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Ascorbate-glutathione-α-tocopherol Triad Enhances Antioxidant Systems in Cotton Plants Grown under Drought Stress

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Abstract: Drought is one of the major abiotic stresses affecting plant growth and development. In the present study, the changes in proline, total phenols, total flavonoids lipid peroxidation rate and antioxidant enzyme activities were determined to investigate the effect of foliar application of ascorbic acid (ASC), glutathione (GSH) and/or α-tocopherol (α-TOC) and their interactions on cotton plants grown under normal and drought conditions during vegetative growth stage. Plants were subjected to two watering regimes (100% and 50% of field capacity). Drought stress reduced total phenols content, while the contents of proline, malondialdehyde (MDA), a product of lipid peroxidation, total flavonoids and the activities of catalase and ascorbate peroxidase were increased in comparison with control. The results showed that treatments of cotton plants under drought stress with ASC, GSH, α-TOC and their interaction caused enhancement of proline content, total phenols, total flavonoids, and antioxidant enzyme activities, while lipid peroxidation was reduced. Finally, it can be concluded that foliar application of ASC, GSH, α-TOC and their interaction improved the drought tolerance of cotton plants by enhancing the antioxidant mechanism.
Key words: Cotton **(***Gossypium barbadense* L.), Drought stress, Glutathione, Ascorbic Acid, α-Tocopherol, Antioxidant enzymes.

Introduction

Drought is considered as one of the most important environmental stresses limiting plant growth and crop productivity[1]. Up to 45% of the world agricultural lands are subject to continuous or frequent drought stress, wherein 38% of the world human population resides[2]. Drought can be defined as the absence of adequate soil moisture necessary for a plant to grow normally and complete its life cycle[3]. When plants are subjected to various abiotic stresses, some reactive oxygen species (ROS) such as superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) are produced. However, under various abiotic stresses the extent of ROS production exceeds the antioxidant defense capability of the cell, resulting in cellular damages[4]. One of the main reasons why environmental stress inhibits growth and photosynthetic abilities of plants is the breakdown of the balance between the production of reactive oxygen species (ROS) and the antioxidant defense[5]. These activated oxygens injure the cellular components of proteins, membrane lipids and nucleic acids[6]. To mitigate and repair damage initiated by ROS, plants have developed a complex antioxidant system[7]. Water deficit is also known to alter a variety of biochemical and physiological processes ranging from photosynthesis to protein synthesis and solute accumulation[8]. Exogenous applications of osmoprotectants, plant growth regulators, fertilizers, and antioxidants have been reported to successfully mitigate the adverse effects of drought on plants. Of these, exogenous application of antioxidants has recently gained a ground as a very

promising means of mitigating the adverse effects of drought on plant growth and metabolism[9]. Number of enzymatic and non-enzymatic antioxidants is produced in plants in response to abiotic stresses which save plant from oxidative damage caused by ROS[10]. Major enzymatic antioxidants reported are superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) whereas, ascorbic acid (vitamin C), tocopherols and glutathione are the main non-enzymatic antioxidants exploited by plants under stressful conditions to ameliorate the adverse effects imposed by ROS[11]. A few studies report that exogenously applied ascorbic acid, glutathione and α-tocopherols ameliorates adverse effects of drought[12,13,14]. From the earlier mentioned reports it is evident that each of ascorbic acid, glutathione and α-tocopherol plays a key role in the regulation of a number of metabolic processes in plants exposed to drought stress. However, information on how non-enzymatic antioxidants regulate physiological/biochemical processes in cotton plants subjected to drought stress is not much available in the literature.

Accordingly, this investigation aimed to study the effect of exogenous application of ascorbic acid, glutathione and α-tocopherol (separately and interactions) on some chemical analysis of cotton plants grown under normal and drought conditions.

