The effects of NaCl on antioxidant enzyme activities in callus tissue of salt-tolerant and salt-sensitive cotton cultivars (Gossypium hirsutum L.)

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Summary. To determine NaCl effects on callus growth and antioxidant activity, callus of a salt-tolerant and a salt-sensitive cultivar of cotton was grown on media amended with 0, 75, and 150 mM NaCl. Callus of the salt-tolerant cultivar, Acala 1517-8, grown at 150 mM NaCl, showed significant increases in superoxide dismutase, catalase, ascorbate peroxidase, peroxidase and glutathione reductase activities compared to callus tissue grown at 0 mM NaCl. In contrast, callus tissue of the salt-sensitive cultivar, Deltapine 50, grown at 0, 75, and 150 mM NaCl, showed no difference in the activities of these enzymes. At the 150 mM NaCl treatment, peroxidase was the only antioxidant enzyme from Deltapine 50 with an activity as high as that observed in Acala 1517-88. The NaCl-induced increase in the activity of these enzymes in Acala 1517-88 indicates that callus tissue from the more salt-tolerant cultivar has a higher capacity for scavenging and dismutating superoxide, an increased ability to decompose $\text{H}_2\text{O}_2$, and a more active ascorbate-glutathione cycle when grown on media amended with NaCl.

Key words: Antioxidant enzymes - Environmental stress - Gossypium hirsutum - Oxidative stress - Salt tolerance

Introduction

Activated oxygen species such as superoxide ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), and the hydroxyl radical ('$\text{OH}$'), can be produced during normal aerobic metabolism. Since these activated oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids (Fridovitch 1986; Wise and Naylor 1987), protein (Halliwell and Guteridge 1985; Davies 1987) and nucleic acids (Fridovitch 1986; Imlay and Linn 1988), plants possess a number of antioxidant enzymes that protect them from these potential cytotoxic effects. Superoxide dismutase (SOD) is a major scavenger of $\text{O}_2^-$, and its enzymatic action results in the formation of $\text{H}_2\text{O}_2$. Catalase, ascorbate peroxidase (AP) (Chen and Asada 1989) and a variety of general peroxidases (Chang et al. 1984) catalyze the breakdown of $\text{H}_2\text{O}_2$. In an ascorbate-glutathione cycle, the enzymatic action of AP produces monodehydroascorbate radicals which can dismutate spontaneously or be enzymatically reduced to dehydroascorbate by NADPH-dependent monodehydroascorbate radical reductase (Hossain et al. 1984). Dehydroascorbate is reduced back to ascorbate non-enzymatically by reduced glutathione or enzymatically in a reaction mediated by dehydroascorbate reductase (Polle et al. 1992). The resulting oxidized glutathione is then converted back to the reduced form by NADPH-dependent glutathione reductase (GR) (Foyer et al. 1991).

When plants are subjected to environmental stress such as temperature extremes, drought, herbicide treatment and mineral deficiency, the balance between the production of reactive oxygen species and the quenching activity of the antioxidants is upset, often resulting in oxidative damage (Dhindsa and Matowe 1981; Harper and Harvey 1978; Wise and Naylor 1987; Monk and Davies 1989; Spychalla and Desborough 1990; Cakmak and Marschner 1992; Polle et al. 1992). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative...

While a large volume of papers and several review articles have been published on the subject of plant responses to elevated saline levels, salt tolerance is a complex phenomenon, and the specific mechanism which imparts tolerance to non-halophytic plants has eluded definition. Although cotton (*Gossypium hirsutum* L.) is classified as a salt-tolerant plant, variation in salt tolerance has been observed among different cultivars (Lauchli *et al.* 1981; Gossett *et al.* 1991, 1992). In previous studies, Gossett *et al.* (1993) examined the relationship between antioxidant enzymes and salt tolerance in eight-week-old cotton leaves of two salt-tolerant cultivars (cv Acala 1517-88 and cv Acala 1517-SR2), and two salt-sensitive cultivars (cv Deltapine 50 and Stoneville 825). The more salt-tolerant Acala cultivars contained significantly higher constitutive levels of catalase and induced levels of peroxidase and GR. Studies with other non-halophytes (Smith and McComb 1981a, 1981b) have shown that the degree of salt tolerance observed in the whole plant is also exhibited in callus tissue. These results suggest a salt tolerance mechanism operating at the cellular level.

This study was designed to determine (a) if there are differences in salt tolerance at the cellular level in cotton, (b) if so, are these differences correlated with antioxidant enzyme activity, and (c) to what degree, if any, does salt tolerance and antioxidant response in callus tissue compare with that previously established in the whole plant.

