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Evaluation of NaCl tolerance in the callus cultures of *Suaeda nudiflora* Moq.

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Abstract

Salt tolerance was studied in the callus cultures of *Suaeda nudiflora* Moq. a dicotyledonous succulent halophyte. Growth was significantly inhibited at 50, 100, 150 and 200 mM NaCl. Inorganic ions and proline accumulated in response to salinity. Ion accumulation pattern reflected the utilization of Na^+ as an osmoticum. Na^+/K^+ ratio rose steadily as a function of external NaCl concentration. Salt stress enhanced the activity of peroxidase, whereas it decreased activities of superoxide dismutase and catalase.

Additional key words: catalase, *Chenopodiaceae*, halophyte, peroxidase, proline, salinity, superoxide dismutase.

Introduction

Salinity is a major problem in irrigated agriculture especially in the arid and semiarid environments. While glycophytes undergo growth inhibition in saline environments either due to a decline in water uptake caused by the increase in external osmotic pressure or due to ionic imbalance in the cytosol, halophytes evolved mechanisms for controlled influx of Na^+ and/or Cl^- to be used for their benefit in a saline environment. Although many data are available from whole plant studies the mechanisms of tolerance to high NaCl concentration have not been well clarified (Yeo and Flowers 1986, Reddy *et al.* 1993, Cherian and Reddy 2000, Taha *et al.* 2000, Ouerghi *et al.* 2000, Lee *et al.* 2001).

Cell cultures have served as a very useful tool in trying to elucidate mechanisms of salt tolerance operating at the cellular level. Plant cell and tissue culture are also relevant to crop improvement strategies because they offer a means of rapid selection on a mass scale and useful for the development of breeding techniques for salinity resistant crops. Accumulating evidence suggest that oxidative stress, which include the superoxide ($O_2^{\cdot-}$) and hydroxyl ($OH^{\cdot}$) free radicals, as well as hydrogen peroxide (H_2O_2), is a major damaging factor in plants exposed to different environmental stresses such as drought and salinity. However, little is known of the effect of salt stress on activated oxygen metabolism in cultured plant cells.

Suaeda nudiflora, a member of the family *Chenopodiaceae*, is a succulent halophyte that grows well on the saline marshes of the Gulf of Cambay, Gujarat, India. In recent years several species of *Suaeda* have been selected and developed as an alternative for traditional oil seed crop for regions under saline irrigation (Pasternak *et al.* 1985). We have already studied the possible mechanisms of salt tolerance at the whole plant level (Cherian and Reddy 2000). Therefore, the present study

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; NBT - nitroblue tetrazolium salt; SOD - superoxide dismutase; MS medium - Murashige and Skoog (1962) medium; EDTA - ethylenediaminetetraacetic acid.

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was designed to analyse if there are differences in salt tolerance at cellular level in callus cultures of *S. nudiflora* and possible correlation of these differences with activity of antioxidative enzymes and accumulation of inorganic ions or proline.

Materials and methods

Suaeda nudiflora callus cultures were initiated from leaf explants. The basal medium for the calli initiation consisted of Murashige and Skoog (1962) salts with modified trace elements, vitamins, and 2,4-D and kinetin (1 mg dm^{-3} each), sucrose (30 g dm^{-3}), agar (6 g dm^{-3}) and casein hydrolysate (100 mg dm^{-3}), pH 5.8. Friable calli were sub-cultured every four weeks after initiation and grown for a further eight weeks before being used for salinity tolerance studies. At the beginning of each experiment, callus (500 ± 25 mg) was transferred to fresh medium amended with different concentrations (0, 50, 100, 150 and 200 mM) of NaCl. The calli were grown at 25 ± 2 °C in the dark and were harvested after 21 d of growth.

Growth was determined by taking both fresh and dry mass measurements. Callus was weighed after washing with de-ionized water under sterile or non-sterile conditions at the end of the culture period. Dry mass was determined after drying the callus at 60 °C for 48 h to a constant mass. Ions were extracted from dry callus by wet digestion in 0.1 M nitric acid and 10 % perchloric acid (3:1; v/v). Na^+, K^+ and Ca^{2+} contents were measured by flame photometry. Soluble protein was extracted in 50 mM phosphate buffer (pH 7.5) and estimated by the Folin phenol reagent (Lowry *et al.* 1951). Proline content was determined by the method of Bates *et al.* (1973).