Materials and Methods

A pot experiment was carried out during summer season 2014 in the greenhouse of National Research Centre, Dokki, Giza, Egypt in order to investigate the effect of ascorbic acid (ASC), glutathione (GSH) and/or α-tocopherol (α-TOC) and their interactions on alleviation of drought stress in cotton (*Gossypium barbadense* L.) plants. Seeds of cotton cv. Giza 86 were sown in plastic pots (40 cm diameter and 40 cm depth) filled by clay soil and arranged in factorial experiment in complete randomize design with 5 replicates for each treatment. The soil texture is clay, field capacity (FC %), 38.0, pH 7.95, EC dSm^{-1} 0.96, $CaCO_3$% 0.72, OM% 2.89, available N 155.0 ppm, P 5.50 and K 265.0 were carried out according the methods described by Jackson[15] (1970). Phosphorus and potassium fertilizers were added before sowing at a rate of 6.0 and 3.0 g/pot of calcium super phosphate (15.5% P_2O_5) and potassium sulphate (48-50% K_2O), respectively. Thinning was done twice at 21 and 35 days after planting (DAP) to leave one plant per pot till picking time. Nitrogen fertilizer was applied as two equal portions at a rate of 0.60 g/pot for each in the form of ammonium nitrate (33.5% N) at 30 and 60 days after planting. At 45 days after planting (DAP), the plants were subjected under two irrigation regimes e.g. plants irrigated with 100 % full field capacity (as normal irrigation) and the other plants irrigated after depletion of 50 % field capacity. At 60 days after planting the exogenous application of antioxidant compounds was applied as follows:

1. Without application (Control)
2. Ascorbic acid (ASC) (1mM).
3. Glutathione (GSH) (1mM).
4. Alpha-tocopherol (α-TOC) (1mM).
5. ASC + GSH
6. ASC + α-TOC
7. GSH + α-TOC
8. ASC + GSH + α-TOC

At 90 days after planting a representative sample was taken from each treatment for determining some chemical analyses as the following:

Proline

Proline accumulation was determined according to Bates *et al.*[16].

Total phenolics content

Total phenolics content was determined by the method described by Savitree *et al.*[17] and Pourmorad *et al.*[18].

Total flavonoids content

Total flavonoids content in the cotton plant was determined according to method described by Adom and Liu[19].

Lipid peroxidation

The level of lipid peroxidation was measured by determining the levels of malonadialdehyde (MDA) content using the method of Hodges *et al.*[20]. The absorbance of supernatant was recorded at 532nm by Agilent

Technologist Cary Series UV-VIS spectrophotometer. The value for non– specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 $nmol^{-1}$ cm^{-1} and expressed as $nmol^{-1}g^{-1}$ fresh weight.

Enzyme extraction

Enzyme extract was prepared according to the method of Mukherjee and Choudhuri[21].

Super oxide dismutase (SOD) assay

SOD activity was measured according to the method of Dhindsa and Matowe[22]. The absorbance was measured at 560 nm, using VEB Carl Zeiss UV-VIS spectrophotometer. One unit of SOD activity was defined as the amount of the enzyme that caused 50% inhibition of NBT to blue formazan.

Peroxidase (POD) assay

POD activity was measured according to the method of Bergmeyer[23]. The absorbance was measured within 60 s at 470 nm, using VEB Carl Zeiss UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the conversion of one micromole of H_2O_2 per minute at 25°C[24].

Ascorbate peroxidase (APX)

APX assay was performed using the method of Koricheva *et al.*[25]. The decrease rate in absorbance as ascorbate oxidized was monitored at 290 nm using VEB Carl Zeiss UV-VIS spectrophotometer (ε= 2.8 mM^{-1} cm^{-1}). One unit of enzyme activity was calculated as the amount of the enzyme that catalyzed the conversion of micromole of H_2O_2 per minute at 25°C.

Catalase (CAT, EC 1.11.1.6) assay

CAT activity was assayed according to the method of Chen *et al.*[26]. CAT activity was determined by measuring the rate change of H_2O_2 absorbance in 60 s at 250 nm using VEB Carl Zeiss UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of the enzyme that reduced 50% of the H_2O_2 in 60 s at 25°C[24].

