SUPEROXIDE LEVELS IN NaCl STRESSED COTTON CALLUS

Stephen W. Banks, John A.H. Braud, M. Cran Lucas and Dalton R. Gossett
Louisiana State University in Shreveport
Shreveport, LA

Abstract

Superoxide detection in time course studies showed increases in superoxide radicals in response to exposure to NaCl and paraquat. Both control callus and 150 mM NaCl acclimated callus were treated with paraquat and NaCl. Both the paraquat and NaCl treatments resulted in significant increases in superoxide levels, but the paraquat induced increases were significantly higher than the NaCl-induced increases. In all cases the NaCl acclimated callus tissue exhibited significantly lower levels of superoxide production than the control callus. Increases in superoxide levels directly correspond with earlier observations of increases in antioxidant enzyme levels in response to NaCl and paraquat stress.

Introduction

During normal aerobic metabolism activated oxygen species such as $O_2^{\cdot-}$ (superoxide), H_2O_2 (hydrogen peroxide) and $OH^{\cdot}$ (the hydroxyl radical) are produced through a series of univalent reductions of ground state oxygen. Electron leakage from electron transport chains in the mitochondria and photosystems I and II in the chloroplast (Asada 1994) can react with O_2 to produce the superoxide ($O_2^{\cdot-}$) radical. When plants are subjected to physiological stress such as drought, temperature extremes, herbicide treatment or mineral deficiency, the production of reactive oxygen species may exceed the capacity of the scavenging reactions of the antioxidant system. This leads to cellular oxidative damage to lipids (Fridovich, 1986; Liebler *et al*, 1986; Price and Hendry 1987; Wise and Naylor, 1987), proteins (Halliwell and Gutteridge 1985; Kim *et al.*, 1985), and nucleic acids (Fridovich, 1986; Imlay and Linn 1988). Plants with higher constitutive, or inducible antioxidant enzyme levels have been reported to have greater resistance to oxidative damage (Dhindsa and Matowe, 1981; Harper and Harvey, 1987; Wise and Naylor 1987; Monk and Davis 1989; Spychalla and Desborough 1990).

Previous studies carried out with salt-stressed whole plants (Gossett *et al.*, 1992), callus tissue (Gossett *et al.*,1994a,b), and ovule cultures (Banks *et al.*, 1997, Rajguru *et al.*, 1999) has revealed a significant increase in the activity of antioxidant enzymes in the more salt-tolerant tissues compared to controls. In addition, it is now known that the increases in antioxidant enzyme activities are transcriptionaly regulated (Banks *et al.*, 1998; Manchandia *et al.*, 1999). 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.*,1994a,b). These results indicate that elevation in the activity of the ascorbate-glutathione cycle probably confers a degree of salt tolerance.

It has been inferred from the NaCl effects on the levels of antioxidant enzymes that NaCl stress must be contributing to oxidative stress in whole plants or callus tissue (Gossett, *et al*., 1994a,b; Gossett, *et al*., 1996; Hernandez, *et al*., 1993; Hernandez, *et al*., 1994). To test the hypothesis that NaCl produces oxidative stress in cotton, we have used a reagent, luminol, to directly measure the relative concentrations of superoxide radicals in control and NaCl stressed cotton callus.

Materials and Methods

Growth and Harvest of Callus Tissue

Callus tissue was generated from the Coker 312 cotton cultivar according to the methods of Trolinder and Goodin (1987). The callus was then either maintained as salt-sensitive controls or selected to grow on progressively higher levels of salt according to the method outlined by Gossett *et al*. (1996). This procedure yielded a NaCl-tolerant cell line that grew as well at 150 mM NaCl as the control cell line grew at 0 mM NaCl.

Superoxide Detection

Approximately 2.5 g of either control or NaCl-tolerant callus tissue was weighed and placed in 10 ml of the previously defined growth media. This callus solution was then placed in a 50 ml tube and aerated for 1 hr period prior to treatment with either paraquat or NaCl. When paraquat was used, both cell lines were treated with 0.2 µM paraquat. The control (0 mM NaCl) callus was treated with 150 mM NaCl, while the NaCl-tolerant (150 mM) callus was treated with 250 mM NaCl. At time points of 0, 15, 30, 45, 60, 90, and 120 minutes after treatment, 100 µl of solution was pipetted out and transferred to a pre-weighed luminometer tube. All steps prior to centrifugation were kept at 4°C. This tube was centrifuged for 2 min. at 1,500 g, and the supernatant was discarded. The tube was reweighed to the nearest 0.0001 g to measure the amount of callus tissue transferred. The assay protocol followed the instructions in Stratagene's Lumimax Superoxide Anion Detection kit. Five µl of 4.0 mM luminol, 5.0 µl of 5.0 mM enhancer, and 190 µl of SOA assay medium were added to each tube. The tubes containing the callus and detection solution were slightly shaken and exactly 30 seconds later, the reading from the luminometer was recorded. Each control and experimental data point was replicated 4 times.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:613-615 (2000)
National Cotton Council, Memphis TN

