). Heat stress usually begins to be effective at the squaring stage of cotton growth under field conditions. Changes in ambient temperature occur within hours, unlike drought and salinity stresses. Therefore, plants need to suppress and respond to the adverse effects of heat in a very short time. Because of this quick response time, it was hypothesized that gradual temperature increase in a day could cause some alterations in antioxidant metabolism or in other physiological responses. Most of the earlier studies have reported about the effects of multitude of abiotic stresses; for example, drought and salt, by investigating the changes in the level of several physiological parameters including lipid peroxidation, H₂O₂ production, chlorophyll content and proline accumulation in cotton (Garratt et al., 2002; Meloni et al., 2003; Ratnayaka et al., 2005; Desingh and Kanagaraj, 2007). However, there is a limited amount of information about the effects of heat stress on physiological traits. Better understanding of the mechanism of response of cotton to elevated temperatures would help the development of cotton cultivars that perform better under heat stress. The purpose of this study was the investigation of antioxidant enzyme activities, lipid peroxidation, H₂O₂ production, chlorophyll content and proline accumulation in cotton leaves at squaring stage under heat stress conditions.

MATERIALS AND METHODS

Plant growth

Cotton (*Gossypium hirsutum* L.) cultivar Stoneville-453 was used in this study, which is the most commonly grown cultivar in Sanliurfa province of Southeastern region of Turkey, where hot and semiarid climatic conditions are observed. Plants were grown in a growth chamber under controlled conditions with optimum temperature regime of 30/26°C, humidity of 60%, photoperiod of 16 h and light intensity of 350 µmol/m²/s until the squaring stage with an average of 4 - 5 squares in each plant (that is, 67 day old plants). Plants were irrigated with ½ strength Hoagland solution (Hoagland and

Arnon, 1950). Heat stress treatments and harvesting were carried out at squaring stage (that is, 67 day old plants) and at light period. The top most fully expanded leaves were harvested and immediately frozen in liquid nitrogen and stored at -84°C for subsequent enzyme assays and quantification of physiobiochemical traits.

Stress treatments

Plants were randomly divided into three groups at squaring stage (4 - 5 squares) and heat stress treatments were applied at 30°C (control), 38°C (moderate heat stress) and 45°C (high heat stress). For moderate heat stress, the plants in the growth chamber were exposed to a gradual temperature increase from 30 to 38°C with increments of 1°C/10 min. After the temperature reached 38°C, plants were kept at 38°C for 2 h (Figure 1). High heat stressed plants were exposed to 45°C. For the high heat stress applications, the plants were exposed to same conditions as plants exposed to moderate heat stress until the end of 38°C. Therefore, high heat stressed plants were acclimatized to high temperatures. After the acclimatization, temperature was further increased to 45°C in an hour and 45 min and plants were kept at 45°C for 2 h (Figure 1). After each stress treatment, leaf samples were harvested and immediately frozen in liquid nitrogen for subsequent analyses.

Measurement of MDA, H₂O₂, chlorophyll and proline concentrations

The level of lipid peroxidation was quantified by measuring the amount of malondialdehyde (MDA), which is determined by thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Briefly, leaf samples (0.3 g) were homogenized in 6 ml of 1% (w/v) trichloroacetic acid (TCA) and the homogenates were centrifuged at 10,000 × g for 10 min. After the centrifugation, 1.5 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA were added to 1.5 ml of the supernatants. After the incubation in boiling water for 30 min, the mixture was quickly cooled on ice bath, and centrifuged at 10,000 × g for 5 min. Finally, the absorbance of supernatant was measured at 532 nm. To calculate MDA content, the value for non-specific absorption at 600 nm was subtracted from the readings at 532 nm and extinction coefficient of 155 mM⁻¹ cm⁻¹ was used. The values for MDA content is expressed as µmol/g fw.