Polyphenol oxidase (PPO)

PPO activity was assayed as described by Kumar and Khan[27] by measuring the absorbance of the purpurogallin formed at 495 nm using VEB Carl Zeiss UV-VIS spectrophotometer. PPO activity was expressed in Ug^{-1} FW (U = change in 0.1 absorbance $min^{-1}g^{-1}$ FW). In case of enzyme assay, volume at zero time was taken as blank and the activity of the enzyme/g fresh weight/hour was expressed as (Δ x Tv x 60 min)/t x v x f. wt where, Δ is the absorbance of the sample after incubation minus the absorbance at zero time, TV is the total volume of filtrate, t is the time (minutes) of incubation with substrate and V is the total volume of filtrate taken for incubation and f. wt is the fresh weight used[28].

Statistical Analysis

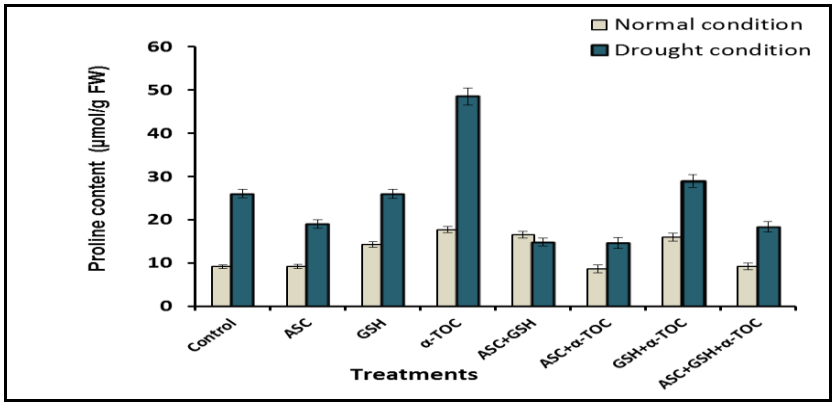
Data of thirty measurements from two independent experiments were analyzed through ± SD values using SPSS statistics data document for Windows, version 17.0 and Excel program, 2007. Each experiment was statistically analyzed according to Snedecor and Cochran[29]. The least significant differences (LSD) at 5% level of probability were calculated to compare the means of different treatments.

Results and Discussion

Proline content

Data presented in Fig. 1 indicated that foliar application of GSH, α-TOC and their interactions on cotton plants grown under normal condition caused pronounced increment in the proline content whereas, other treatments caused a marked decrease in the proline content compared to untreated stressed plants. The highest values of proline content (17.68 and 48.45 µmol/g fw) were achieved in α-TOC treated control and drought-stressed plants, respectively. Meanwhile, foliar application of ASC, GSH, α-TOC and their interactions decreased total free amino acids contents in leaves of cotton plants grown under drought condition compared to control ones. There is a strong correlation between increased cellular proline levels and the capacity to survive the effects of drought stress. It may also, serve as an organic nitrogen reserve[30]. Moreover, the higher level of proline content in cotton leaves may be due to expression of gene encoding key enzymes of proline synthesis

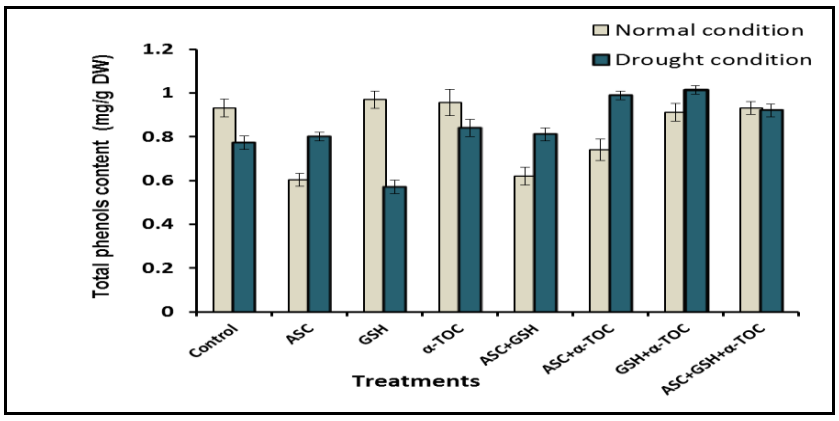
and low activity of the oxidizing enzymes which is controlled by drought stress[31]. Finally, it was also, reported that proline act as free radical scavengers and/or enzyme protectants as well as compatible solutes[32, 33].



