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*Full Length Research Paper*

# Sugar alcohols-induced oxidative metabolism in cotton callus culture

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**Sugar alcohols (mannitol and sorbitol) may cause oxidative damage in plants if used in higher concentration. Our present experiment was undertaken to study physiological and metabolic responses in cotton (*Gossypium hirsutum* L.) callus against mannitol and sorbitol higher doses. Both markedly declined mean values of relative fresh weight growth rates with the increase in their concentration intensities. The overall protein and malonaldehyde (MDA) contents increased in the stressed-shocked cells. Also, the mean values of various antioxidants such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and calalase (CAT) quantitatively improved over their respective controls. As a whole, MDA contents were higher in magnitude than that of different antioxidant enzymes. Also values of relative increase in case of POD were higher as compared to SOD showing the ability of cotton callus culture to scavenge $H_2O_2$ produced as a result of the activity of SOD. Our results show that both agents caused greater damage to the membranous structure in comparison to less activation of the antioxidants. As a whole, the overall change regarding fresh weight growth rates was less after 14-day stress regime, while the mean values of the antioxidant enzymes activities were lower after the 28-day stress period. Such decrease conveys the message that less reactive oxygen species (ROS) might have been produced.**

**Key words:** Antioxidants, callus culture, *Gossypium hirsutum* L., osmotic stress, sugar alcohols.

## INTRODUCTION

Exposure of plant species to various environmental stresses results in the production of reactive oxygen species (ROS) in chloroplasts, mitochondria and microbodies of plant cells. These species can initiate cascade reactions that result in the production of $OH^{\cdot}$ and other toxic species such as lipid peroxides (Bernstein et al., 2010). Due to their highly reactive nature, they result in pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation and DNA mutation in the absence of any protective mechanism (Mittler, 2002; Meloni et al., 2003). Their accumulation in cells causes cyto-toxicity (Scandalios, 2005) and hence results in greater loss of crop productivity worldwide (Mahajan and Tuteja, 2005; Apel and Hirt, 2004).

To avoid and alleviate such damage, plants have evolved specific protective antioxidant enzymes system comprised of: (a) low molecular weight antioxidants (α-tocopheroal and β-carotene), and water-soluble reductants (e.g. glutathione and ascorbate); and (b) antioxidative enzymes like superoxide dismutase, peroxidase, ascorbate peroxidase and calalase. These enzymes are the most important components in the scavenging system of ROS (Meloni et al., 2003). Superoxide dismutase (SOD) provides the first line among the antioxidant defense system as it dismutates

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two $O_2^{\cdot-}$ to $H_2O_2$ and oxygen (Cakmak and Horst, 1991). $H_2O_2$ is broken down by peroxidase (POD) and catalase (CAT) converts $H_2O_2$ to water. CAT can directly dismutate $H_2O_2$ into $H_2O$ and $O_2$ and is unavoidable for ROS detoxification during the stressful conditions. APX provides the ascorbate-dependent $H_2O_2$-scavenging mechanism in the plants. $H_2O_2$ is reduced to $H_2O$ by the activity of APX using ascorbic acid as an electron acceptor. malonaldehyde (MDA) formation is considered to be the general indicator of lipid peroxidation (Somashekaraiah et al., 1992; Chaoui et al., 1997) in membranes.

*In vitro* culture techniques provide a uniformly controlled environment for studying physiological and biological processes in plants, particularly at the cellular level under salt and drought-induced stresses (Hasegawa et al., 1984; Aghaleh et al., 2009). Both have a common osmotic effect (Torabi and Niknam, 2011) that induces the plants to decrease their internal water potential to avoid desiccation. The osmotic stress effects in plant cell and tissue culture can be investigated by using either ionic and penetrating (NaCl and KCl), non-ionic and penetrating (mannitol and sorbitol) or non-ionic and non-penetrating (polyethylene glycol [PEG]) stress agents (Gangopadhyay et al., 1997). The present *in vitro* studies were under taken using various sugar alcohols (that is, mannitol and sorbitol) as osmotic stress causing agents in the embryogenic callus of Coker 312. These are polyols, which are obtained through the reduction of sugar. They are also referred as osmolytes (Zhu, 2001) and exhibit ROS scavenging properties (Xiong and Zhu, 2002).

Cotton being one of the first species used for callus induction and somatic embryogenesis studies is one of the most promising plant species regarding the *in vitro* studies of factors affecting the callus induction, somatic embryogenesis and plant regeneration. However, the least untouched part of the various experiments related to *in vitro* studies of cotton is the biochemical characterization of the callus cultures under these factors. The present study was undertaken using sugar alcohols (mannitol and sorbitol) as osmotic agents. Although, they are being used as carbon sources for *in vitro* growth, however, they may introduce osmotic stress above certain concentrations (Al-Khayri and Al-Bahrany, 2002). So based on these information, we carried out an experiment using mannitol and sorbitol at higher concentrations.

The main aims of the work were to induce osmotic stress and to peep into the physiological and metabolic responses of cotton embryogenic callus culture. Such studies will be useful in identifying the physiological and biochemical responses of the cotton callus culture under these osmolytes when being used at higher concentration. Results obtained will determine the involvement of various antioxidant enzymes to cope with the production of reactive oxygen species. They will also allow further insights into the molecular mechanisms of tolerance/susceptibility of cotton embryogenic callus cultures against these osmotic agents.