The amount of H₂O₂ was quantified as described by Loreto and Velikova (2001). Briefly, leaf samples (0.3 g) were homogenized in 3 ml of 1% (w/v) TCA. The homogenates were centrifuged at 10,000 × g (4°C) for 10 min. Subsequently, 0.75 ml of the supernatants were added to 0.75 ml of 10 mM K-phosphate buffer (pH 7.0) and 1.5 ml of 1 M KI. After that, H₂O₂ content of the supernatant was evaluated by comparison of the absorbance values at 390 nm to a standard calibration curve standard in the range from 10 to 200 nmol/3 ml cuvette. H₂O₂ concentration was expressed as µmol/g fw.

Chlorophyll was extracted as described previously with some minor modifications (Arnon, 1949). In short, cotton leaves (0.3 g) were ground in 10 ml of 80% cold acetone and stored in a tube at -20°C in the dark for overnight. The homogenates were filtered through a Whatman No1 filter paper. By using 80% cold acetone as blank, an aliquot of the extracts were taken and the absorbance values at 645 and 663 nm were measured with a spectrophotometer.

Proline contents were determined according to the slightly modified method of Bates et al. (1973). Briefly, leaf samples (0.3 g) were ground in 10 ml of 3% sulphosalicylic acid and then centrifuged at 10,000 × g for 10 min. The supernatants (2 ml) were mixed with 2 ml of freshly prepared acid-ninhydrin solution (1.25 g

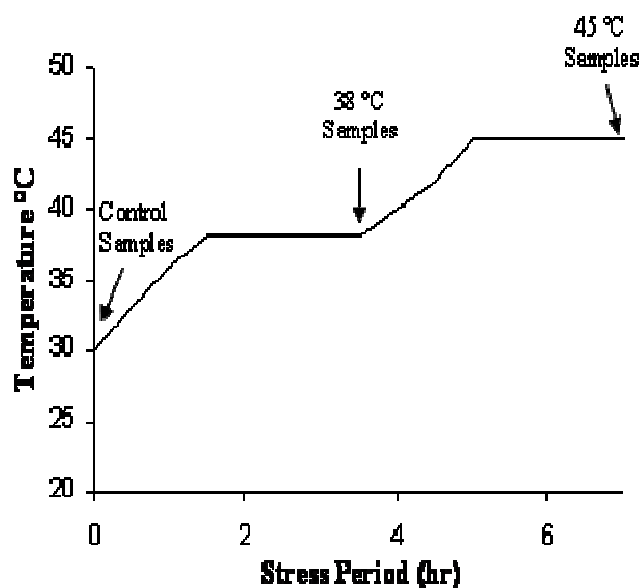


Figure 1. Heat stress treatments and sampling.

of ninhydrin, 30 ml of glacial acetic acid, 20 ml of 6 M ortho-phosphoric acid) and were incubated in boiling water for 30 min. After the termination of the reactions by transferring the samples on ice, the reaction mixtures were extracted with 5 ml of toluene, vortexed for 15 s and the tubes were left undisturbed for at least 20 min at room temperature to allow the separation of toluene and aqueous phases. The toluene phase was then carefully collected and the absorbance values were measured at 520 nm with spectrophotometer. The concentration of proline was calculated using a standard curve.

Enzyme assays

Leaf materials (1 mg) were ground in 6 ml of ice cold 50 mM K-phosphate buffer (pH 7.0) containing 2 mM Na-EDTA and 1% (w/v) polyvinyl-polypyrrolidone (PVP). The homogenates were centrifuged at $10,000 \times g$ (4°C) for 10 min. The tissue extracts were either stored at -84°C or immediately used for subsequent analyses of SOD, CAT, POX and APX. For the quantification of soluble protein content, coomassie blue dye-binding assay was used (Bradford, 1976). Bovine serum albumin was used for the preparation of the standard curve.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in the presence of riboflavin in light (Giannopolitis and Ries, 1977). The reaction mixtures (3 ml) containing 50 mM K-phosphate buffer (pH 7.8), 50 mM NaCO₃, 13 mM methionine, 25 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 100 μl enzyme extract were prepared. One unit of enzyme activity was determined as the amount of the enzyme needed for the inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer. The test tubes were first shaken and subsequently placed in a light box consisting of four 20 W fluorescent lamps for 5 min. The reactions were stopped by switching off the light and placing the test tubes in the dark.

