ANTIOXIDANT RESPONSE TO SALT STRESS DURING FIBER DEVELOPMENT

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Abstract

Ovules from flowers removed 2 days postanthesis (DPA) from greenhouse-grown salt-tolerant [cv. Acala 1517-88 (AC-88)] and salt sensitive [cv.Deltapine-50 (DP-50)], MAR and Coker (CO-312) cotton plants were grown in culture media amended with either 0mM (control conditions) or 100mM (stress conditions) NaCl and at 24 DPA were analyzed for differences in fresh weight and antioxidant enzyme activities. Salt treatment reduced fiber growth in all the cultivars except AC-88. Superoxide dismutase activity increased during salt treatment in AC-88 and MAR, but not in CO-312 or DP-50. Catalase was constitutively high in AC-88 and a salt induced change was recorded in DP-50. Glutathione reductase activity was constitutively high in AC-88, increased in MAR and CO-312 under stress, and decreased significantly in DP-50 when subjected to NaCl stress. Under stress conditions, peroxidase activity increased significantly in MAR and AC-88 and decreased significantly in CO-312 and DP-50. High constitutive levels of ascorbate peroxidase activity were observed in AC-88 and DP-50 while the activity of this enzyme increased in MAR and CO-312 when stressed. Glutathione-S-transferase activity significantly increased in all the cultivars when treated with NaCl. These findings indicate that fibers from the more salt-tolerant ovules did not exhibit a reduction in fresh weight when subjected to salt stress and in addition suggests that the varietal difference in salt tolerance may have been, in part, due to the up regulation of the antioxidant enzymes.

Introduction

Oxidative stress results when plants are exposed to various forms of environmental stress (Asada, 1994; Krause, 1994). Plants possess antioxidants for protection against the cytotoxic species of activated oxygen such as superoxide, ($O_2^{\cdot -}$), hydrogen peroxide, (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). A number of studies investigating the impact of stress conditions including high NaCl levels (Gossett *et al.*, 1994a 1994b, 1994c) drought (Dhindsa and Matowe, 1981;Burke *et al.*, 1985; McCue and Hanson, 1990), temperature and light extremes (Rabinowitch and Fridovich, 1983; Wise and Naylor, 1987;Spychalla and Desborough, 1990; Baker, 1994), mineral deficiencies (Monk and Davies 1989; Cakmak and Marschner, 1992; Polle *et al.*, 1992),and herbicide treatment (Harper and Harvey, 1978; Dodge 1994) have revealed that oxidative stress often disrupts the homeostasis between the production of the reactive oxygen species and the quenching activity of the antioxidant enzymes. A plant may be said to be under oxidative stress when the production of the reactive oxygen free radicals exceeds the scavenging capacity of the antioxidant defense system. Cotton is classified as a salt-tolerant plant and a comparison between the cultivars shows varietal differences in the levels of salt tolerance (Gossett *et al.*, 1992, 1994a). In cotton high salt concentrations have been shown to reduce yield as well as growth, stomatal conductance, stomatal resistance and transpiration rates (Gossett, 1991). Previous studies carried out with whole plants (Gossett *et al.*,1992,1993, 1994a) and callus tissue (Gossett *et al.*,1994a,b) exposed to NaCl have revealed a significant increase in the activity of antioxidant enzymes compared to controls. Additional work monitored the activities of catalase, peroxidase, ascorbate peroxidase, and glutathione reductase in a control and a salt-tolerant cell line. The NaCl-tolerant cultivars and cell line had higher antioxidant enzyme activities as well as a significantly lower ascorbate/oxidized ascorbate ratio and a significantly higher GSH/GSSG ratio, (Gossett *et al.*,1994c). These results indicate that elevation in the activity of the ascorbate-glutathione cycle probably confers a degree of salt tolerance. Fiber strength and fiber fineness are among the most important cotton fiber qualities for more effective processing and end-use. However, investigating cotton fiber development under field conditions at the biochemical or molecular level is not practical due to the problems associated with microbial contamination and the unpredictable temperature fluctuations (Haigler *et al.*, 1991; Haigler, 1992). Fortunately fiber can be grown on ovules cultured *in vitro* from greenhouse grown plants. The *in vitro* culture method allows investigators to supply developing fibers with nutrients including unlimited glucose, which is the preferred carbon source, and the correct plant growth regulators. This approach also allows the influence of various stress factors such as cold stress (Gossett *et al.*, 1996) to be examined. The objective of this project was to culture ovules and subject them to salt stress to see if antioxidant responses similar to those observed in vegetative tissue occur during fiber development.