## MATERIALS AND METHODS

### Plant materials and establishment of embryogenic callus

Seeds of upland cotton (*Gossypium hirsutum*) cv. Coker 312 were obtained from the Cotton Research Institute, Chinese Academy of Agricultural Sciences, Anyang, Henan, China. They were surface sterilized by 70% (v/v) ethyl alcohol for 3 min, followed by 0.1% (w/v) $HgCl_2$ for 8 min. The sterile seeds were then inoculated on Murashige and Skoog (1962) basal medium (1962) supplemented with 1.5% (w/v) glucose and 0.25% (w/v) phytagel for germination. Seeds were cultured at 28 ± 2°C in the dark for three days and then transferred to the culture room (28 ± 2°C) under a 14:10 day: night photoperiod with light provided by cool-white fluorescent lamps at an irradiation of 135 L mol $m^{-2}$ $s^{-1}$ for five to seven days. The hypocotyls of the sterile seedlings were cut into about 3 to 4 mm cuttings and inoculated on $MSB_5$ callus induction medium (MS medium plus $B_5$ vitamins (Gamborg et al., 1968)), adding 0.5 mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.15 mg $L^{-1}$ KT, 3% (w/v) glucose and 0.25% (w/v) phytagel. Induced calli were subcultured on fresh $MSB_5$ callus induction medium for three to four rounds (about three months). Then well-proliferated non-embryogenic calli were transferred to $MSB_5$ embryogenic callus induction medium possessing 0.5 mg $L^{-1}$ IBA, 0.15 mg $L^{-1}$ KT, 1 g $L^{-1}$ glutamine, 0.5 g $L^{-1}$ asparagines, 3% (w/v) glucose, and 0.25% (w/v) phytagel. After subculturing three to four times (about three months), the embryogenic calli of parrot green color were produced. Moreover, throughout the experiment, the pH of all types of media was 5.8. Each sub-culturing was done after three to four weeks, and after seven to eight months, embryogenic callus with high proliferation rate was obtained, which was subjected to sugar alcohols (mannitol and sorbitol)-mediated osmotic stress.

### Supplementation of the mannitol and sorbitol as sugar alcohols

The parrot green and highly proliferating embryogenic calli were used osmotic stress experiments induced by mannitol and sorbitol known as sugar alcohols. Both the stresses were singularly applied in the $MSB_5$ embryogenic callus induction and proliferating medium prior to autoclaving. The data were taken after 14 and 28 day stress period. For both stresses, five different treatment levels were used including their controls. For mannitol stress, there were 0, 50, 110, 170 and 230 mM stress levels while for that of sorbitol stress the levels were 0, 30, 80, 140 and 190 mM.

### Determination of relative fresh weight growth rate (RFWGR)

Relative fresh weight growth rate of both unstressed and stressed calli of Coker 312 was determined based on the initial fresh weight and the final fresh weight in case of both stresses after 14 and 28-day stress regimes. The percentage values of the relative fresh weight growth rate were determined according to the following formula:

$$RFWGR = (FW_f - FW_i)/FW_i \times 100$$

Where, $FW_i$ = initial fresh weight; $FW_f$ = final fresh weight.

### Determination of lipid peroxidation

The level of lipid peroxidation was expressed as MDA content and was determined as 2-thiobarbituric acid (TBA) reactive metabolites as previously described by Zhou and Leul (1998). Briefly, cotton calli (0.5 g) were homogenized and extracted in 10 ml of 0.25% TBA made in 10% trichloroacetic acid (TCA). Extract was heated at 95°C for 30 min and then quickly cooled on ice. After centrifugation at 5000 *g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Nonspecific absorbance at 600 nm was subtracted from that at 532 nm. The level of lipid peroxidation was expressed as µmol $g^{-1}$ fresh weight by using an extinction coefficient of 155 mM $cm^{-1}$.

### Sample extraction for determination of soluble proteins and antioxidant enzymes

For the determination of soluble proteins and various antioxidant enzymes, 0.5 g of cotton calli was homogenized with a mortar and pestle under chilled conditions in the extraction buffers specific for each assay. The homogenate was filtered through four layers of muslin cloth and was centrifuged at 10 000 *g* for 20 min at 4°C and the supernatants were used for various assays. An aliquot of the extract was used to determine protein contents following the method of Bradford (Bradford, 1976) using bovine serum albumin as standard. SOD (EC 1.15.1.1) activity was determined by using the photochemical nitroblue tetrazolium (NBT) method. The samples (0.5 g) of cotton callus culture were homogenized in 5 ml extraction buffer consisting of 50 mM phosphate, pH 7.8. The assay mixture in 3 ml contained 50 mM phosphate buffer, pH 7.8, 26 mM L-methionine, 750 µM NBT, 1 µM ethylenediaminetetraacetic acid (EDTA), and 20 µM riboflavin. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm and an inhibition curve was made against different volumes of extract. One unit of SOD is defined as being present in the volume of extract that causes inhibition of the photo-reduction of NBT by 50%.