Activities of catalase (CAT; EC 1.11.1.6) and peroxidase (POX; EC 1.11.1.7) enzymes were measured as described by Chance and Maehly (1955). For assaying CAT activity, the decomposition of

H₂O₂ was followed by decline in the absorbance at 240 nm. The reaction mixtures (3 ml) containing 50 mM phosphate buffer (pH 7.0), 10 mM H₂O₂ and 50 μl of enzyme extract were prepared. The reactions were initiated by adding enzyme extracts (Osswald et al., 1992). CAT activity was determined by following the consumption of H₂O₂ (extinction coefficient, 39.4 mM⁻¹ cm⁻¹) at 240 nm over a 3 min interval. As for POX activity, the oxidation of guaiacol was measured by the determination of the increase in the absorbance values at 470 nm. The assay mixtures contained 0.05 ml of guaiacol (20 mM), 2.88 ml of K-phosphate buffer (10 mM, pH 7.0) and 50 μl of enzyme extract. The reactions were initiated by the addition of 20 μl of H₂O₂ (40 mM) (Osswald et al., 1992). POX activity was determined by measuring the oxidation of guaiacol in the presence of H₂O₂ (extinction coefficient, 26.6 mM⁻¹ cm⁻¹) at 470 nm over a 2 min interval.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was assayed by following the decrease in the amount of ascorbate and determining the change in the absorbance values at 290 nm at 3 min interval. The reaction mixtures containing 50 mM K-phosphate buffer (pH 7.0), 1 mM EDTA-Na₂, 0.5 mM ascorbic acid, 0.1 mM H₂O₂ and 50 μl of crude enzyme extract were prepared (Nakano and Asada, 1981). The activity of ascorbate peroxidase was calculated using the extinction coefficient (2.8 mM⁻¹ cm⁻¹).

Statistical analysis

The experiment was conducted in a completely randomized design. There were 9 plants in each treatment and a total of 27 plants in the experiment. The uppermost fully expanded leaves were collected randomly as three replicates for each treatment. Data were analyzed using one-way analysis of variance (one-way ANOVA) and subsequently the means were compared with LSD test at 5% significance. The standard errors were given in histograms.

RESULTS

Effects of heat stress on MDA and H₂O₂

Lipid peroxidation levels of the samples, which are equal to the amount of MDA, are given in Figure 2A. The increase of growth temperatures to 45°C resulted in a slight increase, about 14.4%, in MDA content relative to the control plants. MDA levels were measured as 21.00, 20.90 and 24.03 μmol/g fw in the leaves of the plants exposed to no heat stress (that is, control) and the plants, treated with 38 and 45°C (see the materials and methods for the details of heat treatments), respectively. Although there was an increase in MDA accumulation at elevated temperatures, differences in the levels of MDA concentrations were not statistically significant.

The results showed that H₂O₂ content in cotton leaves linearly increased with the increase in temperature (Figure 2B). H₂O₂ contents of the leaves were 0.786, 0.839 and 0.931 μmol/g fw for the control, 38 and 45°C treated plants, respectively. Mild heat stress (38°C) and extreme heat stress (45°C) resulted in an increase of 6.71 and 18.44% in H₂O₂ content as compared to the untreated plants. Differences with respect to H₂O₂ content were not statistically significant among the treatments.