Materials and Methods

Seeds of a putative salt-tolerant cultivar [cv. Acala 1517-88 (AC88)] and three salt sensitive [cv.Deltapine-50 (DP-50)], MAR (Multiple Adversity Resistance) and Coker (CO 312)] were planted in 30cm pots in November, 1995 and placed in a temperature controlled greenhouse. Following emergence, the plants were watered every other day with nutrient solution. Flowers were removed 2 DPA, and under a sterile transfer hood, the ovaries were surface sterilized by

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submerging them in 100% ethanol and flaming. The ovaries were opened by making an incision along each suture line and opening each locule. The ovules were removed and placed in 250ml flasks containing 100ml of sterile culture medium (Beasley *et al.,* 1984) amended with either 0mM (control) or 100mM NaCl. The ovules were incubated in the dark at a constant temperature of 30°C.for 14 days. Considerable trichome development was evident in both control and experimental replicates. The ovules were blotted dry, weighed and stored -70°C. for subsequent antioxidant enzyme analysis. Samples were prepared for SOD, catalase, glutathione reductase peroxidase, glutathione S-transferase and total protein analysis by homogenizing 1 gram of the frozen material in 15 mls of extraction buffer (Anderson *et al.,* 1992), as modified by Gossett *et al.,* (1994a,b). Catalase activity was determined by monitoring the disappearance of H_2O_2 according to the method of Beers and Sizer (1952). Total SOD activity was measured by determining the amount of enzyme required to produce 50% inhibition of the reduction of cytochrome C by superoxide generated by xanthine oxidase as described by Forman and Fridovich (1973). Glutathione reductase (GR) activity was determined by monitoring the glutathione-dependent oxidation of NADPH as described by Schaedle and Bassham (1977). Peroxidase activity was measured by the H_2O_2-dependent oxidation of reduced 2,3',6-trichloroindophenol according to the method of Nickel and Cunningham (1969). Ascorbate peroxidase (AP) activity assayed by monitoring the ascorbic acid-dependent reduction of H_2O_2 as described by Anderson *et al.,* (1992) Glutathione-S transferase (GST) activity was assayed according to the method of Habig *et al*., (1974). For catalase, peroxidase, and AP, one unit of enzyme activity was defined as the amount necessary to decompose 1 *m*mole of substrate min^{-1} at 25°C. One unit of GR and GST was defined as the amount of enzyme required to reduce 1 nmole of substrate min^{-1} at 25°C. One unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of cytochrome C by 50%.Total protein was measured by the Pierce BCA method of Smith *et al.,* (1985). Since there was no significant difference in total protein between treatments or within cultivars, enzyme results are expressed as units gram fresh $weight^{-1}$. Data points are based on a mean of a minimum of four replicates and subjected to a one-way analysis of variance. Significance was determined at the 95% confidence limits.