Peroxidase (POD) activity (E.C. 1.11.1.7) was measured using guaiacol as the substrate in a total volume of 3 ml. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% $H_2O_2$ and enzyme extract. Increase in the absorbance due to oxidation of guaiacol was measured at 470 nm. Enzyme activity was calculated in terms of absorbance on 470 nm per gFW per min at 25 ± 2°C. Assay for ascorbate peroxidase (APX) activity was carried out in a reaction mixture in 3 ml containing 100 mM phosphate (pH 7.0), 0.1 mM EDTA-$Na_2$, 0.3 mM Ascorbic acid, 0.06 mM $H_2O_2$ and 100 µL enzyme extract. The change in absorption at 290 nm was recorded 30 s after the addition of $H_2O_2$. Enzyme activity was quantified using the molar extinction coefficient for ascorbate ($E$= 2.8 $mM^{-1}cm^{-1}$) expressed as µmol per gFW. Catalase (EC 1.11.1.6) activity was measured according to (Radwan et al., 2006). Briefly, the disappearance of $H_2O_2$ was monitored by measuring the decrease in absorbance at 240 nm (E = 0.036$mM^{-1}$ $cm^{-1}$) of a reaction mixture consisting of 25 mM potassium phosphate buffer (pH 7.0), 10 mM $H_2O_2$ and enzyme extract. The final activity was expressed as U per gFW.

### Statistical analyses

The data obtained from the present experiment were subjected to one-way analysis of variance (ANOVA) using SAS (Version 9) software for statistical significance at $P < 0.05$. All the results were the mean ± SE of three replications. Means were separated by least significant difference (LSD) test at 5% level of significance.

## RESULTS

### Relative fresh weight growth rate of cotton callus culture

In order to study the influence of sugar alcohols stresses on cotton callus growth, the percent values of relative fresh weight growth rate of cotton callus culture were analyzed (Table 1). These stressful conditions caused progressive decline in the relative growth rates of the callus culture at all levels over their respective controls after 14-day treatment. Relative to normal conditions, similar trend was also obvious after 28-day treatment at all treatment levels except at 50 mM mannitol and 30 and 80 mM sorbitol stress levels. Moreover, lower decrease was found at 110 mM mannitol stress level after 14-day stress regime, while higher decrease was experienced by cotton calli at 190 mM sorbitol stress after 28-day stress duration. Regarding the percent time-course changes, also the time-dependent changes were found to be greatly varied. As compared with their relevant controls, highest percent change (33%) was observed for 30 mM sorbitol treatment, while the lowest value (14%) was found at 140 mM sorbitol treatment levels.

### Soluble protein contents of cotton callus culture

Table 2 illustrates the total soluble protein contents in the cotton callus culture grown for 14 and 28-day mannitol and sorbitol stresses, respectively. Compared to their respective controls, the cell protein contents of Coker 312 tend to increase under both stresses after 14 and 28-day stress treatment. However, in case of mannitol stress relative decrease over the control was noticed both at 170 and 230 mM after 14-day stress treatment, while in case of sorbitol stress decrease at all levels as compared with control observed after 28-day stress period. Moreover, the highest relative increase (83, 79%) was noticed respectively at 110 mM mannitol and 80 mM sorbitol after 14-day stress duration. Considering the percentage time-course change, as a whole the highest percentage change (468%) was observed for 140 mM sorbitol treatment, while the lowest value (13%) was found at 230 mM mannitol treatment level.

### MDA contents of cotton callus culture

In order to estimate the influence of mannitol and sorbitol stresses on lipid peroxidation, MDA contents of cotton cell culture were determined (Table 3). Compared to their respective controls, the MDA contents of the stress-shocked callus culture obviously increased except at 80, 140 and 190 mM sorbitol stress levels after 28-day treatment. Among the applied stress conditions, highest relative rise (187%) was observed at 110 mM mannitol stress after 14-day treatment period, while the highest

**Table 1.** Percent values of relative fresh weight growth rate of cotton callus culture (cv. Coker 312) grown under sugar alcohols stresses.

| Stresses | Treatments (mM) | 14-day | 28-day | Time-course Change (%) |
|---|---|---|---|---|
| | 0 | 100.00 ±0.00$^{a}$ (100.00) | 100.00± 0.00$^{b}$ (100.00) | 100.00 |
| | 50 | 56.00 ± 2.25$^{b}$ (-44.00) | 150.34 ± 2.50$^{a}$ (50.34) | -38.34 |
| Mannitol | 110 | 42.22 ± 2.21$^{c}$ (-57.78) | 97.31 ± 3.22$^{b}$ (-2.69) | -12.87 |
| | 170 | 24.66 ± 2.05$^{d}$ (-75.34) | 53.42 ± 2.48$^{c}$ (-46.58) | -4.10 |
| | 230 | 21.27 ± 1.51$^{d}$ (-78.73) | 19.81 ± 1.79$^{d}$ (-80.19) | 22.74 |
| | 0 | 100.00 ± 0.00$^{a}$ (100.00) | 100.00 ± 0.00$^{b}$ (100.00) | 100.00 |
| | 30 | 79.56 ± 1.93$^{b}$ (-20.44) | 125.69 ± 1.76$^{a}$ (25.69) | 33.44 |
| Sorbitol | 80 | 49.15 ± 1.59$^{cd}$ (-50.85) | 101.02 ± 1.39$^{b}$ (1.02) | -2.73 |
| | 140 | 25.51 ± 1.05$^{d}$ (-74.49) | 37.26 ± 0.96$^{d}$ (-62.74) | 13.75 |
| | 190 | 13.50 ± 1.82$^{e}$ (-86.50) | 45.02 ± 0.61$^{c}$ (-54.98) | -18.03 |

Values are the means ± SE of three replications. Variants possessing same letters are not statistically significant at 5% probability level. Values in parenthesis show the relative increase (+) or decrease (-) over their respective controls. Percentage time-course change: was calculated based on difference in per day change in the enzyme activity that is, percentage time course change = (per day change after 14 day stress-per day change after 28 day stress)/respective control*100).