Materials and Methods

**Plant material.** Seeds of a relatively salt-tolerant cultivar of cotton (cv Acala 1517-88) and a relatively salt-sensitive one (cv Deltapine 50) were obtained from the Louisiana State University Agriculture Center Red River Research Station in Bossier City, Louisiana. Seeds of both cultivars were surface-sterilized and germinated on Stewart’s media (Stewart and Hsu 1977) according to the method outlined by Trolinder and Goodin (1987). After 10 days, the sterile hypocotyls were sectioned into 5 mm segments and placed on callus generation media consisting of MS salts (Murashige and Skoog 1962) supplemented with Gamborg’s vitamins (Gamborg 1978), 0.75 g/l MgCl₂, 0.1 mg/l 2,4-D, 0.1 mg/l kinetin, 30g/l glucose, 2g/l phytoel and adjusted to pH 5.8 (Trolinder and Goodin 1987). The resulting callus tissue was sub-cultured every 4 to 6 weeks. At the beginning of each experiment, approximately 500 mg of callus tissue was transferred to fresh media amended with either 0, 75, or 150 mM NaCl. The callus was then grown at 30°C in a lighted incubator with a photoperiod of 15 h (70µE/M²/s supplied by fluorescent lights) and a 9 h dark period. After 42 days of salt treatment, the cultures were harvested, weighed, and stored at -70°C for subsequent antioxidant enzyme analyses.

**Protein extraction.** Samples were prepared for SOD, catalase, peroxidase and GR analyses by a modification of the method outlined by Foster and Hess (1980). One g of frozen callus tissue was homogenized in 1 ml of an ice-cold solution containing 100 mM Tris (pH 7.0), 10 mM D-isoascorbic acid, 2% PVP-10, 0.1 mM EDTA, 0.2% Trition X-100 and 1 drop of antifoam A emulsion. The homogenate was then centrifuged for 2 min at 15,000g in a microcentrifuge. One ml of the supernatant was centrifuge-desalted through a 10 ml bed of Sephadex G50-300 (Helmerhorst and Stokes 1980). A portion of the eluent was analyzed immediately for catalase activity, and the remainder was stored at -70°C for subsequent analysis of SOD, GR and peroxidase activities. AP was extracted by homogenizing 1 g of the frozen callus tissue in 1 ml of 50 mM Pipes buffer (pH 6.8), 6 mM L-cysteine hydrochloride, 10 mM D-isoascorbate, 1 mM EDTA, 0.3% Triton X-100, 1% (w/v) PVP-10 and 1 drop of antifoam A emulsion (Anderson *et al.* 1992). The homogenate was centrifuged for 2 min at 15,000g in a microcentrifuge, and 1 ml of the supernatant was centrifuge-desalted following the procedure outlined by Anderson *et al.* (1992).

**Enzyme Determinations.** Catalase activity was determined by monitoring the disappearance of H₂O₂ by measuring the decrease in absorbance at 240 nm of a reaction mixture containing 1.9 ml H₂O, 1 ml 0.059 M H₂O₂ in KPO₄ buffer (pH 7.0), and 0.1 ml extract (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 in a reaction mixture consisting of 900 µl SOD cocktail which contained 0.1 mM EDTA, 0.01 mM ferricytochrome C, and 0.05 mM xanthine in 50 mM KPO₄ buffer (pH 7.8); 50 µl catalase at 0.05 units/ml; 20 to 50 µl extract; and 50 µl xanthine oxidase at 0.05 units/ml (Forman and Fridovich 1973). GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm in a reaction mixture containing 950 µl of 0.15 mM NADPH, 0.5 mM GSSG, and 3 mM MgCl₂ in 50 mM Tris (pH 7.5) and 50 µl extract (Schaeidle and Bassham 1977). Peroxidase activity was measured by monitoring at 675 nm the H₂O₂-dependent oxidation of reduced 2,3,6 trichloroindophenol in a reaction mixture containing 950 µl of 120 mM H₂O₂, 17 mM Na₂S₂O₃, and 0.3 mM 2,3,6 trichloroindophenol in 40 mM NaPO₄ buffer (pH 6.0), and 0.5 µl of extract (Nickel and Cunningham 1969). AP activity was assayed by monitoring the ascorbic acid-dependent reduction of NADPH at 340 nm in a reaction mixture consisting of 950 µl of 1.9 ml H₂O, 1 ml 0.059 M H₂O₂, and sample (Anderson et al. 1992). For catalase, peroxidase and AP, one unit of enzyme was defined as the amount necessary to decompose 1 nmole of substrate/min at 25°C. One unit of GR was defined as the amount on enzyme required to reduce 1 nmole of substrate/min at 25°C. One unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of cytochrome C by 50%.

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