Results and Discussion

Fresh weight data for the four cultivars indicates no significant difference in fiber fresh weight among MAR, DP-50, or AC-88 or among MAR, DP-50 or CO-312 under control conditions (O mM NaCl). However a significant difference in fresh weight was observed between CO-312 and AC-88 **(Fig. 1).** The salt stress data (100mM NaCl) reveals a 65% decrease in growth in MAR, a 73% decrease in CO-312 and a 57% decrease in DP-50. However, no significant decrease was recorded for AC-88, the most salt tolerant of the cultivars tested in this study. The activity of SOD was not significantly different among any of the cultivars tested under control conditions, but under salt stress SOD activity increased by 768% in AC-88 and in MAR a 390% increase was recorded. Significant changes in SOD activity were not observed under salt stress conditions for CO-312 or DP-50 **(Fig 2).** The activity of catalase was significantly lower in MAR than the other three cultivars in controls. Although significant increases in catalase activity were not observed in AC-88 or DP-50 when subjected to salt stress, catalase was seen to undergo significant increases in two of the cultivars, MAR and CO-312 where 236% and 25% increases were observed respectively. The regulation of catalase in MAR is of particular interest in that it has the lowest constitutive activity of the four cultivars tested but undergoes the greatest increase during salt stress **(Fig 3).** The same general trends recorded for catalase were also recorded for glutathione reductase under control conditions. MAR and CO-312 exhibited a significant increase in GR activity 212% and 54% respectively under salt stress conditions, but no significant increase was observed in AC-88, and GR activity in DP-50 underwent a 58% decrease under these conditions **(Fig. 4).** The assay of ascorbate peroxidase **(Fig. 5)** from control tissue showed MAR to have significantly lower activity for this enzyme than the other three cultivars. During salt stress AC-88 and DP-50 underwent no significant change, whereas increases were recorded for MAR (92% increase) and CO-312, (33% increase). High constitutive activities of peroxidase were recorded for all cultivars. The activity of this enzyme undergo inconsistent changes in response to salt stress. Significant decreases were seen in DP-50 and CO-312 (35% and 44% respectively) as compared to a 152% increase in MAR and a smaller (24%) increase in AC-88 **(Fig.6)**. Of the four cultivars MAR had the highest constitutive level of glutathione S-transferase activity and all four cultivars underwent increases in response to salt treatment;68%, 343%, 120% and 159% in MAR, CO-312, AC-88 and DP-50 respectively **(Fig.7).**

The reductions in fiber fresh weight recorded for MAR, CO-312, and DP-50 are consistent with reductions in leaf area and plant fresh weight observed in whole plant studies with cotton grown under salt stress conditions. Notably AC-88 showed no significant decrease in fiber fresh weight when subjected to salt stress. The antioxidant data indicate that the dismutating abilities of the cultivars at 0mM NaCl were similar but that under salt stress conditions, the activity of SOD differed significantly. Once again the increases in SOD activity in salt-tolerant AC-88 is in contrast to the lack of increase in salt sensitive DP-50 and CO-312. The elevated levels of SOD activity during salt stress in AC-88 and MAR suggest that NaCl exposure elicits an increase in the production of superoxide radicals and that the increase in SOD activity indicates an increased capacity to scavenge superoxide free radicals. Similar increases in SOD activity have also been observed in NaCl-tolerant cotton callus tissue (Gossett, *et al.,* 1994b) developing cotton fibers subjected to cold stress (Gossett *et al.*,1996), maize (Malan