**Table 2.** Soluble protein (mg $g^{-1}$FW) in cotton callus culture (cv. Coker 312) grown under sugar alcohols stresses.

| Stresses | Treatments (mM) | 14-day | 28-day | Time-course Change (%) |
|---|---|---|---|---|
| | 0 | 22.49 ± 1.50$^{b}$ (100.00) | 32.27 ± 0.86$^{b}$ (100.00) | 100.00 |
| | 50 | 38.22 ± 1.15$^{a}$ (69.93) | 37.78 ± 0.86$^{a}$ (17.08) | 304.09 |
| Mannitol | 110 | 41.25 ± 0.72$^{a}$ (83.41) | 39.72 ± 2.26$^{a}$ (23.09) | 336.58 |
| | 170 | 22.40 ± 1.27$^{b}$ (-0.40) | 36.56 ± 1.14$^{ab}$ (13.28) | 64.84 |
| | 230 | 20.28 ± 0.85$^{b}$ (-9.83) | 38.88 ± 1.20$^{a}$ (20.49) | 13.21 |
| | 0 | 23.93 ± 0.48$^{c}$ (100.00) | 33.89 ± 1.03$^{a}$ (100.00) | 100.00 |
| | 30 | 39.21 ± 0.46$^{b}$ (63.89) | 23.84 ± 0.90$^{bc}$ (-29.64) | 390.81 |
| Sorbitol | 80 | 42.84 ± 0.74$^{a}$ (79.05) | 20.23 ± 0.41$^{d}$ (-40.31) | 468.61 |
| | 140 | 40.02 ± 0.70$^{b}$ (67.24) | 25.00 ± 0.83$^{b}$ (-26.23) | 394.01 |
| | 190 | 39.89 ± 1.27$^{b}$ (66.70) | 22.50 ± 0.59$^{cd}$ (-33.59) | 410.02 |

Values are the means ± SE of three replications. Variants possessing same letters are not statistically significant at 5% probability level. Values in parenthesis show the relative increase (+) or decrease (-) over their respective controls. Other information is same as Table 1.

**Table 3.** MDA contents (nmol $g^{-1}$FW) in cotton callus culture (cv. Coker 312) grown under sugar alcohols stresses.

| Stresses | Treatments (mM) | 14-day | 28-day | Time-course Change (%) |
|---|---|---|---|---|
| | 0 | 5.33 ± 0.68$^{c}$ (100.00) | 11.88 ± 0.08$^{d}$ (100.00) | 100.00 |
| | 50 | 14.96 ± 0.03$^{a}$ (180.91) | 12.67 ± 0.65$^{d}$ (6.65) | -1399.33 |
| Mannitol | 110 | 15.26 ± 0.55$^{a}$ (186.62) | 17.28 ± 0.78$^{b}$ (45.40) | -1075.00 |
| | 170 | 9.35 ± 0.37$^{b}$ (75.67) | 20.04 ± 0.36$^{a}$ (68.61) | 107.60 |
| | 230 | 7.77 ± 0.66$^{b}$ (45.99) | 15.45 ± 0.35$^{c}$ (30.03) | -7.90 |
| | 0 | 9.61 ± 0.59$^{c}$ (100.00) | 12.48 ± 0.36$^{b}$ (100.00) | 100.00 |
| | 30 | 19.93 ± 1.92$^{a}$ (107.45) | 19.36 ± 0.38$^{a}$ (55.10) | 304.52 |
| Sorbitol | 80 | 22.53 ± 0.67$^{a}$ (134.52) | 7.54 ± 0.33$^{d}$ (-39.60) | 557.38 |
| | 140 | 12.56 ± 0.25$^{bc}$ (30.80) | 10.39 ± 0.19$^{c}$ (-16.78) | 219.02 |
| | 190 | 14.15 ± 0.52$^{b}$ (47.28) | 8.49 ± 0.38$^{d}$ (-32.00) | 294.27 |

Values are the means ± SE of three replications. Variants possessing same letters are not statistically significant at 5% probability level. Values in parenthesis show the relative increase (+) or decrease (-) over their respective controls. Other information is same as Table 1.

relative decrease (40%) was found to be at 80 mM sorbitol level after 28-day treatment period. Furthermore, regarding the percentage time-course change, the highest percentage positive change (557%) was observed for 80 mM sorbitol treatment, while the lowest percentage negative change (1399%) was found at 50 mM mannitol treatment level.

