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PHYSIOLOGY

Antioxidant Response to Salt Stress During Fiber Development in Cotton Ovules

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INTERPRETIVE SUMMARY

This paper reports the results of studies on the effect of salt stress on the activities of antioxidant enzymes during the development of cotton fibers. Previous research on cultured cotton tissues and on whole plants has established a correlation between salt-stress resistance and the activities of the group of chemicals known as antioxidant enzymes that operate by reducing the concentration of harmful, stress-induced, reactive oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radical. Reactive oxygen species are generated in living cells during normal metabolism. The constitutive levels of antioxidant enzymes in plants are usually sufficient to scavenge the free radicals and convert them to harmless metabolites.

During physiological stress, the production of reactive oxygen species is known to increase to such an extent that the normal levels of antioxidant enzymes are not sufficient to prevent the damaging effects of the highly reactive radicals. Cell damage and a reduction in growth rate result. Numerous studies have shown a positive correlation between the activity of antioxidant enzymes and stress caused by temperature extremes, drought, ultraviolet light, mineral deficiencies, herbicide treatment, and excess salt.

The leaves and callus tissue of some cultivars of cotton exhibit an increase in antioxidant enzyme activity in response to an increase in reactive oxygen species concentrations and, thus, demonstrate some stress resistance. This study examined the salt-stress resistance properties and antioxidant enzyme activities in four cotton cultivars at the site of cotton fiber production. Experiments were carried out on three salt-sensitive cultivars, MAR-LBCBHGDPIS-1-91, Coker 312, Deltapine 50, and one putatively salt-tolerant cultivar, Acala 1517-88. Ovules were grown in culture media amended with either no salt (control conditions) or 100 m*M* (approximately 5850 ppm) salt (stress conditions) and were subsequently analyzed for differences in ovule fresh weight and antioxidant enzyme activities following 24 days growth.

Salt treatment reduced ovule fresh weight in the three salt-sensitive cultivars, but not in the salt-tolerant Acala 1517-88. This research also showed that varietal differences in salt-stress resistance are correlated with differences in the activities of antioxidant enzymes. The normal levels of glutathione reductase and ascorbate peroxidase and the inducible activity of superoxide dismutase were higher in Acala 1517-88 than in the other three cultivars. These research results can be used in the future designing of genetically engineered varieties of cotton with enhanced salt-stress resistance properties.

ABSTRACT

Ovules from flowers were removed 2 days post anthesis from greenhouse-grown plants of the salt-tolerant cotton (*Gossypium hirsutum* L.) cultivar 'Acala 1517-88' and the salt-sensitive cotton cultivars: 'Deltapine 50', 'MAR-LBCBHGDPIS-1-91', and 'Coker 312'. The ovules were grown in culture media amended with either 0 m*M* (control conditions) or 100 m*M* (stress conditions) NaCl and analyzed at 24 days post anthesis for differences in ovule fresh weights and antioxidant enzyme activities. Salt treatment reduced ovule fresh weight in all cultivars except Acala 1517-88. Superoxide dismutase activity increased under salt treatment in Acala 1517-88 and MAR-LBCBHGDPIS-1-91, but not in Coker

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312 or Deltapine 50. Catalase activity was constitutively high in Acala 1517-88, and NaCl-induced changes were recorded in MAR-LBCBHGDPIS-1-91 and Coker 312, but not in Deltapine 50. Glutathione reductase activity was constitutively high in Acala 1517-88, increased significantly in MAR-LBCBHGDPIS-1-91 and Coker 312 under NaCl stress, and decreased significantly in Deltapine 50 ovules subjected to a high concentration of NaCl. Under stress conditions, peroxidase activity increased significantly in MAR-LBCBHGDPIS-1-91 and Acala 1517-88 and decreased significantly in Coker 312 and Deltapine 50. High constitutive levels of ascorbate peroxidase activity were observed in Acala 1517-88 and Deltapine 50. The activity of this enzyme increased in MAR-LBCBHGDPIS-1-91 and Coker 312 ovules subjected to NaCl stress. Glutathione-S-transferase activity significantly increased in all the cultivars treated with NaCl. These findings indicate that ovules from the more salt-tolerant cultivar did not exhibit a reduction in growth when subjected to NaCl stress and furthermore suggest this variation may be partially due to the varietal differences in the activities of the antioxidant enzymes.