et al., 1990), paraquat-resistant tobacco (Schalteil *et al.,* 1988), and chilling resistant spinach (Schoner and Krause, 1990). Analysis of the enzymes which break down the hydrogen peroxide generated by SOD revealed no significant difference in catalase activity among CO-312, AC-88 or DP-50 grown under non-stress conditions, but catalase activity in MAR was significantly lower. The increase in catalase MAR and CO-312 during salt stress and the constitutively high levels of this enzyme in AC-88 under control and salt stress conditions are consistent with whole plant data from cotton (Gossett *et al.,* 1994a) and with data from paraquat resistant cell lines of perennial rye grass Harper and Harvey, (1978). The profiles of peroxidase activity in AC-88 and MAR are similar to catalase for these cultivars. Both enzymes were constitutively high in AC-88 and increased in MAR and CO-312 during salt stress. Salt induced enhancement of catalase and peroxidase in the ovules of the putative salt tolerant cultivar AC-88 suggests that this tissue as well as the vegetative tissue has a higher capacity for the decomposition of hydrogen peroxide generated by SOD. Similar increases in peroxidase activity have been reported in salt-tolerant cultivars of cotton (Gossett *et al.,* 1994a), and stored potato tubers (Spychalla *et al.,* 1990). Salt stress induced changes in GR activity have been reported previously for cotton ovules exposed to cold stress (Gossett *et al.,* 1996), in NaCl tolerant cultivars, (Gossett *et al.,* 1994a,b), in NaCl tolerant cotton cell lines (Gossett *et al.,* 1994c), in turf grass, in *Tortula ruralis* during drought stress (Dhindsa, 1991), in maize during elevated O_2 stress (Foster and Hess 1982*), Pisum sativum* during O_2, NO_2 and SO_2 stress (Melhorn *et al.,* 1987), *Pinus strobilus* during cold stress (Anderson *et al.,* 1992) and during heat stress in tomatoes (Rainwater *et al.,* 1995). Ascorbate peroxidase underwent increases in activity in MAR and CO-312 during salt stress and was maintained at a high level in AC-88 in control and salt stress conditions. This high activity of AP maintains a high rate of ascorbate turnover. AC-88 maintains a high activity profile for both AP and GR as compared to the other cultivars studied. The high levels of AP activity may serve to maintain a high ascorbate turnover rate and a high GR activity may serve to maintain a high glutathione turnover rate. DP-50. A salt sensitive cultivar exhibited a reduction in GR activity when stressed. MAR, which is a relatively NaCl-tolerant cultivar exhibited an increase in the activities in both enzymes when subjected to salt stress conditions, however overall activity was less than those observed in AC-88. This strongly suggests that the more salt-tolerant cultivar has a much higher ascorbate turnover rate and a more active ascorbate-glutathione cycle than the less tolerant cultivars. Glutathione S-transferase, the enzyme responsible for the detoxification of xenobiotics, showed a high level of activity in MAR and AC-88 under control conditions. Significant increases were recorded during salt stress. Similar results have been recorded for this enzyme in cotton ovules during cold stress (Gossett *et al.,* 1996). The results presented in this paper show that varietal differences in salt stress resistance are correlated with differences in antioxidant enzyme activities. This together with data from cold stress, heat stress, drought stress and oxygen stress conditions from both cotton and other plants point to an important role for antioxidant enzymes in the stress physiology of plants.

Acknowledgments

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References

Anderson, J.V., B.I. Chevone, and J.L. Hess. 1992. Seasonal variation in the antioxidant system of eastern white pine needles. Plant Physiol. **98**:501-508.

Baker, N. 1994. Chilling stress and photosynthesis. In C.H. Foyer and P.M. Mullineaux, eds. Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC. Boca Raton, FL. 127-154.

Beasley, C.A., I.P. Ting, A.E. Linkins, E.H. Birnbuam, and D.P. Delmer. 1984. Cotton ovule culture: A review of progress and a preview of potential. In H.E. Street, ed. Tissue Culture and Plant Science. Academic Press, New York, 169-192.

Beers, R.F., and I. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195**:133.

Burke, J. J., P. E. Gamble, J. L. Hatfield, and J. E. Quisenberry. 1985. Plant morphological and biochemical responses to field water deficits. I. Responses of glutathione reductase activity and paraquat sensitivity. Plant Physiol. **79**:415.

Cakmak, I. and H. Marschner. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol. **98:**1222-1227.

Dhindsa, R. S. and W. Matowe. 1981. Drought tolerance in two moses: correlated with enzymatic defense against lipid peroxidation. J. Exp. Bot. **32**:79-91.

Dhindsa, R.S. 1991. Drought stress, enzymes of glutathione metabolism, oxidation injury, and protein synthesis in Tortula ruralis. Plant Physiol**. 95**:648-651.

Dodge, A. 1994. Herbicide action and effects on detoxification processes. In C. H. Foyer and P.M. Mullineaux, eds. Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC. Boca Raton, FL. 219.

Forman, H.J., and I. Fridovich. 1973. Superoxide dismutase: A comparison of rate constants. Arch. Biochem. Biophys**. 158**:396-400.