Aggregates of callus cells were separated by treatment with 15 % chromic acid at 65 °C for 30 min (Iraki *et al.* 1989a). The cells were viewed with a phase contrast microscope (*Nikon*, Kangawa, Japan). Mean width of at least twenty samples were taken.

For extraction and assay of enzymes 500 mg each fresh callus tissue was homogenized in 5 cm^3 of 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 1 % insoluble polyvinylpyrrolidone and 0.5 % (v/v) Triton X-100. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 15 000 *g* for 15 min and the supernatant was assayed for enzyme activity. All steps in the preparation of enzyme extract were carried out at 0 - 4 °C. An aliquot of the extract was used to determine protein concentration.

Peroxidase activity was measured according to the method of Shannon *et al.* (1966). The assay mixture consisted of 2.8 cm^3 of *O*-dianisidine buffer, 0.1 cm^3 H_2O_2 (1 % m/v) and 0.1 cm^3 enzyme extract. The absorbance was recorded by a spectrophotometer (*Shimandzu UV-160A*, Kyoto, Japan) at 460 nm at every 30 s upto total 90 s. The amount of enzyme required changing the absorption by 0.01 min^{-1} mg^{-1}(protein) was taken as unit enzyme activity.

Catalase activity was assayed from the rate of H_2O_2 decomposition (extinction coefficient 39.4 mM cm^{-1}) as measured by the decrease of absorbance at 240 nm, following the procedure of Aebi (1974). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and the appropriate volume of enzyme extract. The reaction was initiated by adding 10 mM H_2O_2. One unit of catalase was defined as the amount of enzyme which liberates half the peroxide oxygen from 10 mM H_2O_2 solution in 100 s at 25 °C.

The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The 3 cm^3 reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μm NBT, 0.1 mM EDTA and 0.1 cm^3 enzyme extract. Riboflavin (2 μm) was added and the tubes were shaken and placed 30 cm below a light bank consisting of two 15 W fluorescent lamps. The absorbance of the reaction mixture was read at 560 nm. A non-irradiated reaction mixture that did not develop color served as control. Log A_{560} was plotted as function of the volume of enzyme extract used in the reaction mixture (Giannopolitis and Ries 1977). From the resultant graph the volume of enzyme extract corresponding to 50 % inhibition of the reaction was considered as one enzyme unit (Beauchamp and Fridovich 1971).

The data are based on a mean of minimum five replicates. All data were subjected to two way analysis of variance and significance was determined at 95 % confidence limits.

Results

Growth of callus cultures of *S. nudiflora* measured either as fresh or dry mass accumulation decreased at all salinities. When callus was cultured at 50 mM NaCl, a reduction of 44 % below control by fresh mass and 74 % by dry mass was observed. Further reductions of 69, 82 and 92 % on fresh mass and 87, 89 and 94 % on dry mass

occurred at 100, 150 and 200 mM NaCl salinity, respectively. Callus maintained in medium without NaCl served as control. The relative growth rate and water content also showed a similar trend (Table 1). The callus cells were spherical in shape and the cell size decreased considerably at all salinity levels tested (Table 1). When compared with control almost 4 times reduction in cell size was noticed in 200 mM NaCl.

Moderate salinity stress resulted in accumulation of soluble proteins: an increase of 23 and 32 % was observed at 50 and 100 mM NaCl, respectively. At higher salinity (150 and 200 mM NaCl), the protein content declined to 33 and 35 %, respectively of that measured in the control (Table 1). Compared to control proline accumulation increased steadily and an increase of about 2-, 4-, 6- and 9-fold was observed at 50, 100, 150 and 200 mM NaCl, respectively (Table 1). Na^+ accumulated in all cultures and the accumulation increased as a function of external salinity. K^+ content decreased with increase in salinity. Ca^{2+} concentration increased significantly at 50 mM NaCl and then decreased at high NaCl. Na^+/K^+ ratio ranged from 0.59 in control to 1.94 in 200 mM NaCl (Table 2).