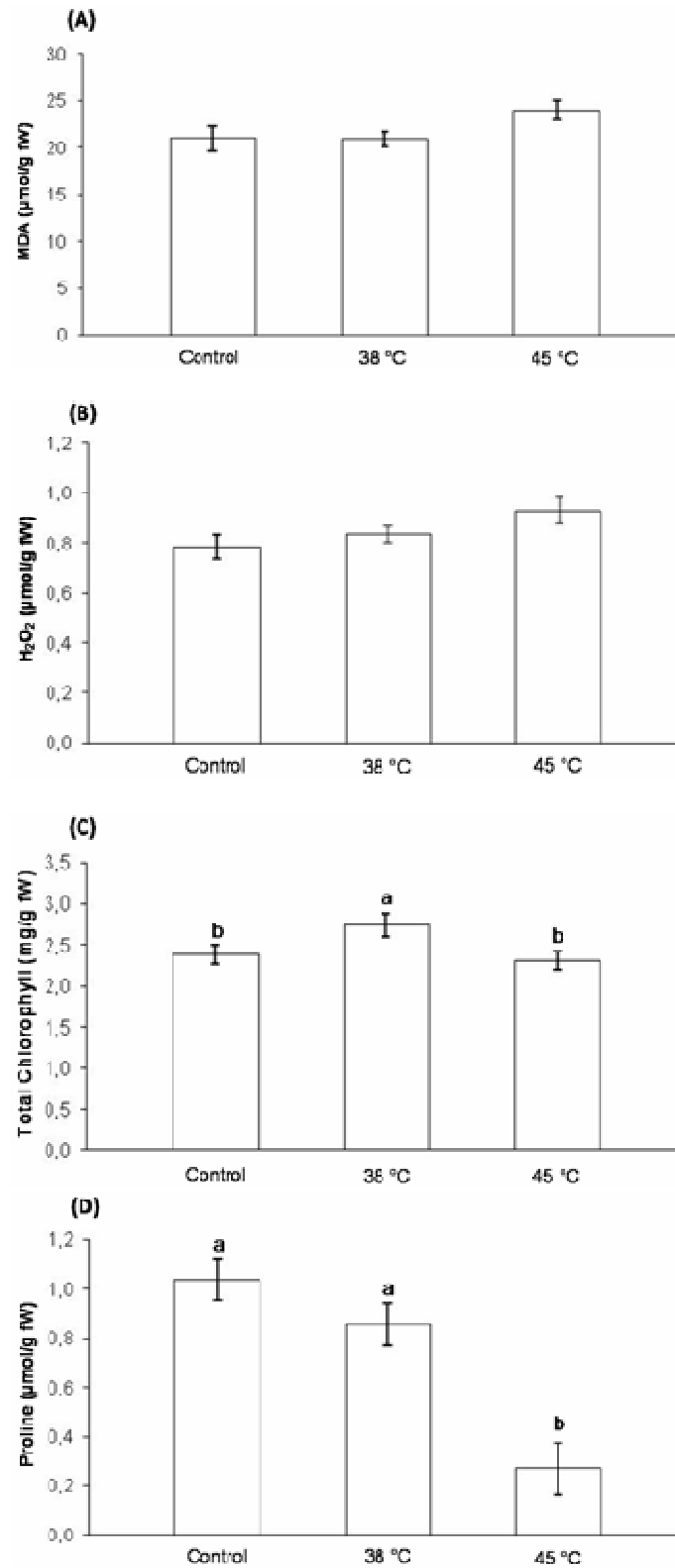


Figure 2. Effects of heat stress treatments on MDA (A), H_2O_2 (B), total chlorophyll (C) and proline (D) in the leaves of cotton. Letters on bars indicate results of LSD test. Different letters on the histograms indicate that the means differ significantly ($P < 0.05$). Error bars refer to standard error (S.E. \pm).