Foster, J.G., and J.L. Hess. 1980. Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen. Plant Physiol. **66:**482-487.

Gossett, D.R., M.C. Lucas, E.P. Millholon, W.D. Caldwell and S. Munday 1991 Isozyme variation among salt tolerant and salt sensitive varieties of cotton. Proc Beltwide Cotton Prod Res Conf National Cotton Council Memphis TN 556-559.

Gossett, D.R., M.C. Lucas, E.P. Millholon, W.D. Caldwell and A.Barclay 1992 Isozyme variation among salt tolerant and salt sensitive varieties of cotton. Proc Beltwide Cotton Prod Res Conf National Cotton Council Memphis TN 1036-1039.

Gossett D.R., E.P. Millhollon, M.C. Lucas, and M.M. Marney. 1993. Antioxidant enzymes and salt tolerance in cotton. Proceedings of the Beltwide Cotton Confeence, Cotton Physiology Symposia. 1262-1266.

Gossett, D. R., E. P. Millhollon, and M. C. Lucas. 1994a. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci. **34:**706-714.

Gossett. D. R., E. P. Millhollon, M. C. Lucas, S. W. Banks, and M. M. Marney. 1994b. The effects of NaCl on antioxidant enzyme activities in callus tissue of salt-tolerant and salt-sensitive cultivars of cotton. Plant Cell Reports**. 13**:498-503.

Gossett. D.R., E.P. Millhollon, S.W. Banks, M.C. Lucas, and M.M. Marney. 1994c. Antioxidant status in cotton cell lines selected for salt tolerance. Proceedings of the Beltwide Cotton Confeence, Cotton Physiology symposia. 1314-1317.

Gossett, D.R., S.W. Banks, M.C. Lucas, and E.P. Millhollon. 1996. Antioxidant response to cold stress during fiber development. Proceedings of the Beltwide Cotton Confeence, Cotton Physiology Symposia 1175-1177.

Habig, W.H., M.J. Pabst, and W.B. Jacoby. 1974. Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. **249:**7130-7139.

Haigler, C.H. 1992. The crystallinity of cotton cellulose in relation to cotton improvement. In cotton fiber cellulose: Structure, Function, and Utilization Conference. Savannah, GA: National Cotton Council, Memphis, TN. 211-226.

Haigler, C.H. 1991. Relationship between polymerization and crystallization in microfibril biogenesis, In: Biosynthesis and biodegradation of cellulose, Haigler, C.H. and P.J. Weimer, eds. Marcel Dekker, Inc.:New York. 99-124.

Harper, D.B., and B.M.R. Harvey. 1978. Mechanism of paraquat tolerance in perennial ryegrass II. Role of superoxide dismutase, catalase, and peroxidase. Plant Cell Environment**. 1:**211-215.

Malan, C., M.M. Greyling, and J. Gressel, 1990. Correlation between Cu, Zn superoxide dismutase and glutathione reductase and environmental and xenobiotics stress tolerance in maize inbreds. Plant Sci**. 60:** 187.

McCue, K. F. and A. D. Hanson. 1990. Drought and salt tolerance: towards understanding and application. Tibtech**. 8:**358.

Melhorn, H., D.A. Cottam, P.W. Lucas, and A.R. Welburn. 1987. Induction of ascorbate peroxidase and glutathione reductase activities by interaction of mixtures of air pollutants. Free Rad. Res. Commun. **3:**193-199.

Monk, L.S. and H.V. Davies. 1989. Antioxidant status of the potato tuber and Ca^{++} deficiency as a physiological stress. Physiol. Plant. **75:**411-416.

Nickel, R.S., and B.A. Cunningham. 1969. Improved peroxidase assay method using leuco 2,3',6 trichloroindophenol and application to comparative measurements of peroxidase catalysis. Ann. Biochem. **27:** 292-299.