Peroxidase activity generally increased as a function of external salinity whereas catalase and superoxide dismutase activities declined. The exception was 50 mM NaCl, where the peroxidase activity decreased to 60 % of control and then increased at higher salinities (150 and 200 mM). Whereas, the total SOD activity increased non-significantly at 50 mM NaCl and decreased to 32, 35 and 49 % at 100, 150 and 200 mM NaCl, respectively (Table 3).

Table 1. Effect of different NaCl concentrations on growth, and protein and proline contents in *S. nudiflora* callus cultures. Means ± SE, $n = 5$.

NaCl [mM]	Fresh mass [g]	Dry mass [g]	Protein [mg g^{-1}(f.m.)]	Proline [μg g^{-1}(f.m.)]	Water content [g(water) g^{-1}(d.m.)]
0	16.48 ± 0.78	1.03 ± 0.04	12.37 ± 0.24	54.82 ± 4.01	15.50 ± 0.74
50	9.23 ± 0.71	0.27 ± 0.02	15.30 ± 0.61	103.33 ± 8.12	8.23 ± 0.71
100	5.11 ± 0.47	0.13 ± 0.01	16.39 ± 0.57	236.54 ± 18.39	4.02 ± 0.55
150	1.99 ± 0.21	0.11 ± 0.01	8.37 ± 0.21	333.44 ± 14.95	0.99 ± 0.21
200	1.33 ± 0.09	0.06 ± 0.01	8.09 ± 0.02	508.19 ± 19.42	0.34 ± 0.08

Table 2. Effect of different NaCl concentrations on cell size and ion contents in *S. nudiflora* callus cultures. Means ± SE, $n = 5$.

NaCl [mM]	Cell size [μm]	Na^+ [mg g^{-1}(d.m.)]	K^+ [mg g^{-1}(d.m.)]	Ca^{2+} [mg g^{-1}(d.m.)]	Na^+/K^+
0	42	60.23 ± 1.45	102.02 ± 3.51	110.59 ± 4.0	0.59
50	38	74.48 ± 1.83	93.42 ± 2.73	128.22 ± 0.40	0.79
100	27	82.30 ± 1.83	86.38 ± 1.56	98.97 ± 0.40	0.95
150	20	119.54 ± 1.83	73.48 ± 1.56	82.14 ± 1.20	1.62
200	15	130.81 ± 0.22	67.23 ± 1.56	67.31 ± 1.20	1.94

Table 3. Effect of different NaCl concentrations on peroxidase, catalase and superoxide dismutase activity of *S. nudiflora* callus cultures. Mean ± SE, $n = 5$

NaCl [mM]	Peroxidase [U mg^{-1}(protein) min^{-1}]	Superoxide dismutase [U mg^{-1}(protein)]	Catalase [U mg^{-1}(protein)]
0	86.79 ± 2.36	3.78 ± 0.07	4.12 ± 0.05
50	34.85 ± 3.77	3.98 ± 0.13	3.95 ± 0.12
100	82.56 ± 2.45	2.57 ± 0.11	3.57 ± 0.15
150	350.47 ± 6.92	2.47 ± 0.20	2.50 ± 0.13
200	230.32 ± 5.72	1.94 ± 0.24	1.65 ± 0.11

Discussion

The results from the present study on *in vitro* salt tolerance of *S. nudiflora* suggest that salt tolerance in intact halophytic plants is not entirely based on cellular mechanisms as the callus cells grew poorly on saline media. Our results indicate that at 170 and 340 mM NaCl corresponding to optimum salinity for whole plants (Cherian and Reddy 2000), growth of *S. nudiflora* callus cultures were strongly inhibited. This supports the observation of Strogonov (1973) on *Salicornia herbacea* and Smith and McComb (1981) on *Atriplex undulata* and *Suaeda australis*. Growth inhibition (50 %) was reported at 170 mM NaCl in callus cultures of *Suaeda maritima* (Hedenstrom and Breckle 1974) and at 200 mM NaCl in cell suspensions of *Atriplex nummularia* (Casas *et al.* 1991). Cell suspension cultures of the halophytic grasses *Distichlis spicata* and *Spartina pectinata* showed tolerance with 50 % growth inhibition occurring at 320 and 280 mM NaCl, respectively (Warren and Gould 1982, Warren *et al.* 1985). The inconsistency in the *in vitro* and *in vivo* response to NaCl may be due to the multiplicity of mechanisms for whole plant salt tolerance (McCoy 1987).