Results and Discussion

Previous studies have indicated that the superoxide anion is produced in plants under salt stress (Hernandez *et al.*, 1993; Hernandez *et al.*, 1994). Superoxide anions, until now, have not been directly measured in NaCl stressed cotton callus. To test the hypothesis that NaCl treatment produces oxidative stress, the relative levels of the superoxide anion in control callus and salt-adapted callus was measured after NaCl and paraquat treatment. Paraquat is known to produce superoxide *in vivo* (Harper and Harvey, 1978).

Under control conditions the NaCl-sensitive (0 mM NaCl) callus was observed to have much higher superoxide levels than NaCl-tolerant (150 mM NaCl) callus with as much as a 20-fold difference observed between the two (Fig. 1). Both the NaCl-sensitive and NaCl-tolerant callus tissue stressed with paraquat showed significant increases in superoxide concentration at 30 min. and 45 min., but the superoxide levels dropped faster in the 150 mM NaCl adapted callus (Fig. 2). The 0 mM NaCl control callus continued to produce higher levels of $O_2^{\cdot-}$ for up to 60 minutes. When the salt-sensitive and salt-tolerant callus lines were stressed with 150 mM and 250 mM NaCl respectively, there was a significant increase in superoxide levels in the 0 mM NaCl callus at 30-45 minutes (Fig. 3). However, during the same time frame the 150 mM NaCl adapted callus tissue showed no significant change in superoxide concentration (Fig. 3).

There was a dramatic difference in the control levels of superoxide in the NaCl-sensitive and NaCl-tolerant callus (Fig. 1). Both NaCl and paraquat induced increases in the levels of the superoxide anion in cotton callus tissue (Fig. 2 and Fig. 3). The differences between the NaCl-sensitive and NaCl-tolerant callus were even more dramatic under stress conditions. The lower levels of superoxide in NaCl-adapted cotton was most likely due to the enhanced oxidative stress response machinery in this cell line. There were also differences in the speed at which the superoxide production returned to near control levels when the NaCl-tolerant cell line was stressed with paraquat versus the rate at which superoxide levels declined in the NaCl-sensitive cell line. The most striking difference between the two cell lines occurred under NaCl stress (Fig. 3). The NaCl-induced superoxide generation in the NaCl-adapted cell line was minuscule in comparison with NaCl-induced superoxide in the NaCl-sensitive cell line (Fig. 3). This again suggests that the NaCl-adapted cotton callus had acquired the ability to prevent NaCl-induced oxidative stress. This conclusion is supported by numerous studies involving salt stress, H_2O_2 stress, osmotic stress, and studies involving differential regulation of SOD (superoxide scavenger) in sensitive cultivars versus stress tolerant cultivars (Gossett *et al.*, 1994b; Gossett *et al.*, 1996; Guan and Scandalios, 1998; Manchandia *et al.*, 1999; Matters and Scandalios, 1986; Tsang *et al.*, 1991).

Acknowledgements

We gratefully acknowledge the support of Cotton Incorporated and the Louisiana Board of Regents Support Fund (BORSF) for support of this research.

References

Asada, K. (1994) Production of active oxygen species in photosynthetic tissue. In, C.H. Foyer and P.M. Mullineaux, eds. Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Boca Raton FL pp77-104.

Banks, S.W., Rajguru, S.N., Gossett, D.R., Lucas, M.C., (1997) Antioxidant Response to Salt Stress During Fiber Development *Proceedings of the Beltwide Cotton Conference.* National Cotton Council. Memphis, TN. Pages 1422-1424.

Banks, S.W., Gossett, D.R., Manchandia, A., Bellaire, B., Lucas, M.C., Millhollon, E.P. (1998) The Influence of α-Amanitin on the Induction of Antioxidant Enzymes during Salt Stress. Proceedings of the Beltwide Cotton Conference. National Cotton Council Memphis, TN.Pages 1393-1395

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

Fridovich, I., (1986) Biological effects of the superoxide radical. *Arch Biochem.Biophys* **247:** 1-11.