**SOD activity of cotton callus culture**

In our present experiment, the SOD activity in the cotton callus culture exposed to mannitol and sorbitol stresses was also studied (Table 4). The tabulated data reveals that its activity improved relative to their respective controls. However, in case of mannitol stress, there was 5% decrease in its activity at 230 mM after 14-day treatment. And there were found 9 and 10% decrease at 50 and 230 mM mannitol, respectively at 28-day treatment. Moreover, 12% decrease was observed at 30 mM sorbitol after 14-day treatment, while there were 7 and 6% decrease, respectively at 140 and 190 mM sorbitol treatment after 28-day treatment process. Taken into account of the relative increase or decrease, the highest increase and decrease were found to be 23 and 10% in case of mannitol stress treatment under the 14 and 28-day stress treatment periods, respectively. Similarly, under the sorbitol treatment, 31 and 6% were the highest respective increase and decrease after 14 and 28-day stress treatment. Furthermore, regarding the percent time-course change, the highest percent change (166%) was observed for 190 mM sorbitol treatment, while the lowest value (63%) was found at 230 mM mannitol treatment level.

**POD activity of cotton callus culture**

POD activity of the cotton callus culture (Coker 312) revealed that both stresses caused progressive increase at all stress levels relative to normal conditions (Table 5). However, at 50 mM mannitol stress level its activity decreased by 20% over the control after 28-day stress period. Moreover, the highest percent relative increases (243, 129%) were observed respectively at 170 mM mannitol and 80 mM sorbitol concentration after 14-day stress period. Regarding the percent time course change, enhanced (586, 529%) time-course changes were noticed at 170 mM mannitol and 80 mM sorbitol stress levels.

**APX activity of cotton callus culture**

The ascorbate per oxidase (APX) activity also greatly varied in the experimental cotton callus culture under the influence of mannitol and sorbitol stresses (Table 6). Its activity ascended first over their relevant controls up to their second highest treatment level and then descended but was higher than their controls except at 230 mM mannitol treatment. At 230 mM mannitol treatment, the decrease was almost 6%. Regarding the relative increase/decrease in the mean values, the highest relative increase (41%) was observed at mannitol highest concentration, while in case of sorbitol treatment the highest increase (39%) in the APX activity was found to be at its 140 mM concentration after 14-day treatment. As a whole, the percent relative increase was higher under the mannitol stress after 28-day but the same trend was observed under sorbitol stress after 14-day stress regime. Also the percent time-course change was studied based on the difference in the activity of APX under the both stresses.

**CAT activity of cotton callus culture**

The CAT activity was also obvious under the stressful effects of these osmolytes (Table 7). In comparison to their respective controls, its mean values in the stress-shocked callus culture increased except at 230 mM mannitol stress levels after 14-day treatment. At this treatment level, there was 61% relative decrease over its control. Moreover, the highest relative rise 106 and 186% were respectively observed at 170 mM mannitol and 80 mM sorbitol stress after 14-day treatment period. Furthermore, regarding the time-dependent changes, the highest percentage positive change (1070%) and the lowest percent negative change (3087%) were noticed for 30 and 190 mM sorbitol treatment level, respectively.

**DISCUSSION**

Plants may experience drought (Simova-Stoilova et al., 2009), cold (Van Heerden et al., 2003), high temperature (Reynolds-Henne et al., 2010), salinity (Meloni et al., 2003), heavy metals (Daud et al., 2009; Smeets et al., 2009) and ultraviolet radiation (Gao and Zhang, 2008) stresses during their entire growth phase. These stresses may lead to the overproduction of reactive oxygen species (ROS), which are highly reactive and can cause oxidative cellular damage (Gill and Tuteja, 2010). To prevent such damage, plants have developed a complex antioxidant enzymes system (Joseph and Jini, 2011) being comprised of enzymatic (SOD, POD, APX and CAT) and non-enzymatic antioxidants (GR and AsA) (Foyer et al., 1991). In this study, we analyzed the physiological and biochemical response reactions of the cotton callus culture to mannitol and sorbitol-mediated osmotic stress.

**Growth of cotton callus culture**

Callus growth in terms of growth rate is the first physiological response against any external stimuli. In our present experiment, the applied increasing

**Table 4.** SOD activity (U $g^{-1}$FW) in cotton callus culture (cv. Coker 312) grown under sugar alcohols stresses.

| Stresses | Treatments (mM) | 14-day | 28-day | Time-course Change (%) |
|---|---|---|---|---|
| | 0 | 342.85 ± 2.27$^{c}$ (100.00) | 315.93 ± 5.18$^{b}$ (100.00) | 100.00 |
| | 50 | 368.21 ± 4.86$^{bc}$ (7.40) | 288.67 ± 5.38$^{c}$ (-8.63) | 121.09 |
| Mannitol | 110 | 387.22 ± 3.15$^{b}$ (12.94) | 320.83 ± 3.07$^{b}$ (1.55) | 122.68 |
| | 170 | 422.99 ± 7.96$^{a}$ (23.38) | 365.68 ± 2.58$^{a}$ (15.75) | 129.89 |
| | 230 | 257.47 ± 21.23$^{d}$ (-4.90) | 283.25 ± 3.04$^{c}$ (-10.34) | 62.66 |
| | 0 | 329.53 ± 0.93$^{c}$ (100.00) | 323.50 ± 12.62$^{bc}$ (100.00) | 100.00 |
| | 30 | 290.87 ± 9.93$^{d}$ (-11.73) | 338.55 ± 4.25$^{b}$ (4.65) | 72.47 |
| Sorbitol | 80 | 391.23 ± 3.31$^{b}$ (18.73) | 364.61 ± 6.24$^{a}$ (12.71) | 124.53 |
| | 140 | 404.73 ± 5.31$^{b}$ (22.82) | 299.78 ± 8.64$^{c}$ (-7.33) | 151.90 |
| | 190 | 431.63 ± 4.21$^{a}$ (30.98) | 304.97 ± 6.33$^{c}$ (-5.73) | 166.38 |

Values are the means ± SE of three replications. Variants possessing same letters are not statistically significant at 5% probability level. Values in parenthesis show the relative increase (+) or decrease (-) over their respective controls. Other information is same as Table 1.