Oxidative stress occurs when plants are exposed to various forms of environmental stress (Asada and Takahashi, 1987; Asada, 1994; Krause, 1994). Plants can produce 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 ($OH^{\cdot}$). Studies of the effects of environmental stresses (i.e. high NaCl levels (Gossett et al., 1994a, 1994b), 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, Rainwater et al., 1996), mineral deficiencies (Monk and Davies 1989; Cakmak and Marschner, 1992; Polle et al., 1992), and herbicide treatment (Harper and Harvey, 1978; Dodge 1994)) have shown that oxidative stress often disrupts the homeostasis between production of reactive oxygen species and the quenching activity of antioxidant enzymes.

Cotton is classified as a salt-tolerant plant (Ayers and Westcot, 1977). Comparisons among cultivars have shown varietal differences in the levels of salt tolerance (Lauchi et al., 1981; Gossett et al., 1992, 1994a). High salt concentrations have been shown to reduce cotton yield (Fowler, 1986), growth, stomatal conductance, stomatal resistance, and transpiration rates (Gossett et al., 1991). Previous studies carried out with whole plants (Gossett et al., 1992, 1993, 1994a) and callus tissue (Gossett et al., 1994b, 1996) exposed to NaCl have revealed significant increases in the activities of antioxidant enzymes. The NaCl-tolerant cultivars and callus tissue lines acclimated to grow on media amended with 150 m*M* NaCl had higher antioxidant enzyme activities as well as a significantly lower ascorbate/oxidized ascorbate ratio and a significantly higher reduced glutathion/oxidized glutathione ratio (Gossett et al., 1994a, 1994b).

Increases in antioxidant enzyme activities are regulated via transcription in cotton callus (Banks et al., 1998; Manchandia et al., 1999). These results indicate that elevation in the activity of the ascorbate-glutathione cycle probably confers a degree of salt tolerance. The objective of this project was to determine if cotton ovule cultures subjected to NaCl stress during fiber development would produce antioxidant responses similar to those observed in vegetative tissue.

Investigating antioxidant enzyme activity during cotton fiber development under field conditions at the biochemical or molecular level is not practical because of the problems associated with microbial contamination and the unpredictable temperature fluctuations (Haigler 1991, 1992). However, fiber can be grown on ovules cultured *in vitro* from greenhouse-grown plants. *In vitro* culture allows investigators to supply developing fibers with nutrients, including unlimited glucose, the preferred carbon source, and the correct plant growth regulators. An approach *in vitro* also provides the means whereby the influence of various stresses such as cold stress (Gossett et al., 1996) can be isolated and studied.

MATERIALS AND METHODS

Seeds of a putatively salt-tolerant cultivar (cv. Acala 1517-88) and three salt sensitive cultivars (Deltapine 50, MAR-LBCBHGDPIS-1-91 (line from Texas A&M's Multiple Adversity Resistance breeding program), and Coker 312) were planted in 30-cm pots in November 1995 and placed in a temperature-controlled greenhouse. Following

seedling emergence, the plants were watered every other day with full-strength Hoagland's nutrient solution. Flowers were removed 2 days post anthesis. Under a sterile transfer hood, the ovaries were surface sterilized by submersion in 100% ethanol and flaming. The ovaries were opened with an incision along the suture line of each locule. The ovules were removed and placed in 250-mL flasks containing 100 mL of sterile culture medium (Beasley et al., 1984) amended with either 0 or 100 m*M* NaCl. Preliminary studies in which ovules were cultured at 0, 75, 100, and 150 m*M* NaCl had indicated that 100 m*M* was the lowest salt concentration at which a reduction in growth was observed.