LSD 0.05 for drought= 0.63 , for treatments = 1.3, for interaction =1.8, Vertical bars indicate ± SD.

Figure 1. Effect of foliar application of ASC, GSH, α- TOC and their interactions on proline content in leaves of cotton plants under normal irrigation and drought stress conditions.

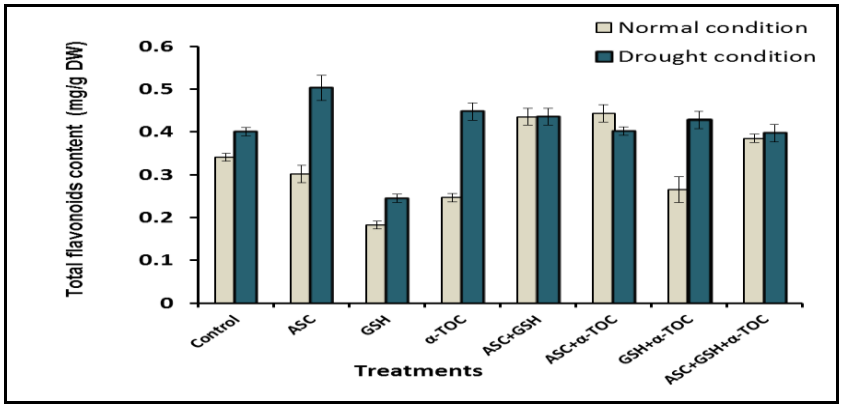
Total phenolics and flavonoids contents



LSD 0.05 for drought= 0.04, for treatments = 0.06, for interaction =0.09, Vertical bars indicate ± SD.

Figure 2. Effect of foliar application of ASC, GSH, α-TOC and their interactions on tota phenols content in leaves of cotton plants under normal irrigation and drought stress conditions.

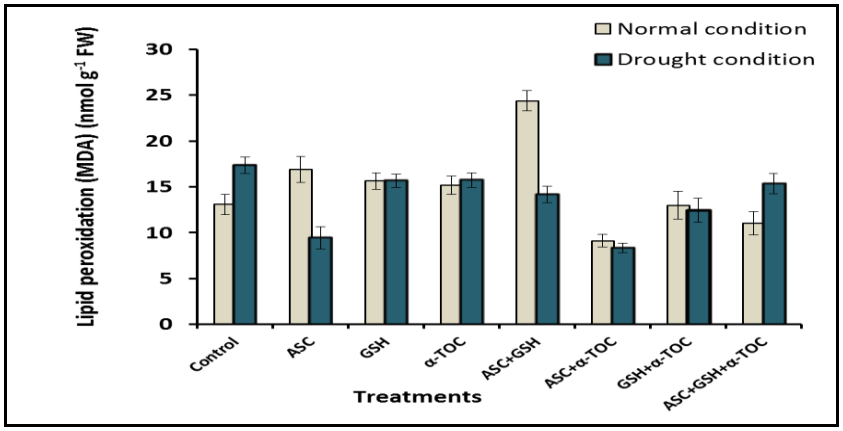
Data in Figs. 2 and 3 revealed that application of GSH or α-TOC increased total phenols and flavonoids in cotton plants grown under normal irrigation compared to control ones. Meanwhile, the results showed that all treatments, except GSH, caused pronounced increment in the total phenolics and total flavonoids content in cotton plants grown under drought condition compared to untreated stressed plants. The highest value of total phenols was recorded (1.05 mg/g dw) in GSH+α-TOC treated drought stressed plants. Meanwhile, the highest value of total flavonoids was recorded (0.50 mg/g dw) in ASC treated drought stressed plants. Total phenols play a significant role in the regulation of plant metabolic processes and overall plant growth as well as lignin synthesis[34]. On the other hand, phenols act as substrates for many antioxidant enzymes, so, it mitigates the drought stress injuries. In the recent study, the total phenolics of cotton plants were significantly lower compared to that of control unstressed plants. The reduction in phenols levels under drought stress may be due to its oxidation by the antioxidant enzymes which withdraw phenols as their substrate and may also, due to the decline in its biosynthesis. Phenols protect the cells from potential oxidative damage and increase stability of cell membrane[35, 36, 37].