Polle, A., K. Chakrabarti, S. Chakrabarti, F. Seifert, P. Schramel, and H. Renneberg. 1992. Antioxidants and manganese deficiency in needles of norway spruce (Picea abies L.) trees. Plant Physiol. **99:** 1084-1089.

Rabinowitch, H. D., and I. Fridovitch. 1983. Superoxide radicals, superoxide dismutase, and oxygen toxicity in plants. Photochem. Photobiol. **37**:679-790.

Rainwater, D.T., D.R. Gossett, H.H. Hanna, E.P. Millhollon, S.W. Banks, and M.C. Lucas. 1996. The relationship between yield and the antioxidant defense system in tomatoes grown under heat stress. Free Radical Research **25:** (5) 421-435.

Schaedle, M., and J.A. Bassham. 1977. Chloroplast glutathione reductase. Plant Physiol. **59**:1011-1012.

Schoner, S., and G.H. Krause. 1990. Protective systems against active oxygen species in spinach responses to cold acclimation in excess light. Planta. **180:**383-389.

Shaaltiel, Y., A. Glazer, P.F. Bocion, and J. Gressel. 1988. Cross tolerance to herbicidal environmental oxidants of plant biotypes tolerant to paraquat, sulfur dioxide, and ozone. Pest. Chem. Physiol. **13:**13-21.

Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.M. Gartner, M.D. Provenzano et al. 1985. Measurement of protein using bicinchonic acid. Anal. Biochem**. 150**:76-85.

Spychalla, J.P. and S.L. Desborough. 1990. Superoxide dismutase, catalase, and alpha-tocopherol content of stored potato tubers. Plant Physiol. **94**:1214-1218.

Wise, R. R. and A. W. Naylor. 1987. Chilling-enhanced photooxidation: Evidence for the role of singlet oxygen and endogenous antioxidants. Plant Physiol. **83:**278-282.

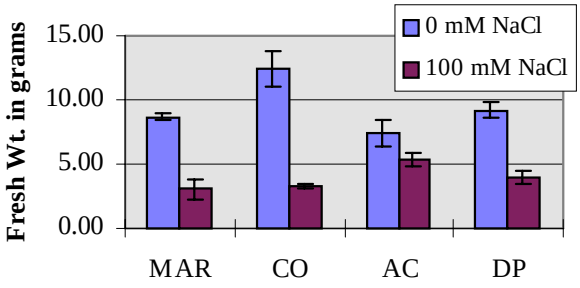

Figure 1. Fresh weight of cotton ovules from MAR, Coker (CO-312), Acala (AC-88), and Deltapine (DP-50) cultured under non-stressed (0 mM NaCl) and stressed (100 mM NaCl) conditions.

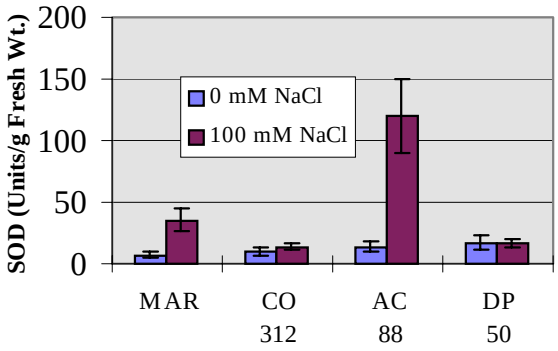

Figure 2. Superoxide dismutase activity (units/g fresh weight) of cotton ovules from MAR, Coker (CO-312), Acala (AC-88), and Deltapine (DP-50), cultured under non-stressed (0 mM NaCl) and stressed (100 mM NaCl) conditions.