Ovules were incubated in the dark at a constant temperature of 30°C for 24 days at which time trichome development was evident in both control and experimental replicates. The ovules from four replicate flasks were blotted dry, weighed, and stored at 70°C for subsequent antioxidant-enzyme analyses. Samples were prepared for superoxide dismutase, catalase, glutathione reductase, peroxidase, glutathione S-transferase, and total protein analysis by homogenizing 1 g of the frozen material in 15 mL of extraction buffer (Anderson et al., 1992), as modified by Gossett et al. (1994a, 1994b).

Catalase activity was determined by monitoring the disappearance of H_2O_2 according to the method of Beers and Sizer (1952). Total superoxide dismutase activity was measured by determining the amount of enzyme required to produce 50% inhibition in the reduction of cytochrome *c* by superoxide generated by xanthine oxidase (Forman and Fridovich, 1973). Glutathione reductase 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 activity was assayed by monitoring the ascorbic acid-dependent reduction of H_2O_2, as described by Anderson et al. (1992). Glutathione S-transferase activity was assayed according to the method of Habig et al. (1974).

For catalase, peroxidase, and ascorbic peroxidase, one unit of enzyme activity was defined as the amount necessary to decompose 1 m*M* of substrate min^{-1} at 25°C. One unit of glutathione reductase and glutathione S-transferase was defined as the amount of enzyme required to reduce 1 nmol of substrate min^{-1} at 25°C. One unit of superoxide dismutase 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 were no significant differences in total protein between treatments or within cultivars, enzyme results are expressed as units gram-fresh $weight^{-1}$ (g FW^{-1}). Data points are based on a mean of four replicate flasks subjected to a one-way analysis of variance. Significance was determined at the 95% confidence limits.

RESULTS AND DISCUSSION

Fresh weights of ovules cultured at 0 m*M* NaCl did not vary significantly among Acala 1517-88, MAR-LBCBHGDPIS-1-91, and Deltapine 50. Coker 312 had a higher ovule fresh weight than did the other three cultivars, when grown on media without NaCl (Fig. 1). When grown on media amended with 100 m*M* NaCl, ovule fresh weight decreased 65% in MAR-LBCBHGDPIS-1-91, 73% in Coker 312, and 57% in Deltapine 50 (Fig. 1). However, no significant fresh weight decrease was recorded for Acala 1517-88, the most salt tolerant of the cultivars tested in this study.

There were no significant differences in superoxide dismutase activity among any of the cultivars grown under control conditions. However, in the 100 m*M* NaCl treatment, superoxide dismutase activity increased 768% in Acala 1517-88 and 390% in MAR-LBCBHGDPIS-1-91 (Fig. 2). No significant changes in superoxide dismutase activity were observed for Coker 312 or Deltapine 50 after NaCl treatment.

The activity of catalase was significantly lower in the MAR-LBCBHGDPIS-1-91 controls than in the controls of the other three cultivars (Fig. 3). No significant increases in catalase activity were observed when Acala 1517-88 or Deltapine 50 ovules were subjected to the salt treatment, but catalase activity increased significantly in MAR-LBCBHGDPIS-1-91 and Coker 312 (236% in MAR-LBCBHGDPIS-1-91 and 25% in Coker 312, respectively). Even though MAR-LBCBHGDPIS-1-

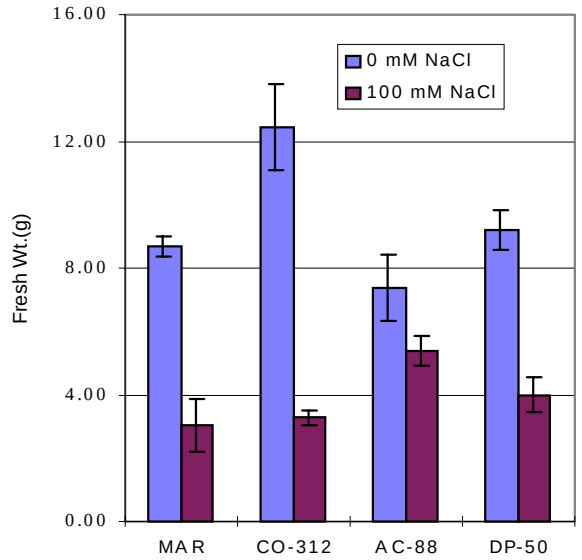

Fig. 1. Fresh weight ± SE of cotton ovules from MAR-LBCBHGDPIS-1-91 (MAR), Coker 312 (CO-312), Acala 1517-88-88 (AC-88), and Deltapine 50 (DP-50) cultured under nonstressed (0 m*M* NaCl) and stressed (100 m*M* NaCl) conditions.