**Table 5.** POD activity (OD470 $gFW^{-1}$ $min^{-1}$) in cotton callus culture (cv. Coker 312) grown under sugar alcohols stresses.

| Stresses | Treatments( mM) | 14-day | 28-day | Time-course Change (%) |
|---|---|---|---|---|
| | 0 | 9.43 ± 0.10$^{d}$ (100.00) | 10.48 ± 1.07$^{c}$ (100.00) | 100.00 |
| | 50 | 11.42 ± 0.62$^{d}$ (21.01) | 8.34 ± 0.88$^{c}$ (-20.36) | 172.67 |
| Mannitol | 110 | 16.86 ± 1.42$^{c}$ (78.73) | 21.10 ± 1.43$^{a}$ (101.36) | 150.48 |
| | 170 | 32.33 ± 2.06$^{a}$ (242.76) | 15.51 ± 0.30$^{b}$ (48.08) | 585.86 |
| | 230 | 22.57 ± 1.71$^{b}$ (139.22) | 15.64 ± 0.42$^{b}$ (49.25) | 351.57 |
| | 0 | 12.14 ± 1.61$^{b}$ (100.00) | 18.04 ± 0.59$^{d}$ (100.00) | 100.00 |
| | 30 | 14.75 ± 1.50$^{b}$ (21.43) | 20.83 ± 0.82$^{cd}$ (15.50) | 138.55 |
| Sorbitol | 80 | 27.84 ± 1.45$^{a}$ (129.27) | 22.64 ± 1.23$^{c}$ (25.53) | 528.60 |
| | 140 | 24.76 ± 1.76$^{a}$ (103.86) | 31.98 ± 0.65$^{b}$ (77.32) | 280.43 |
| | 190 | 25.55 ± 0.88$^{a}$ (110.40) | 28.83 ± 1.24$^{a}$ (59.87) | 356.22 |

Values are the means ± SE of three replications. Variants possessing same letters are not statistically significant at 5% probability level. Values in parenthesis show the relative increase (+) or decrease (-) over their respective controls. Other information is same as Table 1.

**Table 6.** APX activity (µM $g^{-1}$FW) in cotton callus culture (cv. Coker 312) grown under sugar alcohols stresses.

| Stresses | Treatments (mM) | 14-day | 28-day | Time-course Change (%) |
|---|---|---|---|---|
| | 0 | 80.86 ± 1.63$^{b}$ (100.00) | 71.79 ± 0.87$^{d}$ (100.00) | 100.00 |
| | 50 | 83.79 ± 0.99$^{b}$ (3.62) | 80.75 ± 0.67$^{c}$ (12.49) | 96.54 |
| Mannitol | 110 | 87.93 ± 0.75$^{a}$ (8.75) | 88.67 ± 0.77$^{b}$ (23.53) | 96.95 |
| | 170 | 90.46 ± 1.20$^{a}$ (11.87) | 100.29 ± 0.74$^{a}$ (39.70) | 89.66 |
| | 230 | 76.07 ± 0.74$^{c}$ (-5.92) | 101.36 ± 0.62$^{a}$ (41.19) | 56.47 |
| | 0 | 64.02 ± 2.11$^{c}$ (100.00) | 71.79 ± 0.87$^{d}$ (100.00) | 100.00 |
| | 30 | 82.40 ± 1.61$^{b}$ (28.71) | 90.42 ± 1.56$^{c}$ (25.96) | 132.21 |
| Sorbitol | 80 | 88.58 ± 1.46$^{a}$ (38.35) | 93.59 ± 1.93$^{bc}$ (30.38) | 148.53 |
| | 140 | 89.27 ± 1.86$^{a}$ (39.43) | 99.81 ± 1.26$^{a}$ (39.04) | 139.92 |
| | 190 | 82.93 ± 1.36$^{b}$ (29.53) | 96.80 ± 1.36$^{ab}$ (34.85) | 122.74 |

Values are the means ± SE of three replications. Variants possessing same letters are not statistically significant at 5% probability level. Values in parenthesis show the relative increase (+) or decrease (-) over their respective controls. Other information is same as Table 1.