Gossett, D.R., Lucas, M.C., Caldwell, W.D., Millhollon., E.P. and Barclay, A., (1992). Antioxidant status in salt stressed cotton. *Proceedings of the Beltwide Cotton Research Conference.* National Cotton Council Memphis, TN.1036-1039.

Gossett, D.R., Millhollon, E.P., Lucas, M.C., Banks, S.W., and Marney, M-M. (1994a). Antioxidant response to NaCl Stress salt-tolerant and salt sensitive cultivars of cotton (*Gossypium hirsutum* L.) *Crop Science* **9:** 339-341.

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

Gossett, D.R., Banks, S.W., Millhollon, E.P., Lucas, M.C., (1996) Antioxidant response to NaCl stress in a control and an NaCl-tolerant cotton cell line grown in the presence of paraquat, butathione sulfoximine and exogenous glutathione. *Plant Physiology* 112: 803-809.

Guan, L., and Scandalios, J.G., (1998) Two structurally similar maize cytosolic dismutase genes sod4 and sod4a respond differently to abscissic acid and hogh osmoticum. *Plant Physiology* **117:**217-224

Harper, D.B., and Harvey, BMR. (1978) Mechanisms of paraquat tolerance in perenial ryegrass II role of Superoxide dismutase, catalase and peroxidase. *Plant Cell Environ.* **1:** 211-215.

Halliwell, B., and Gutteridge, J.M.C. (1985) Free radicals in biology and medicine. Clarendon Press, Oxford p.29.

Hernandez, J. A., Corpas, F. J., Gomez, M., Del Rio, L. A., and Sevilla, F. (1993) Salt-induced oxicative stress mediated by activated oxygen species in pea leaf mitochondria. *Physiologia Plantarum* **89**: 103-110.

Hernandez, J. A., Del Rio, L. A., and Sevilla, F. (1994) Salt stress-induced changes in superoxide dismutase isozymes in leaves and mesophyll protoplasts from *Vigna unguiculata* (L.) Walp. *New Phytol*. **126**: 37-44.

Imlay, J.A., and Linn, S. (1988) DNA damage and oxygen radical toxicity *Science* **240:** 1302-1309.

Kim, K., Rhee, S.G., Stadtman, E.R. (1985) Non-enzymatic cleavage of proteins by reactive oxygen species generated by dithioretinol and iron. *J. Biol. Chem*. **260:**15394-15397.

Liebler, D.C., King, D.S., and Kling D.J. (1986) Antioxidant protection of phospholipid bilayers by α-tocopherol. Control of α-tocopherol status and lipid peroxidation by ascorbic acid and glutathione *J.Biol. Chem.* **261:** 12114-12119.

Manchandia, A.M., Banks, S.W., Gossett, D.R., Bellaire, B.A., Lucas, M.C., Millhollon, E.P., (1999) "The Influence of α-Amanitin on the Induction of Antioxidant Enzymes during Salt Stress" *Free Radical Research* **30:**429-438.

Matters, G.L., and Scandalios, J.G., (1986) Effect of the free-radical generating herbicide paraquat on the expression of super oxide dismutase (Sod) genes in maize *Biochim Biophys Acta* **882:**29-38

Monk, L.S., and Davies, H.V. (1989) Antioxidant status of the potato tuber an Ca^{2+} deficiency as a physiological stress *Physiologia Plant.* **75:** 411-416.

Price, A., and Hendry, G. (1987) The significance of the tocopherols in stress survival in plants. Free Radicals, Oxidant Stress and Drug Action. C.Rice-Evans, (ed). Richelieu Press London 433-450.

Rajguru, S.N., Banks, S.W., Gossett, D.R., Lucas, M.C., Millhollon, E.P., (1999) Antioxidant Response to Salt Stress During Fiber Development in Cotton Ovules. *The Journal of Cotton Science* **3:**11-21.
Online at: http://www.jcotsci.org/1999/issue01/phys/art01/article.pdf

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

Trolinder, N.L.,and J.R. Goodin. 1987. Somatic embryogenesis and plant regeneration in *Gossypium hirsutum* L. *Plant Cell Rep* **6:**231-234.