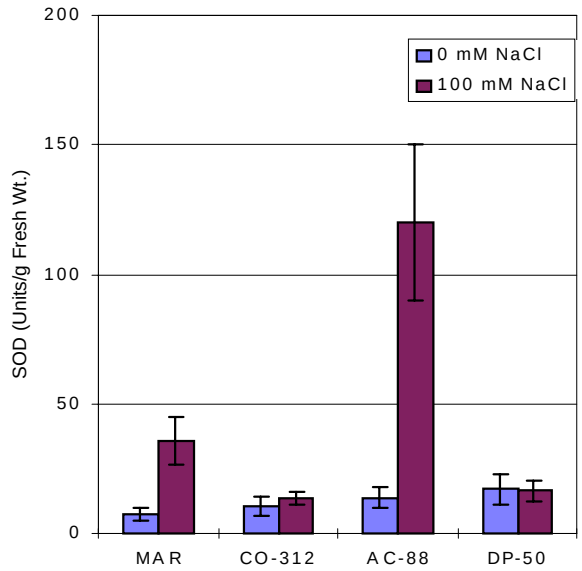

Fig. 2. Superoxide dismutase (SOD) activity (units g FW^{-1} ± SE) of cotton ovules from MAR-LBCBHGDPIS-1-91 (MAR), Coker 312 (CO-312), Acala 1517-88-88 (AC-88), and Deltapine 50 (DP-50) cultured under nonstressed (0 m*M* NaCl) and stressed (100 m*M* NaCl) conditions.

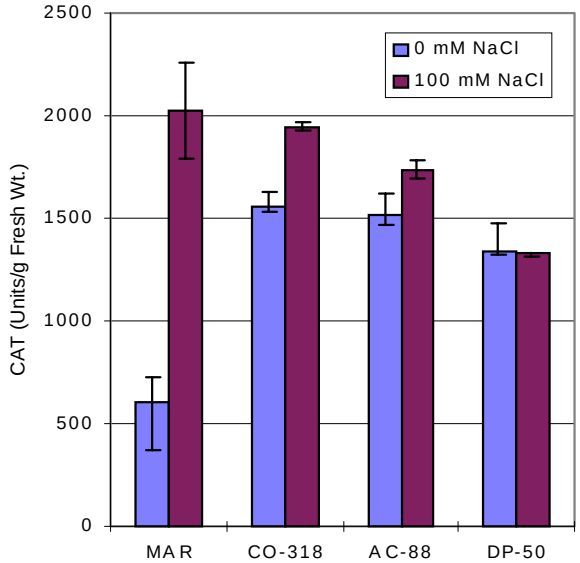

Fig. 3. Catalase (Cat) activity (units g FW $^{-1}$ ± SE) of cotton ovules from MAR-LBCBHGDPIS-1-91 (MAR), Coker 312 (CO-312), Acala 1517-88-88 (AC-88), and Deltapine 50 (DP-50) cultured under nonstressed (0 m*M* NaCl) and stressed (100 m*M* NaCl) conditions.