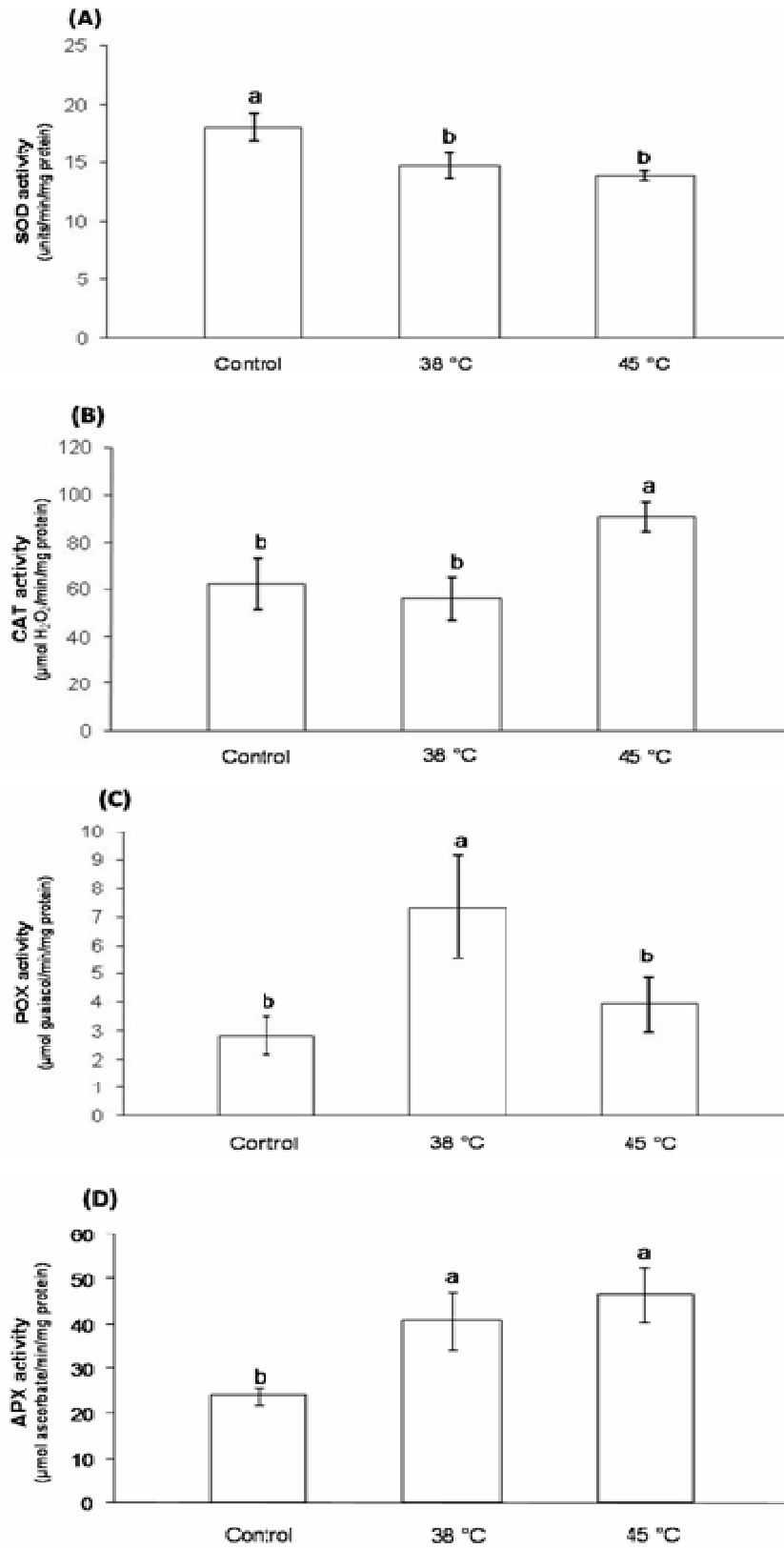


Figure 3. Effects of heat stress treatments on SOD (A), CAT (B), POX (C) and APX (D) activities in the leaves of cotton. Letters on bars indicate results of LSD test. Different letters on the histograms indicate that the means differ significantly ($P < 0.05$). Error bars refer to standard error (S.E. \pm).

Chlorophyll contents and proline accumulation

There was an increase in the quantity of chlorophyll-a (chl-a), chlorophyll-b (chl-b) and total chlorophyll (chl) contents in the leaves of the plants at 38°C, but the chl content of the plants treated 45°C dropped with respect to control plants. Significant differences were observed for chl-a, chl-b and total chl values. Total chl values were 2.38, 2.74 and 2.31 mg/g fw (Figure 2C); chl-a values were 1.77, 2.05 and 1.72 mg/g fw; and chl-b values were 0.61, 0.69 and 0.59 mg/g fw for the control, 38 and 45°C treated plants, respectively.

There was a decline in the level of proline content in the leaves of plants subjected to 38 and 45°C temperatures as compared to the control plants (Figure 2D). Proline values were 1.04, 0.86 and 0.27 $\mu\text{mol/g}$ fw for control, 38 and 45°C treated plants, respectively. As compared to the control plants, proline content dropped by 17.36 and 74.00% in the plants subjected to 38 and 45°C, respectively and the differences between treatments were statistically significant.