LSD 0.05 for drought= 0.01, for treatments = 0.02, for interaction = 0.03, Vertical bars indicate ± SD.

Figure 3. Effect of foliar application of ASC, GSH, α-TOC and their interactions on total flavonoids content in leaves of cotton plants under normal irrigation and drought stress conditions.

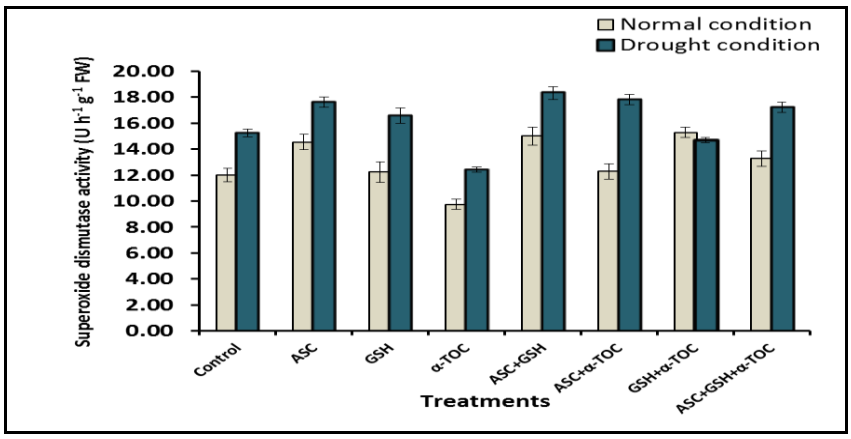
Lipid peroxidation



LSD 0.05 for drought= 0.67, for treatments = 0.80, for interaction =1.20. Vertical bars indicate ± SD.

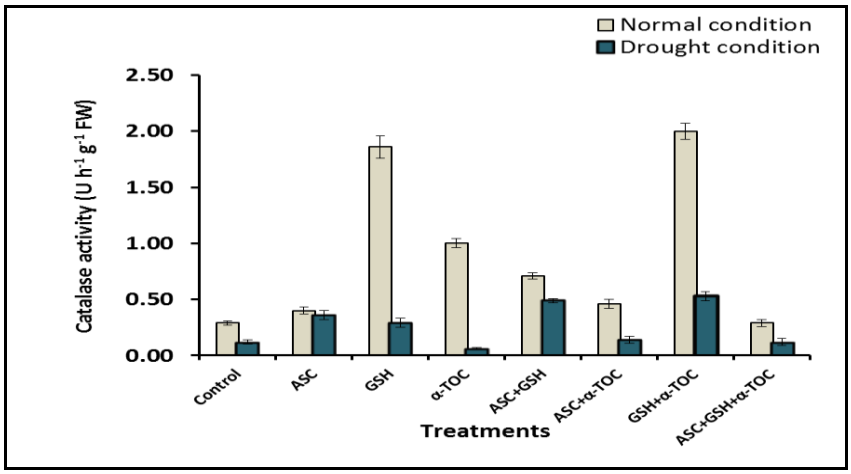
Figure 4. Effect of foliar application of ASC, GSH, α- TOC and their interactions on malondialdhyde contents in leaves of cotton plants under normal irrigation and drought stress conditions.