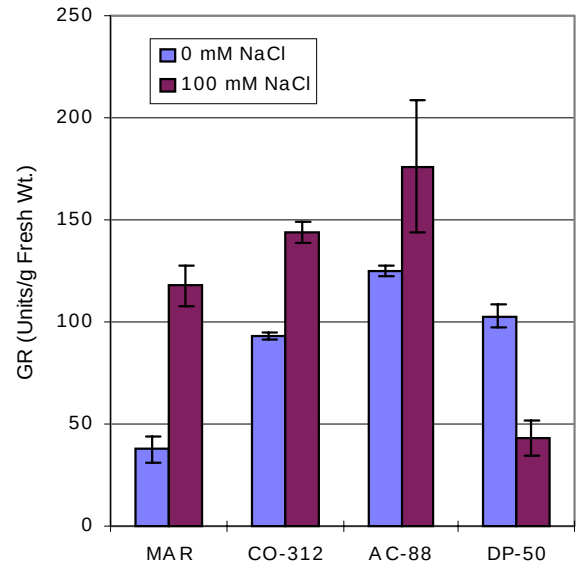

Fig. 4. Glutathione reductase (GR) activity (units g FW^{-1} ± SE) of cotton ovules from MAR-LBCBHGDPIS-1-91 (MAR), Coker 312 (CO-312), Acala 1517-88-88 (AC-88), and Deltapine 50 (DP-50) cultured under nonstressed (0 m*M* NaCl) and stressed (100 m*M* NaCl) conditions .

91 exhibited the greatest salt-induced increase in catalase activity, this increase did not result in catalase activity that was significantly higher than the activity in Acala 1517-88 subjected to NaCl treatment.

Under control conditions, varietal differences in glutathione reductase activity (Fig. 4) were similar to those observed for catalase. MAR-LBCBHGDPIS-

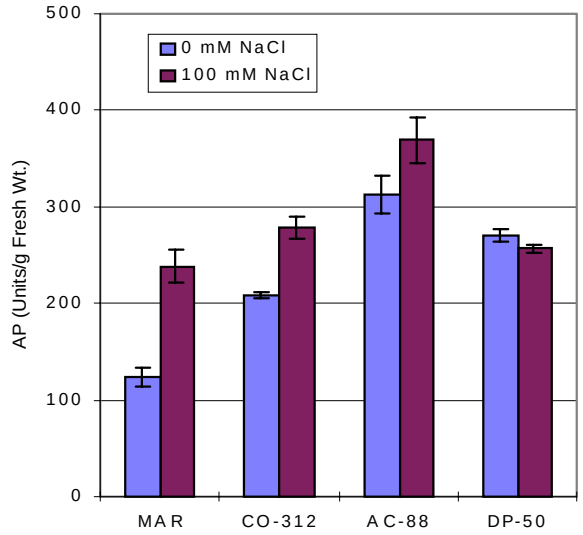

Fig. 5. Ascorbate peroxidase (AP) activity (units g FW $^{-1}$ ± SE) of cotton ovules from MAR-LBCBHGDPIS-1-91 (MAR), Coker 312 (CO-312), Acala 1517-88-88 (AC-88), and Deltapine 50 (DP-50) cultured under nonstressed (0 m*M* NaCl) and stressed (100 m*M* NaCl) conditions.

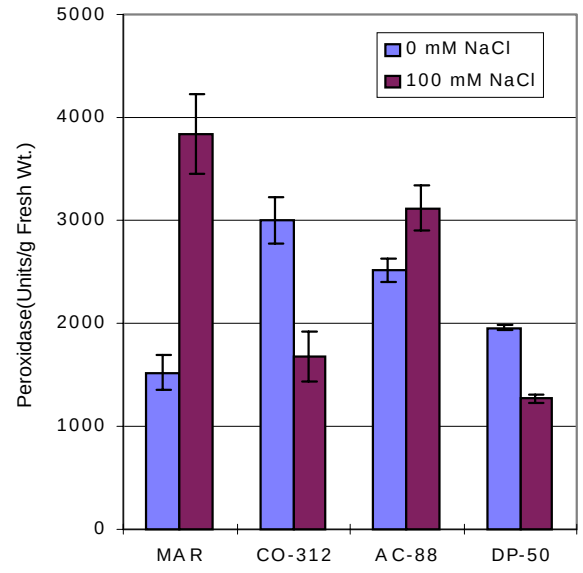

Fig. 6. Peroxidase activity (units g FW $^{-1}$ ± SE) of cotton ovules from MAR-LBCBHGDPIS-1-91 (MAR), Coker 312 (CO-312), Acala 1517-88 88 (AC-88), and Deltapine 50 (DP-50) cultured under nonstressed (0 m*M* NaCl) and stressed (100 m*M* NaCl) conditions.