**Table 7.** CAT activity (U $g^{-1}$FW) in cotton callus culture (cv. Coker 312) grown under sugar alcohols stresses.

| Stresses | Treatments (mM) | 14-day | 28-day | Time-course Change (%) |
|---|---|---|---|---|
| Mannitol | 0 | 7.33 ± 0.9$^{bc}$ (100.00) | 7.70 ± 0.98$^{c}$ (100.00) | 100.00 |
| | 50 | 10.67 ± 2.25$^{ab}$ (45.59) | 9.08 ± 0.84$^{bc}$ (17.86) | 176.26 |
| | 110 | 12.18 ± 1.46$^{ab}$ (66.13) | 12.42 ± 1.26$^{ab}$ (61.33) | 171.43 |
| | 170 | 15.10 ± 2.22$^{ab}$ (106.00) | 14.83 ± 1.25$^{a}$ (92.60) | 220.83 |
| | 230 | 2.84 ± 0.53$^{c}$ (-61.25) | 9.33 ± 1.57$^{bc}$ (21.17) | -52.42 |
| Sorbitol | 0 | 3.67 ± 0.57$^{b}$ (100.00) | 7.67 ± 1.16$^{b}$ (100.00) | 100.00 |
| | 30 | 4.75 ± 0.86$^{b}$ (29.58) | 13.08 ± 1.52$^{a}$ (70.60) | 1070.10 |
| | 80 | 10.50 ± 1.88$^{a}$ (186.27) | 11.17 ± 1.85$^{ab}$ (45.66) | -2937.54 |
| | 140 | 9.17 ± 1.21$^{a}$ (150.06) | 14.25 ± 1.19$^{a}$ (85.81) | -1222.92 |
| | 190 | 10.33 ± 0.96$^{a}$ (181.73) | 10.33 ± 1.63$^{ab}$ (34.78) | -3087.38 |

Values are the means ± SE of three replications. Variants possessing same letters are not statistically significant at 5% probability level. Values in parenthesis show the relative increase (+) or decrease (-) over their respective controls. Other information is same as Table 1.

concentrations of both osmotica caused obvious declines in the relative callus growth rates. These findings are in consistent with those of Torabi and Niknam (2011), who found no visible phytotoxic symptoms and growth retardation in the calli of *Salicornia persica* and *Salicornia europaea* grown on 1 M mannitol. However, we also observed some deviations from the normal trend. For example, at 50 mM mannitol and 30 and 80 mM sorbitol stress levels after 28-day treatment, the mean growth rate was higher over their controls. Moreover, the growth was efficient at each level of both stresses at 28-day treatment in comparison with the 14-day treatment. The reason behind this may be that the growth of the freshly transferred calli is usually very slow in their first few days after being transferred to the fresh medium (first author's personal observations). Also this decline may be due to progressive loss of water from the cells as a result of an osmotic gradient created by increasing concentrations of both osmotica as has been previously hypothesized by Garrat et al. (2002) during their studies on cotton cultures response under salinity stress.

## Soluble protein contents of cotton callus culture

Statistical analysis also showed that the cell protein contents of Coker 312 were significantly influenced under both stresses. Their over all response was the increasing one with few exceptions. For example, over their controls, their mean values relatively decreased both at 170 and 230 mM mannitol stress after 14-day stress treatment, while there was decreasing trend for sorbitol stress after 28-day stress period. Regarding the relative increase or decrease in comparison to the related controls, the highest relative increase (83, 79%) was noticed respectively at 110 mM mannitol and 80 mM sorbitol after 14-day stress. Moreover, for the percent time-course change, 336% was higher value for 110 mM mannitol treatment and 468% was higher value for 140 mM sorbitol treatment. There can be several reasons regarding this increased synthesis of proteins. For example; (1) under stressful conditions, plants can synthesize new proteins that may provide a storage form of nitrogen which is reutilized when stress is over (Torabi and Niknam, 2011) such was also in our experiment.
Based on the fact that when the cotton callus culture was transferred to fresh non-stressed MS after stress period, it profusely grew (data not shown), (2) these osmotic stress causing agents are also used as carbon sources in the MS media, which may help in the increased synthesis of proteins, and (3) Coker 312 being a salt tolerant line (Gossett et al., 1996) and hence can resist osmotic stress also. Our present results are not in line with the experimental findings of Ge et al. (2006).

## MDA contents of cotton callus culture

Membrane damage is due to the peroxidation of its polyunsaturated fatty acids under various stresses (Gill and Tuteja, 2010; Meloni et al., 2003), which is expressed by an increase in its MDA contents. In our present experiment, the MDA contents of the stressed callus culture significantly increased over their controls with few exceptions. Under both osmotic agents, greater relative increase was found at 14-day stress period. Taking into account the percent time-course change, the highest percent change was found to be at 170 mM mannitol while that for sorbitol treatment, was at 80 mM treatment level. Based on relative increase and decrease values in MDA contents, the degree of membrane damage was higher in comparison with the activities of various antioxidants being studied in our present experiment. Similar increase in MDA content has also been noted in *Cicer arietinum* L. and *Phaseolus vulgaris*, respectively by Zlatev et al. (2006) and Eyidogan and Oz (2005) under water and saline stressed conditions

respectively. Pan et al. (2006) also reported an increase in MDA contents by subjecting liquorice seedlings under salt and drought stresses.