It is known that the accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen species. Malondialdhyde content (MDA), as one of the major products of lipid peroxidation can be regarded as a sink for oxidative radical. As an indicator of lipid peroxidation, the content of malondialdhyde (MDA) was enhanced in leaves of cotton plants exposed to drought stress compared with that of the control (Fig. 4). Unexpectedly, the results revealed that exogenous application of ASC separately or in combination with GSH on cotton plants grown under normal irrigation caused marked increments in MAD content compared to control ones. All applied treatments reduced significantly MDA content in leaves of drought stressed cotton plants compared to the control. The lowest values of MDA was obtained in response to the application of ASC and +α-TOC treatment compared to the control value. There is a positive relation among the amount of lipid peroxidation products and the degree of membrane damages resulted from the injurious drought stress. Glutathione is a water soluble antioxidant which reacts directly or indirectly with the reactive oxygen species so, reduces stress injurious effects on membrane. Moreover, decreases in lipid peroxidation by antioxidant treatments showed more tolerance to drought stress. Our results may be also, due to their effects on the activities of antioxidant enzymes (Figs. 5-9). In addition, the presence of oxidation products such as MAD in biological systems is also, related to the beginning of peroxidation of unsaturated fatty acids. The increase in lipid peroxidation may be due to the incapability of antioxidants to neutralize and scavenge all the active oxygen species results from drought stress. The present results were agreed with the results of Ben Amor *et al*.[38], Demiral and Türkan[39] and Chaperzadeh *et al*.[40].



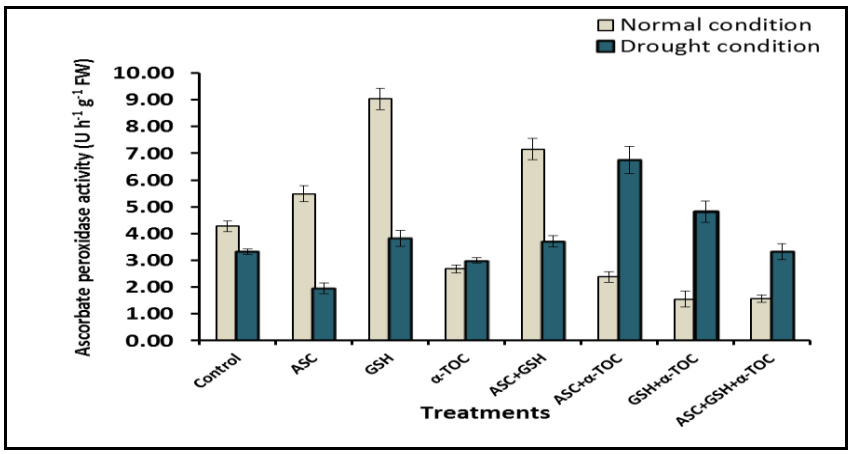
LSD 0.05 for drought= 0.40, for treatments = 0.73, for interaction =1.00, Vertical bars indicate ± SD.

Figure 5. Effect of foliar application of ASC, GSH, α-TOC and their interactions on SOD activity in leaves of cotton plants under normal irrigation and drought stress conditions.



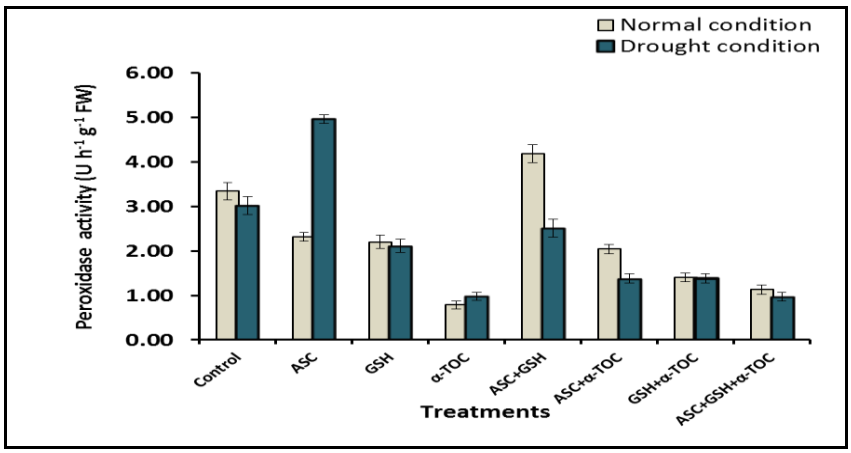
LSD 0.05 for drought= 0.01, for treatments = 0.05, for interaction =0.67. Vertical bars indicate ± SD.