1-91 and Coker 312 exhibited significant increases in glutathione reductase activity (212 and 54%, respectively) under NaCl-stress conditions, but no increase in glutathione reductase activity was observed in Acala 1517-88, which constitutively

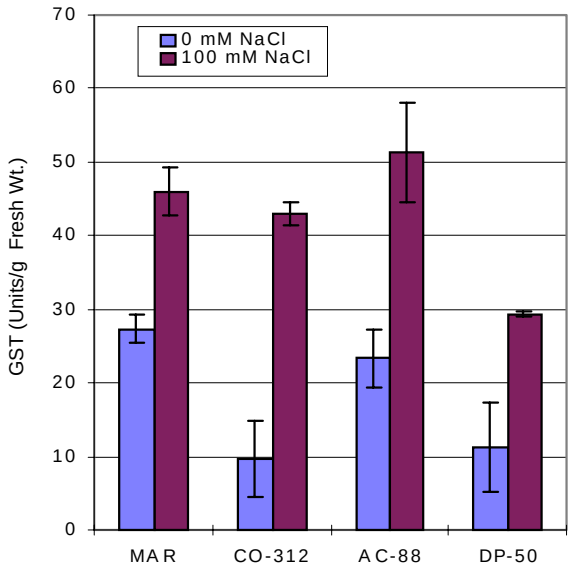

Fig. 7. GST activity (units g FW $^{-1}$ ± SE) of cotton ovules from MAR-LBCBHGDPIS-1-91 (MAR), Coker (CO-312), Acala 1517-88 (AC-88), and Deltapine (DP-50) cultured under nonstressed (0 m*M* NaCl) and stressed (100 m*M* NaCl) conditions.

expressed glutathione reductase at high levels. Glutathione reductase activity in Deltapine 50 underwent a 58% decrease under salt-stress conditions.

The assay for ascorbate peroxidase in control tissue showed MAR-LBCBHGDPIS-1-91 to have significantly lower ascorbate peroxidase activity than the other three cultivars (Fig. 5). After NaCl stress, no significant changes in ascorbate peroxidase activity were observed for Acala 1517-88 and Deltapine 50. However, increases in ascorbate peroxidase activity were recorded in stressed ovules of MAR-LBCBHGDPIS-1-91 (92%) and in Coker 312 (33%).

In response to NaCl stress, changes in the activity of peroxidase were cultivar dependent (Fig 6). Significant decreases in peroxidase activity were observed in Deltapine 50 and Coker 312 (35 and 44%, respectively); while significant increases were recorded for MAR-LBCBHGDPIS-1-91 (152%) and in Acala 1517-88 (24%).

Of the four cultivars, MAR-LBCBHGDPIS-1-91 and Acala 1517-88 had the highest constitutive level of glutathione S-transferase activity (Fig. 7). The salt treatment induced increased glutathione S-transferase activity in all four cultivars tested, (68, 343, 120, and 159% for MAR-LBCBHGDPIS-1-91,

Coker 312, Acala 1517-88, and Deltapine 50, respectively).

The reductions in ovule fresh weight recorded for MAR-LBCBHGDPIS-1-91, Coker 312, and Deltapine 50 are comparable to reductions in leaf area and plant fresh weight observed in whole plant studies of salt-sensitive cotton cultivars grown under NaCl-stress conditions (Gossett et al., 1994a). After the 100 m*M* NaCl treatment, Acala 1517-88 showed no decrease in ovule fresh weight and maintained the highest overall activity profile for antioxidant enzymes. Treatment of Acala 1517-88 ovules with 100 m*M* NaCl resulted in (i) significantly higher catalase, glutathione reductase, and glutathione S-transferase activities than those found in salt-stressed Deltapine 50, (ii) significantly higher peroxidase activity than found in Deltapine 50 or Coker 312, (iii) significantly higher ascorbate peroxidase activity than in MAR-LBCBHGDPIS-1-91 and Deltapine 50, and (iv) significantly higher superoxide dismutase activity than in MAR-LBCBHGDPIS-1-91, Coker 312, and Deltapine 50. There was no instance in which antioxidant enzyme activity was significantly lower in Acala 1517-88 ovules than in ovules of the other cultivars grown under NaCl stress.

