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Regular Article

Salinity induced changes in ascorbic acid, hydrogen peroxide, lipid peroxidation and glutathione content in leaves of salt tolerant and salt-susceptible cultivars of cotton (G*ossypium hirsutum* L.)

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The present study was carried out to investigate the effect of NaCl salinization on ascorbate content, hydrogen peroxide content and lipid peroxidation in seedlings of salt-sensitive (H-1236) and salt-tolerant (H-1226) American cotton genotypes. A non-significant change was observed in ascorbate content in salt-tolerant (H-1226) genotype when exposed to salt stress, while there was a significant increase of 33.7% in sensitive genotype (H-1236). Enhancement in hydrogen peroxide content was observed in both the genotypes upon NaCl treatment. The basal level of hydrogen peroxide content was higher in sensitive genotype (87.2 µmoles/gFW) as compared to the tolerant genotype (67.9 µmoles/gFW). The leaves of both the genotypes showed higher level of malondialdehyde content when subjected to salt stress, however, the extent of enhancement was more in sensitive genotype (182%) as compared to that in tolerant genotype (140%).

Key words: Ascorbate, hydrogen peroxide, malondialdehyde, NaCl, salt-stress

Salinity is a widespread problem and it is considered to be one of the most serious constraints facing agriculture today, especially in the arid and semi-arid areas of the world. It affects almost every aspect of the physiology and biochemistry of plants. High exogenous salt concentration affects seed germination, causes water deficit and ion imbalance of the cellular ions resulting in ion toxicity and osmotic stress (Hajlaoui *et al*., 2009). The reactive oxygen species (ROS) are generated when plants are subjected to harmful stress. The highly damaging reactive oxygen species include superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) (Dionisio-sese and Tobita, 1998). The hydrogen peroxide is one of the major and most stable ROS that regulates basic acclimatization, defense and developmental processes in plants (Slesak *et al*., 2007). It has been shown to act as a signal transduction molecule in several developmental processes (Hung *et al*., 2005). Nevertheless, its high concentration leads to oxidative stress through an increase of lipid peroxidation and modification of membrane permeability. In fact, the modification of membrane fluidity is mediated by changes in profile of fatty acid, as a main component of membrane lipids and thus they are considered to be important in salt tolerance of plants (Upchurch, 2008). Ascorbic acid, an

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important antioxidant, which is concentrated in the chloroplast and the cytosol, protects the photosynthetic apparatus under stress by scavenging excess ROS (Smirnoff, 1995). A correlation between antioxidant capacity and NaCl tolerance has been demonstrated in several plant species like *Solanum tuberosum* (Benavides *et al.*, 2000), *Triticum aestivum* (Mandhania *et al*., 2006) and *Lycopersicon esculentum* (Sklodowska *et al.,* 2009). The present investigations were therefore, undertaken to study the effect of NaCl on ascorbate content, hydrogen peroxide generation and lipid peroxidation in salt-sensitive and salt-tolerant genotypes of American cotton.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of American cotton (*Gossypium hirsutum* L.) cultivars H-1226 (salt-tolerant) and H-1236 (salt-sensitive) obtained from the Cotton Section, Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar were grown in earthen pots lined with polyethylene bags and filled with 6 kg sandy loam soil. The sandy loam soil was saturated with water alone (control) and with 100 mM NaCl. After 8 days of sowing, leaves of cotton seedlings were taken for the further studies.

Preparation of extracts

One gm of the plant tissue was ground in 6 ml of chilled 0.8 N $HClO_4$ and centrifuged at 10000 rpm for 30 minutes. The clear supernatant was decanted and neutralized with 5M K_2CO_3. It was again centrifuged at 10000 rpm for 30 minutes. The clear supernatant thus obtained was carefully decanted and the corresponding volume of each preparation was recorded. This supernatant was used for estimation of ascorbic acid content, hydrogen peroxide content and malondialdyde content.

Determination of Ascorbic acid

The slightly modified procedure of Oser (1979) was followed for estimating total ascorbic acid. For estimation of total ascorbate, 1 ml extract was treated with equal volume of 10% TCA. It was then allowed to stand in ice for 5 minutes. During estimation of total ascorbate, 5 M NaOH, 150 µM sodium phosphate buffer (pH-7.4), 10 mM dithiothreitol (DTT) and 0.5% (w/v) N-ethyl maleimide (NEM) in a final volume of 7 ml were added. 2% dinitrophenyl hydrazine in 9N H_2SO_4 and a drop of 10% thiourea in 70% ethanol were added. The tubes were kept in boiling water bath for 15 minutes and then cooled. After cooling 80% H_2SO_4 was added to the tubes at 0^oC and mixed on vortex shaker. Their absorbance at 530 nm was recorded against reagent blank. A reference curve was prepared with (0-100 nmoles) of ascorbate.