Tsang, E.W., Bowler, C., Herouart, D., Van Camp, W., Villaroel, R., Genetello, C., Van Montagu, M., Inze, D. (1991) Differential regulation of superoxide dismutases in plants exposed to environmental stress. *Plant Cell* **3:** 783-792.

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

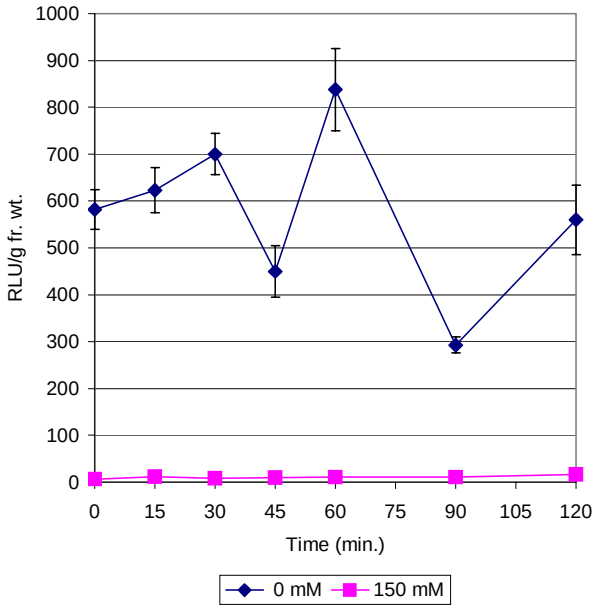

Figure 1. Superoxide generated relative light units (RLU)/g fresh weight over a 2 hour period in NaCl-sensitive (0 mM) and NaCl-tolerant (150 mM) callus tissue.

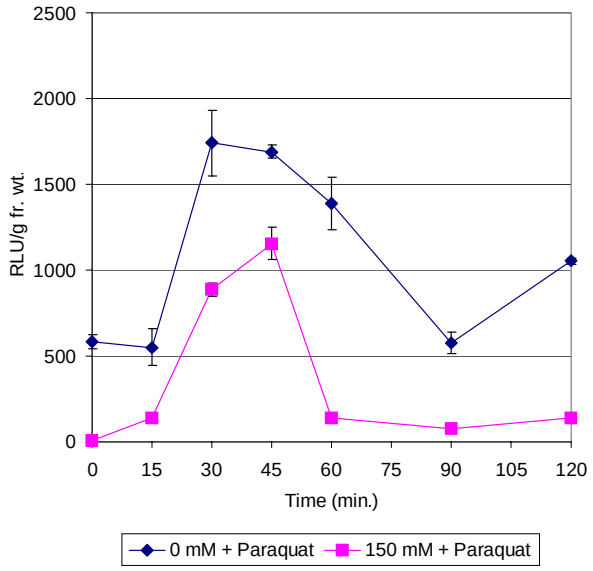

Figure 2. Superoxide generated relative light units (RLU)/g fresh weight over a 2 hour period in NaCl-sensitive (0 mM) and NaCl-tolerant (150 mM) callus tissue treated with 0.2 μM paraquat.

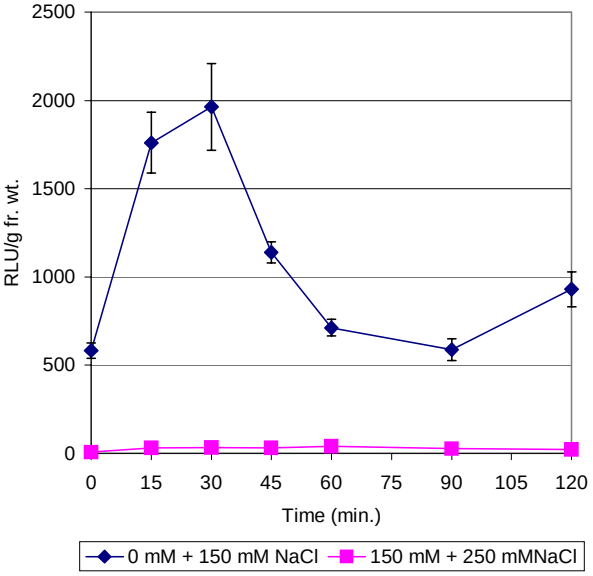

Figure 3. Superoxide generated relative light units (RLU)/g fresh weight over a 2 hour period in NaCl-sensitive (0 mM) callus tissue treated with 150 mM NaCl and NaCl-tolerant (150 mM) callus tissue treated with 250 mM NaCl.