Salinity stress changed the size of the cells of *S. nudiflora* callus cultures. A decrease in cell size following adaptation to NaCl was also reported in tobacco cells (Binzel *et al.* 1985) and may have been due to changes in cell wall properties (Iraki *et al.* 1989a). Iraki *et al.* (1989a,b) and McCann *et al.* (1994) reported occurrence of structural changes in the cell wall accompanied by changes in the composition of pectin and other non-cellulosic polymers and an increase in protein content for cells grown under saline condition. However, the biochemical pathway leading to changes in the cell wall and subsequent reduction in cell expansion is still unclear.

The decrease in protein content at higher concentrations of NaCl observed in our study may be due to the release of some amount of protein to the media due to osmotic shock (Mass *et al.* 1979) or a decrease in the synthesis of protein (Hall and Flowers 1973). Similarly, Handa *et al.* (1983) reported decline in soluble protein content in cultured cells adapted to water stress. Proline content of *S. nudiflora* increased from 2- to 9-fold when cells were exposed to salinity. The high concentration of proline in salinized cells may serve as cytoplasmic osmoticum. The increase in Na^+ content of cells was accompanied by a decrease in K^+ as has been reported in other studies (Watad *et al.* 1983). Blits *et al.* (1993) reported preferential absorption of K^+ over Na^+ in suspension culture of *Kosteletzkya virginica*. In the present study Na^+/K^+ ratio increased as a function of external salinity. The ability to maintain a low Na^+/K^+ ratio is thought to be associated with salt tolerance in plants (Storey and Wyn Jones 1979).

There is much evidence obtained from various plants showing that the amounts and activities of enzymes involved in scavenging active oxygen species are altered by environmental stresses such as drought and salinity. The results obtained with callus tissue were not in total agreement with those observed in whole plant studies of *S. nudiflora*. Salt induced increases in SOD and peroxidase were observed in whole plants while the activities of SOD and catalase remained below control levels in NaCl treated callus tissue. It is possible that oxidative damage is a general response to physiological stress and in light of the fact that increased antioxidant production at whole plants has been implicated in the development of tolerance to several other environmental stresses (Spychalla and Desborough 1990). It may be also pointed out that the much higher irradiance experienced by the naturally grown plants could be accounted for the higher antioxidant enzyme activities in whole plants.

The NaCl induced enhancement of peroxidase activity in salinized cells of *S. nudiflora* indicates that these cells have a higher capacity for the decomposition of H_2O_2 generated by SOD. This view agrees with that of Olmos and Hellin (1996). Our results also agree with the opinion that the high peroxidase activity detected in cells treated with high concentrations of NaCl would cause a change in the mechanical properties of the cell wall and that could eventually determine their final cell size (Sancho *et al.* 1996). Similarly it has been suggested that callus from an NaCl-tolerant cotton cultivar subjected to salt stress exhibits significantly enhanced activities in ascorbate peroxidase, superoxide dismutase, catalase and glutathione reductase, whereas callus tissue from a NaCl-sensitive cultivar exposed to salt stress shows little change in the activities of these enzymes when compared to non-stressed callus tissue (Gossett *et al.* 1994). The decline in SOD activity observed in the present data suggests a lesser $O_2^{\cdot-}$ scavenging and dismutating capacity in the salinized callus cells of *S. nudiflora* and thereby its salt sensitive trait. Day and Kar (1995) observed that SOD and catalase activities were much lower during callus formation than during seedling growth indicating the declining capacity of callus tissue to scavenge $O_2^{\cdot-}$ and H_2O_2.

We assume that the accumulation of H_2O_2 and $O_2^{\cdot-}$ radical in salt treated callus cells could cause damage to membranes and in turn may account for the many-fold increase of proline content of *S. nudiflora* callus cells. Additional analysis, such as isozyme studies and how stresses such as salinity may affect SOD enzyme in callus tissue therefore seem warranted. At present, however, it appears that salt tolerance in *S. nudiflora* depends largely on physiological specialization at whole plant level rather

than on cellular mechanisms. It should also be pointed out that this may not necessarily be the case in non-halophytes where cellular mechanisms could be as important, or even more important, than mechanisms operating at the whole plant level.

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