Determination of Hydrogen peroxide (H_2O_2)

H_2O_2 was estimated by the method of Sinha (1972). 200 µl of above extract prepared was made to 1 ml with 0.1 M phosphate buffer (pH 7.5). 2 ml of 5% potassium dichromate and glacial acetic acid [1:3 v/v], was added to it. The mixture was then heated in boiling water bath for 10 minutes and cooled. Its absorbance was read at 570 nm against reagent blank which was without sample extract. The quantity of H_2O_2 was determined from standard curve prepared with 0-100 µM H_2O_2.

Determination of Malondialdehyde content

The level of lipid peroxidation was measured as malondialdehyde (MDA) content by the procedure described by Dhindsa *et al.* (1981). 2 ml of 20% (w/v) trichloroacetic acid (TCA) containing 0.5% (w/v) thiobarbituric acid (TBA) was added to 0.75 ml of supernatant obtained as above. The mixture was heated at 95^0C for 30 min. and then cooled in ice bucket. After centrifugation for 10 minutes at 10000 rpm; absorbance of the supernatant at 532 nm was read and the value for the non-specific absorption at 600 nm was subtracted from it. The concentration of MDA was calculated using its extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Determination of Glutathione content

Content of oxidized, reduced and total glutathione was estimated by the method of Smith (1985). Total glutathione (GSH+GSSG) was determined by adding 1.6 ml of 0.1 M sodium phosphate buffer (pH7.5), 0.1 ml of 2 mM NADPH, 0.1 ml of 5 mM 5, 5'- dithiobis-(2-nitrobenzoic acid) (DTNB) and 0.2 ml of TCA extract. Added one unit (3.4 µl) of standard glutathione reductase (Sigma), mixed well, incubated for 10 min and recorded absorbance at 412 nm against reagent blank. For estimation of oxidized glutathione, to 1.0 ml of TCA extract added 0.95 ml of potassium phosphate buffer (0.5 M, pH 7.5) and 50 µl of 4-vinylpyridine (Aldrich). The content were mixed well and allowed to stand for 1 h to remove /degrade GSH (reduced glutathione). To 0.4 ml of 4-vinylpyridine treated extract (which was equivalent to 0.2 ml of original TCA extract) added 1.4 ml of 0.1 M sodium phosphate buffer (pH 7.5), 0.1 ml of 2 mM NADPH and 0.1 ml of DTNB. The reaction was started by adding one unit (3.4 µl) of standard GR enzyme, incubated for 10 min and absorbance was recorded at 412 nm against reagent blank. Total and oxidized glutathione were calculated by using the equation $y = 0.0014x$ derived from standard curve of reduced glutathione (100 to 1000 µM). The absorbance of standard GSH was recorded at 412 nm against buffer blank. The concentration of reduced glutathione (GSH) was calculated by subtracting the concentration of GSSG from that of the total glutathione. The results were expressed as µmols g^{-1} f.wt.

RESULTS AND DISCUSSION

Ascorbate

Ascorbate plays an important role in affording protection against active oxygen species, as it acts as electron donor for ascrobate peroxidase. Results depicted in Table 1 shows differential effect of salt-stress on ascorbate content in leaves of salt-sensitive and tolerant genotype of American cotton. A non-significant change was observed in tolerant genotype when exposed to salt stress, while there was an increase of 33.7% in sensitive genotype (H-1236) over their respective controls. Similar findings were reported by Gossett *et al* (1994a) and Benavides *et al* (2000) in NaCl-tolerant cotton and potato. On the contrary, reduction in total ascorbate was observed in leaves of salt-sensitive and salt-tolerant cultivars of *Pisum sativum* L. in response to long term salt stress (Hernandez *et al.*, 2000). Diminished ascorbate content was found in response to other abiotic stresses such as water stress in *Vigna catjang* (Mukherjee and Chaudhari, 1983) and in response to chilling and high irradiation in *Cucumis sativus* (Wise and Naylor, 1987). The diminished ascorbate pool under various stresses may be due to change in glutathione pool that has been implicated in recycling of ascorbate (Foyer *et al.*, 1991).