Figure 6. Effect of foliar application of ASC, GSH, α-TOC and their interactions on CAT activity in leaves of cotton plants under normal irrigation and drought stress conditions.



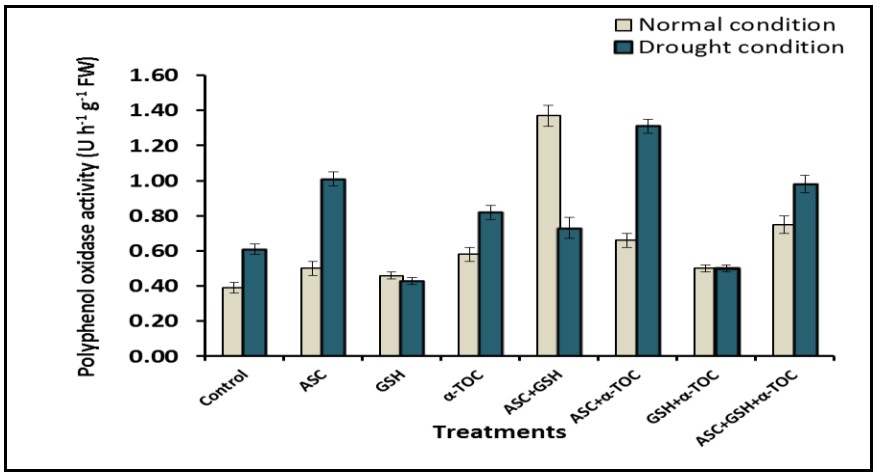
LSD 0.05 for drought= 0.20, for treatments = 0.29, for interaction =0.50,Vertical bars indicate ± SD.

Figure 7. Effect of foliar application of ASC, GSH, α- TOC and their interactions on APX activity in leaves of cotton plants under normal irrigation and drought stress conditions.



LSD 0.05 for drought= 0.10, for treatments = 0.20, for interaction =0.31, Vertical bars indicate ± SD.

Figure 8. Effect of foliar application of ASC, GSH, α-TOC and their interactions on POD activity in leaves of cotton plants under normal irrigation and drought stress conditions



LSD 0.05 for drought= 0.03, for treatments = 0.05, for interaction =0.06, Vertical bars indicate ± SD.

Figure 9. Effect of foliar application of ASC, GSH, α-TOC and their interactions on PPO activities in leaves of cotton plants under normal irrigation and drought stress conditions.

Antioxidant enzymes

The activity of antioxidant enzymes was measured in cotton plants grown under normal and drought conditions. There were many differences in the activity of antioxidant enzymes of cotton plants under normal and drought conditions in response to ASC, GSH and α-TOC and their interaction. Superoxide dismutase (SOD) is the first defense agent against ROS as it is the major scavenger of O_2[41]. Results in Fig. 5 showed that except α-TOC, all treatments markedly enhanced SOD activity in leaves of cotton plants grown under normal irrigation or drought stress at vegetative stage, compared to their corresponding controls. Drought stress stimulates the accumulation of the ROS including H_2O_2 in plants cells. The metabolism of H_2O_2 is dependent on various functionally interrelated antioxidant enzymes such as catalases and peroxidases. These enzymes are involved in elimination of H_2O_2 from stressed cells[42]. Catalase (CAT) is the most effective antioxidant enzyme that scavenges H_2O_2 in cells to preventing oxidative damage. Our results demonstrated that catalase (CAT) activity was significantly decreased under drought stress conditions (Fig. 6).These results are also, supported by the findings of Sahalata *et al.*[44], Kattab[45] and Abedi and Pakniyat[46]. They concluded that the reduction of CAT activity was supposedly due to the inhibition of enzyme synthesis, change in the assembly of enzyme subunits, or protein degradation under drought stress. CAT enzyme activity was significantly increased by all treatments, except the α-TOC in leaves of plants grown under normal irrigation and drought condition compared to the corresponding values of control plants.