Differences in superoxide dismutase activity were of particular interest. While the data indicate that the superoxide-dismutating capacities of the four cultivars were similar when grown at 0 m*M* NaCl, superoxide dismutase activity increased five-fold in Acala 1517-88 under NaCl-stress conditions. This contrasts with the slight increase in superoxide dismutase activity in MAR-LBCBHGDPIS-1-91 and the lack of change in superoxide dismutase activity in Deltapine 50 and Coker 312. These data suggest that tolerance to high concentrations of NaCl in the Acala 1517-88 ovules may be related to the high constitutive levels of ascorbic peroxidase, catalase, glutathione reductase , and peroxidase and to NaCl-inducible increases in the activity of glutathione S-transferase and, in particular, superoxide dismutase.

Increases in superoxide dismutase activity have been reported to play a role in tolerance to cold in potatoes (*Solanum tuberosum* L.) (Spychalla and Desborough, 1990) and maize (*Zea mays* L.), to paraquat in ryegrass (*Lolium perenne* L.) (Harper and Harvey, 1978), to salt in chick pea (*Cicer arietinum* L.) (Hernandez et al., 1994) and resistance to paraquat in tobacco (*Nicotiana tabacum* L.) (Shaaltiel et al., 1988) and to chilling in spinach (*Spinacea oleracea* L.) (Schoner and Krause 1990). It is interesting that results from the present study using ovule culture differ somewhat from the results obtained in whole-plant studies with Acala 1517-88 and Deltapine 50 (Gossett et al., 1994a).

In leaf tissue from Acala 1517-88 and Deltapine 50, the constitutive levels of glutathione reductase and peroxidase in the control plants were similar, and the two cultivars had similar superoxide-dismutating capacities when grown under high NaCl conditions. Sodium chloride treatment resulted in stress-induced increases in glutathione reductase and peroxidase activities in Acala 1517-88, but not in Deltapine 50.

In the current study, ovules from Acala 1517-88 exhibited constitutively higher levels of glutathione reductase and peroxidase activity than did Deltapine 50 ovules. When ovules of Deltapine 50 and Acala 1517-88 were subjected to NaCl treatment, the activities of these two enzymes decreased significantly in Deltapine 50 but did not change appreciably in Acala 1517-88. In contrast, NaCl stress resulted in a five-fold increase in superoxide dismutase activity in Acala 1517-88 and elicited no change in superoxide dismutase activity in Deltapine 50 ovules. These data suggest that both leaf tissue and ovules may use the antioxidant system when tolerating NaCl stress, but the specific processes by which ovules acclimate to high salinity levels differ from the specific processes used in leaf tissue. In leaf tissue, glutathione reductase and peroxidase appear to be limiting factors; but in ovules, the antioxidant response appears to be limited by superoxide dismutase activity. Of course, it is also possible that the response of tissues cultured *in vitro* to salt stress differs from the response *in planta*.

CONCLUSIONS

The results of this study of ovules cultured *in vitro* show that varietal differences in salt tolerance at the site of fiber production are correlated with differences in antioxidant-enzyme activities. Notably Acala 1517-88, a cotton cultivar that has been shown to exhibit salt tolerance, showed no decrease in ovule fresh weight when cultured in the presence of 100 m*M* salt during the 24 day experimental

period. When exposed to salt, the other three cultivars, MAR-LBCBHGDPIS-1-91, Coker 312 and Deltapine 50, all exhibited significant decreases in ovule fresh weights. Enzyme assays indicate that tolerance in Acala 1517-88 may be related to higher constitutive levels of glutathione reductase and ascorbate peroxidase and a greater capacity to up-regulate superoxide dismutase activity than found in the other cultivars tested. These findings are consistent with results obtained from salt-stressed cotton callus and whole cotton plants (Gossett et al., 1991, 1992, 1993, 1994a, 1994b, 1994c, 1996).

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