Antioxidant enzymes

Heat stress treatments caused a significant decrease in SOD activity. Mean value of SOD was 18.07 unit/mg protein in control plants, while it was 14.74 and 13.87 unit/mg protein in 38 and 45°C treated plants, respectively (Figure 3A). A significant reduction in SOD activity was observed in the leaves of high temperature treated plant; that is, 18.46 and 23.26% decrease in the activity after exposure to 38 and 45°C temperatures, respectively.

There was a significant increase (45.86%) in CAT activity in the plants exposed to 45°C temperature, but there were no significant differences between the control plants and plants exposed to 38°C. The values of CAT activity were 62.26, 55.90 and 90.81 $\mu\text{mol H}_2\text{O}_2/\text{min/mg}$ protein in control, 38 and 45°C treated plants, respectively (Figure 3B).

POX activity also showed an escalation in response to heat stress treatments (Figure 3C) and the increase in the activity of this enzyme was statistically significant at 45°C relative to control plants. The increase in POX activity was 159.90 and 38.87% at 38 and 45°C treatments, respectively.

On the other hand, APX activity followed a trend of increase under the heat stress conditions (Figure 3D). That is, APX activity was 71.10% higher at 38°C and 94.39% higher at 45°C. This level of increase in APX activity was statistically significant for both treatments, 38 and 45°C, with respect to control plants.

DISCUSSION

A change in the level of MDA and H_2O_2 in plant cells is an

indicator of oxidative stress. In other words, an increase in the amount of MDA and H_2O_2 is associated with oxidative damage (Sairam and Srivastava, 2000; Larkindale and Knight, 2002; Ozden et al., 2009). In this study, although there was an increase in MDA accumulation at high temperatures, the level of MDA concentrations were not significantly different relative to the non-treated plants.

Furthermore, MDA content was found to be similar to the levels found in control plants even after the 12th day of heat stress treatment in two cool-season turfgrasses (Jiang and Huang, 2001). Likewise, the present results corroborated the previous findings reporting the absence of a significant correlation between high temperatures and MDA amount of cotton seedlings grown under field conditions (Mahan and Mauget, 2005). Therefore, the results may suggest that increase in diurnal gradual temperature caused low damage and low oxidative stress in cotton, which is because antioxidant metabolism could be sufficient to protect cotton plants from damaging effects of oxidative stress caused by elevated temperatures. The efficiency of antioxidant metabolism for the protection of cotton plants from heat stress was also proposed by Mahan and Mauget (2005).

Chlorophyll content in plants is an important trait to assess photosynthetic efficiency under stressed conditions. Leaf chlorophyll content of plants exposed to drought, heat or combined stresses of both was increased above the level obtained from the control plants within 12 days in *Festuca arundinacea* and 6 days in *Poa pratensis*. The prolonged periods of exposure to these stresses caused the complete loss of chlorophyll in *F. arundinacea* and *P. pratensis* (Jiang and Huang, 2001). However, the total chlorophyll content was not significantly affected by high temperature treatments combined with CO_2 or UV-B. On the other hand, it was significantly affected by combination of CO_2 × high temperature × UV-B in soybean cultivars (Koti et al., 2007). In the present study, significant changes were observed in the chlorophyll content of cotton leaves subjected to heat stress at 38°C.

Under the different abiotic stress conditions and H_2O_2 treatments, proline is accumulated in cells as an osmoprotectant (Hare and Cress, 1997; Ozden et al., 2009). However, the present results clearly showed that heat stress caused a decline in the level of proline accumulation in cotton cells. The level of proline, thought to be important for plant protection during drought stress, is strongly suppressed in *Arabidopsis* after heat stress treatments and a combination of drought and heat stresses (Rizhsky et al., 2004). In the same experiment, it was reported that *Arabidopsis* plants subjected to a combination of drought and heat stress accumulated sucrose instead of proline (Rizhsky et al., 2004). Hare and Cress (1997) suggested that proline degradation upon relief from stress may provide sufficient reducing agents, which, in turn, supports mitochondrial oxidative phosphorylation and generation of ATP required for recovery from stress. Since various abiotic stress factors

result in different effects on proline accumulation, a better understanding of the relationship between abiotic stress and proline accumulation in plants could be obtained under controlled conditions by the application of different combinations of abiotic stress factors.