**SOD activity of cotton callus culture**

During oxidative stress, the SOD activity is up-regulated, which eliminates $O_2^{\cdot-}$ by the formation of $H_2O_2$ and $O_2$. Regarding our experiment, significant up-regulation in SOD activity under both osmotica has been noted. However, the mean values of the activity declined over their respective controls at 230 mM mannitol after 14 and 28-day and 190 mM sorbitol after 28-day stress period. Also the highest increase, in case of mannitol was 23 and 16%, while that of sorbitol was 31 and 13% after 14 and 28-day stress period. Moreover, the highest percent time-dependent change value was observed at 190 mM sorbitol treatment and the lowest value (63%) was found at 230 mM mannitol treatment level. Increase in SOD activity was also noted in three cultivars of *P. vulgaris* by Zlatev et al. (2006) and in *O. sativa* by Sharma and Dubey (2005) under drought stress. Similar findings under salt stress in various plants viz. cotton (Gossett et al., 1994; Rajguru et al., 1999), mulberry (Harinasut et al., 2003), *C. arietinum* (Kukreja et al., 2005) and *L. esculentum* (Gapińska et al., 2008) have also been documented. Furthermore, Molassiotis et al. (2006) found that osmotica and salts stresses up-regulated the SOD activity in apple cultivar (MM 106) grown *in vitro*. Here in case of our findings, such increase is probably the indication of the ability of cotton cells to respond to these stress causing agents.

**POD activity of cotton callus culture**

Although SOD functions as the first line of defense against oxidation at the membrane boundaries, its end product is the toxic hydrogen peroxide ($H_2O_2$) (Mittler, 2002). Therefore, an efficient $H_2O_2$-scavening system is required to enable rapid removal of $H_2O_2$. Peroxidase (POD) activities are involved in detoxification of hydrogen peroxide over-produced in stress conditions (Siegel, 1993; Smirnoff, 1993). In case our studies, the peroxidase activity progressive increase relative to normal conditions were noticed. Except at 50 mM mannitol stress level at 28-day stress period, the POD relative activity promoted with the increase in the treatment levels. The highest percent relative increase was noticed after 14-day stress regime and the percent time-dependent changes were higher at 170 mM mannitol and 80 mM sorbitol stress levels. The osmolyte-induced enhancement of POD activity in Coker 312 callus culture indicated that it had a higher capacity for the decomposition of $H_2O_2$ generated by SOD. Similar trend was found by Meloni et al. (2003) in cotton, Molassiotis et al. (2006) in apple, Panda (2001) in green gram and Koji et al. (2009) in rice leaves during their studies on salinity.

**APX activity of cotton callus culture**

Ascorbate per oxidase (APX) has got the most essential role in scavenging ROS because it has higher affinity for $H_2O_2$ (µM range) than CAT and POD (mM range) (Gill and Tuteja, 2010). In our present study, the APX activity showed ascending pattern over their relevant controls up to their second highest treatment level. At the highest treatment levels, the mean values descended but was higher than their controls except at 230 mM mannitol treatment. Considering the relative increase or decrease, the highest relative increase was at 230 mM mannitol after 28-day stress period and that was 39% at 140 mM concentration after 14-day treatment. Moreover, the percent time-course changes were highest (97, 149%) at 50, 140 mM mannitol and sorbitol treatment levels. Similar activity pattern regarding different cultivars of *P. vulgaris* (Zlatev et al., 2006) and *P. asperata* (Yang et al., 2008) under water stress was also noticed. Sharma and Dubey (2005) found that mild drought stressed plants had higher chloroplast-specific APX activity than control, which was declined when the drought stress levels were increased.

**CAT activity of cotton callus culture**

CAT is among the $H_2O_2$-scavenging enzymes. The balance between the activity of $H_2O_2$-producing enzymes and that of $H_2O_2$-scavenging plays an important role in providing a plant defense mechanism against oxidative damage (Torabi and Niknam, 2011). In our experiment, different concentrations of sugar alcohols induced significant changes in the CAT activity in case of osmolytes. And as a whole its activity under sorbitol stress was higher than its activity under the mannitol stress. In comparison to its control, only at 230 mM mannitol level relative decrease was noticed, while at all other levels in case of both stresses, there was observed relative increase. Moreover, the time-bound changes were higher under the sorbitol-induced osmotic stress conditions, which conveys the idea that sorbitol caused greater changes in the CAT activity as compared to mannitol. Increase in the CAT activity in our present experiment reveals that CAT might be actively involved in the removal of $H_2O_2$, which is produced as result of SOD. Our findings are in line with those of Vital et al. (2008) and Simova-Stoilova et al. (2010) who found an increase in the CAT activity under drought and salinity stress, respectively. However, Pan et al. (2006) found a decrease in the CAT activity in liquorice seedlings under the combined effect of salt and drought stress, which are contrary to our findings.

## Conclusions

The greater degree of membrane damage (as revealed by higher relative increase in MDA contents) and comparatively lower activities of SOD, POD, APX, CAT covey the idea that these agents caused greater damage to cellular structure of Coker 312 but have comparatively produced less ROS. Greater membrane damage in comparison to lower antioxidant activities needs to be further studied by exploring the cellular mechanism in order to better comprehend the reasons behind it. The osmolyte-induced enhancement of POD activity in Coker 312 callus culture indicated that it had a higher capacity for the decomposition of $H_2O_2$ generated by SOD and furthermore it provides sound basis for an initial understanding of the cellular responses of the cotton callus cultures, which is important for future studies to develop strategies for selecting drought tolerant cotton cultivars through *in vitro* means.

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