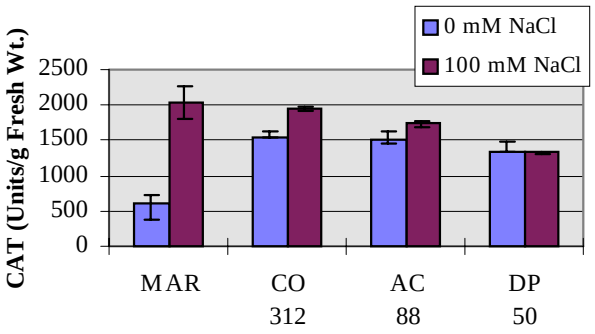

Figure 3. Catalase activity (units/g fresh weight) of cotton ovules from MAR, Coker (CO-312), Acala (AC-88), and Deltapine (DP-50) cultured under non-stressed (0 mM NaCl) and stressed (100 mM NaCl)

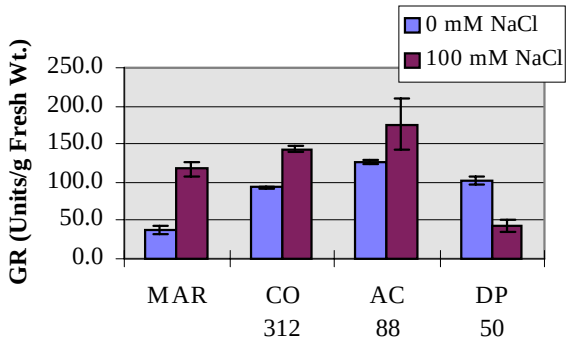

Figure 4. Glutathione reductase activity (units/g fresh weight) of cotton ovules from MAR, Coker (CO-312), Acala (AC-88), and Deltapine (DP-50) cultured under non-stressed (0 mM NaCl) and stressed (100 mM NaCl) conditions.

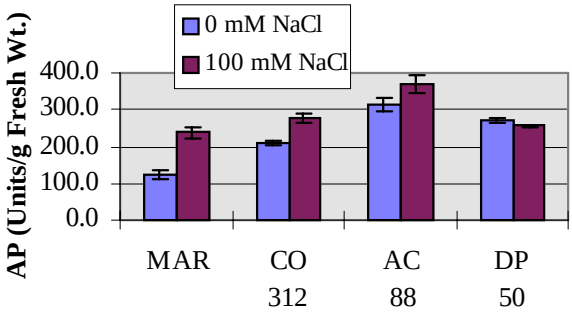

Figure 5. Ascorbate peroxidase activity (units/g fresh weight) of cotton ovules from MAR, Coker (CO-312), Acala (AC-88), and Deltapine (DP-50) cultured under non-stressed (0 mM NaCl) and stressed (100 mM NaCl) conditions.

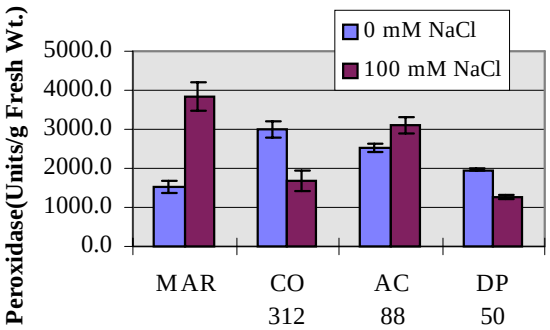

Figure 6. Peroxidase activity (units/g fresh weight) of cotton ovules from MAR, Coker (Co-312), Acala (AC-88), and Deltapine (DP-50) cultured under non-stressed (0 mM NaCl) and stressed (100 mM NaCl) conditions

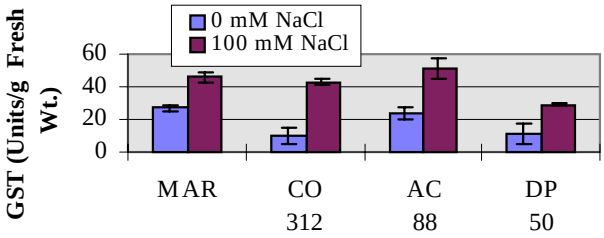

Figure 7. GST activity (units/g fresh weight) of cotton ovules from MAR, Coker (CO-312), Acala (AC-88), and Deltapine (DP-50) cultured under non-stressed (0 mM NaCl) and stressed (100 mM NaCl) conditions.