Table 1. Effect of salt stress on ascorbate (nmole/gFW) content in leaves of salt-susceptible and salt-tolerant genotypes of American cotton

Genotype	Treatment		% change
	Control	100mM NaCl	
H-1226 (T)	212.61±32.20	188.58±28.68	-11.3
H-1236 (S)	160.50±0.72	214.73±1.91	+33.7

Hydrogen peroxide

Direct or indirect formation of oxygen radicals, especially superoxide anion and H_2O_2 can adversely affect plants under salt-stress. Table 2 shows the amount of hydrogen peroxide (H_2O_2) produced in leaves of salt-sensitive (H-1236) which was 87.26 µmoles/gFW and it was significantly higher than that of salt tolerant genotype H-1226 which had 67.99 µmoles/gFW of leaves. Salt stress (100mM) resulted in enhancement in H_2O_2 content in both the cultivars but the amount of H_2O_2 was significantly higher in the sensitive genotype as compared to salt- tolerant genotype. Similar findings have been reported in wheat (Esfandiari *et al*. 2011). This increase in H_2O_2 might be due to enhanced production of O_2^- in mitochondria, and/or H_2O_2 in peroxisomes in response to NaCl (Hernandez *et al* 1993). Foyer *et al.* (1997) hypothesized that H_2O_2

along with glutathione act as signal response to various types of stresses and triggers change in gene expression, thereby playing an important role in inducing stress tolerance.

Table 2. Effect of salt stress on hydrogen peroxide concentration (µmole/gFW) in leaves of salt-susceptible and salt-tolerant genotypes of American cotton

Genotype	Treatment		% change
	Control	100mM NaCl	
H-1226 (T)	67.99±0.05	156.97±0.03	130
H-1236 (S)	87.26±0.02	199.23±0.02	128

Lipid Peroxidation

Malondialdehyde content (MDA) is the final product during lipid peroxidation and is thought to be responsible for membrane deterioration during most type of stresses. The extent of damage to lipid membrane with salinity as measured by malondialdehyde (MDA) content is presented in Table 3 which indicates that the leaves of both the genotypes showed higher level of MDA when subjected to salt stress, however, the extent of enhancement was more in sensitive genotype (182%) as compared to that in tolerant genotype (140%). Similarly it has been reported that significantly higher lipid peroxidation occurred in salt-sensitive genotype of cotton than in salt-tolerant one, as the higher increase of MDA content in sensitive genotype might necessitate greater diversion of their resources towards membrane repair than in the salt-tolerant cultivars (Gossett *et al.*, 1994 a,b).

Table 3. Effect of salt stress on malondialdehyde (nmole /gFW) content in leaves of salt-susceptible and salt-tolerant genotypes of American cotton

Genotype	Treatment		% change
	Control	100mM NaCl	
H-1226 (T)	4.23±0.31	10.15±1.98	140
H-1236 (S)	5.35±0.17	15.13±0.41	182

Dionisio-Sese and Tobita (1998) reported that higher increase in MDA content in sensitive cultivar would be due to greater enhancement in free radical formation and associated lipid peroxidation under salinity stress. On the contrary, Olmos *et al.* (1994) reported that under salt stress conditions, peroxidation was higher in salt-tolerant calli of *Pisum sativum* than in NaCl-sensitive calli.

Glutathione content

Glutathione plays an important role in the protection against oxidative stress. It is involved in the ascorbate / glutatione cycle and in the regulation of protein thiol-disulphid redox status of plants in response to abiotic and biotic stress (Mullineaux and Rausch, 2005). Results presented in Table 4 reveal the effect of salt stress on glutathione content in leaves of salt-sensitive and salt-tolerant genotypes of American cotton. Enhancement in glutathione content was observed in both the genotypes under salinity. At 100 mM NaCl, tolerant genotype showed an elevation of 28.78% increase in glutathione content while sensitive genotype showed an elevation of 39.60% over their respective control. Glutathione is involved in the defense system by increasing the glutathione pool for scavenging H_2O_2. It has been documented that GSH functions as an antioxidant to react none enzymatically with singlet oxygen, superoxide ion and hydroxyl radical and also protect proteins against denaturation caused by the oxidation of protein thiol groups (Noctor *et al.*, 2002).

Table 4. Effect of salt stress on glutathione (µg/gFW) content in leaves of salt-susceptible and salt-tolerant genotypes of cotton

Genotype	Treatment		% change
	Control	100mM NaCl	
H-1226 (T)	28.70±0.93	40.30±2.47	28.78
H-1236 (S)	20.50±1.02	28.62±2.47	39.60

Thus, the present study clearly revealed that American cotton seedlings when exposed to salt stress, a linear increase was observed in ascorbate and hydrogen peroxide content and the basal level of hydrogen peroxide content was

more in sensitive genotype. Moreover, the leaves of both the genotypes showed higher level of malondialdehyde content when subjected to salt stress, however, the extent of increase was more in sensitive genotype as compared to that in tolerant one.

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