Plants evolved both enzymatic and non-enzymatic antioxidant response systems against ROS injury (Vranova et al., 2002). In enzymatic system, SOD converts free O_2^- radicals to H_2O_2 and O_2 (Breusegem et al., 2001). CAT, APX and POX scavenge the accumulated H_2O_2 to nontoxic levels or form water and oxygen (Mittler, 2002).

SOD activity in Kentucky bluegrass significantly increased after 2 h of heat stress application, but decreased to the control levels after 6 h of heat stress application (He et al., 2005). An increase in SOD activities was observed after 12 d of drought, heat or combined stress of both two cool-season turfgrasses (Jiang and Huang, 2001). In the same study, it was reported that the activity of SOD decreased rapidly to a level lower than the control plants after 18 d of heat and drought combined stresses. SOD activity decreased at 35/35°C compared to that at 20/20°C for both of the two different creeping bentgrass cultivars (Huang et al., 2001). In the present study, the observed diminish activity of SOD could be explained by either increased level of enzyme degradation or decline in synthesis of this enzyme. The reduction in SOD activity was in concordance with previous reports about the decline in the transcript level of SOD related genes in the heat stress treated tobacco plants (Rizhsky et al., 2002).

The present study revealed a significant increase (45.86%) in CAT activity in the plants treated with 45°C temperature. Keleş and Öncel (2002) reported that heat treatment increased CAT activities in *T. aestivum* genotypes, but decreased in *T. durum* genotypes. CAT activity reached up to the maximum level after a ½ and 1 h of heat treatments and thereafter decreased in Kentucky bluegrass (He et al., 2005). Because CAT scavenges H_2O_2 to nontoxic levels or catalyze the formation of water and oxygen, an increase in CAT activity could play a role in the protection of the plants from the damages of upward accumulation of H_2O_2 in cotton leaves at 45°C. POX activity can show different responses to abiotic stress treatments in various cultivars. For example, POX activity increased with the increase in NaCl levels in cotton c.v., Pora, whereas it remained essentially constant under all NaCl levels in cotton c.v., Guazuncho (Meloni et al., 2003). POX activity increased as a response to increase in temperatures to 38 and 45°C.

APX is one of the most important antioxidant enzymes. APX uses ascorbic acid as substrate and it catalyzed the conversion of H_2O_2 to monodihydroascorbate (MDHA) and $2H_2O$. Thus, APX helps in the removal of the toxic effects of H_2O_2 (Payton et al., 2001; Blokhina et al., 2003). Mahan and Mauget (2005) investigated anti-oxidant metabolism in cotton seedlings exposed to heat stress under field conditions. They could not find any significant correlation between APX activity and either

minimum or maximum temperature increases. APX activity increased at 34°C after 3 day treatments, whereas short-term heat stress at 37°C caused no significant difference and short-term heat stress at 44°C resulted in a remarkable decline in *Arabidopsis* (Panchuk et al., 2002). However, transcript level of APX gene is increased in heat, drought and combined stress treated tobacco plants (Rizhsky et al., 2002).

In conclusion, diurnal heat stress cause low damage in leaf cells of cotton and it is likely to cause a non significant increase in MDA and H_2O_2 amounts. This low risk of injury could be as a result of scavenging activities of CAT, POX and APX in cotton. In other words, cotton cells could use CAT, POX and APX enzymes to scavenge the increasing H_2O_2 caused by heat stress for protection.

Under heat stress conditions, particularly at reproductive stage, the level gene expression and the upstream regulatory regions of the genes that regulate the antioxidant enzymes such as SOD, CAT, POX and APX needs to be studied in more detail to better understand the mechanism of heat tolerance in cotton. Molecular markers associated with the genes encoding antioxidant enzymes and the genes playing role in heat tolerance would be another step towards developing better performing cotton plants under elevated temperatures. In addition, the effect of prolonged heat stress should also be investigated to better reveal the damage of heat stress in cotton leaf cells and to determine the role of antioxidants in the reduction of the damage caused by heat stress.

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