Ascorbate peroxidase (APX) scavenges peroxidase by converting ascorbic acid to dehydroascorbate[47]. APX enzyme activity was significantly decreased in leaves of plants grown under drought condition compared to that of the corresponding control plants (Fig. 7). Meanwhile, APX enzyme activity was significantly increased in leaves of plants grown under normal condition and treated with ASC, GSH or their combination and the opposite situation was obtained with other treatments compared to the corresponding values of control plants. Moreover, all treatments except, ASC increased APX enzyme activity in leaves of plants grown under drought condition compared to the untreated stressed plants. Higher activity of APX in antioxidant treated plants, suggests a more effective H_2O_2 removal in these plants. Data presented in Fig. 8 showed that POD enzyme activity was significantly decreased in leaves of plants grown under drought condition compared to the untreated stressed plants. These results are also, supported by the findings of Sahalata *et al.*[44] and Kattab[45]. The reductions in peroxidase activities suggest that these enzymes were unable to completely neutralize H_2O_2 resulted from the drought stress. Meanwhile, Of all treatments, ASC+GSH under normal irrigation and ASC under drought conditions, significantly increased POD enzyme activity in leaves of plants compared to the corresponding values of control plants, suggesting a better antioxidant system for removing H_2O_2 by POD. Moreover, increase in POD activity under various stress conditions has been linked with protection from oxidative damage, lignifications, and cross-linking of cell wall to prevent from such adverse conditions[48]. Polyphenol oxidase is among the major antioxidant enzymes involved in scavenging AOS[49]. The activity of phenol oxidase (PPO) was significantly increased in drought stressed plants compared to the unstressed control plants (Fig. 9). The increase in phenol peroxidase activity in stressed plants may be decrease the injurious effect of drought stress as well as it reacts with H_2O_2 and maintain the membrane integrity. Moreover, application of all treatments increased phenol oxidase (PPO) activity in plants grown under normal irrigation compared to the control ones. The highest value was obtained in ASC+GSH treated control plants. In contrast to GSH and GSH+ TOC, other treatments significantly increased POD enzyme activity in leaves of plants grown under drought condition compared to the corresponding values of control plants.

Generally, foliar application of GSH, ASC and α-TOC may be alleviate the harmful effect of reactive oxygen species (ROS) caused by drought stress through inhibiting the lipid photoperoxidation, involving in both electron transport of PS II and antioxidizing system of chloroplasts, and scavenging cytotoxic H_2O_2[50]. Vaidyanathan *et al.*[51] reported that the non-enzymatic antioxidants (ascorbic acid, glutathione, α-tocopherol and flavanoids) showed an accumulation in root tissues in plants subjected to stress. Tocopherols also play a key role as antioxidants because they physically quench or chemically scavenge singlet molecular oxygen ($^1O_2$), the excited molecular oxygen with spin paired valence electrons. One molecule of α-tocopherol can deactivate up to 120 $^1O_2$ molecules by resonance energy transfer[52]. We can deduced also, that application ASC and α-TOC, play a protective role in drought tolerance by increased the activities of the antioxidant enzymes as wells as antioxidant substances. ASC acts at the cellular level as affects the redox status of the cell and it may act on the gene level. The exogenous application of glutathione mitigated the adverse effects of water deficit stress on growth of cotton plants, it may be affected nuclear gene expression which influenced by plant's external environment[45]. Non-enzymatic antioxidant activity is represented by a series of antioxidant molecules that the plant uses against active oxygen species formation[11].

Finally, from the results of this experiment, it can be concluded that all treatments may be alleviate the harmful effect of reactive oxygen species (ROS) caused by drought stress through inhibiting the lipid photoperoxidation, accumulation of some antioxidant (proline, phenols and flavanoids) and activation of antioxidant enzymes (SOD, CAT, POD , APX and PPO) in drought stressed plants.

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