Journal of Plant Biology, December 2000, 43(4) : 217-225

Influence of NaCl Salinity on the Behavior of Hydrolases and Phosphatases in Mulberry Genotypes: Relationship to Salt Tolerance

S. Shaik Mohamed Anas[1]* and M. Vivekanandan[2]

[1]International Centre for Water and Energy Systems, P.O.Box 2623, Abu Dhabi, United Arab Emirates
[2]Department of Biotechnology, School of Life Sciences, Bharathidasan University
Tiruchirapalli - 620 024, Tamil Nadu, India

Two genotypes (Tr4 and Tr10) of mulberry (*Morus indica*) were cultured using aqueous solutions that contained different concentrations of NaCl (0 to 30 mM). Electrical conductivity (EC) values ranged from 0.52 to 4.60 mS cm^{-1}, with a pH of 7.0. Analyses were conducted to envisage the effects of NaCl salinity on some hydrolytic enzymes. Neither nitrate reductase nor nitrite reductase activities were affected by salt treatment in roots and leaves of either genotype. The activities of β- and α-amylases were higher in leaves than in roots. Both alkaline and acid phosphatases exhibited significant increases in the roots and leaves of Tr4; no such trend was observed in Tr10. The levels of peroxidase and superoxide dismutase increased quite significantly in the leaves as well as the roots of both genotypes. Increased enzymatic activities may suggest a stress-tolerant nature for these genotypes against NaCl toxicity. This was also evidenced by the unaffected levels in percent rooting, chloroplast pigments, osmoprotectants, nitrogenous compounds, and some biomolecules, as had been observed in earlier studies.

Keywords: hydroponics, mulberry, saline tolerance, sodium chloride

Salinity is a major factor that reduces field production in arid and semi-arid regions of the world (Franco et al., 1993). Crop yields are limited on nearly one-third of the world's irrigated land, and salinization continues to increase worldwide (Johnson et al., 1992). Reclamation, drainage, and improved irrigation practices might reduce the severity and spread of salinization in some regions, but the costs of these practices are generally prohibitive, especially in developing countries such as India. Therefore, the introduction of saline-tolerant genotypes may provide a relatively cost-effective, short-term solution to the problems associated with low to moderate levels of salinity.

In India, sericulture has become the means of livelihood for more than six million people in over 52,000 villages (Dorcus, 1995). Mulberry (*Morus indica*) is a highly valued tree that is widely advocated for extensive cultivation in Tamil Nadu to boost silk production. Mulberry leaves form the chief source of food for the mulberry silkworm (*Bombyx mori* L.). In general, mulberry does not grow well under either rainy conditions (Susheelamma et al., 1990; Dorcus and Vivekanandan, 1991) or salinity (Ramanjulu et al., 1993, 1994; Shaik Mohamed Anas and Vivekanandan, 1994a). Nevertheless, it has become increasingly necessary to cultivate mulberry even in saline soils. Silk production may be improved by employing saline-tolerant mulberry genotypes in the vast stretches of available coastal regions of our country.

The present study was undertaken to envisage the behaviors of nitrate and nitrite reductases, catalase, superoxide dismutase, peroxidase, protease, amylases, as well as acid and alkaline phosphatases in two promising mulberry genotypes, Tr4 and Tr10. These two genotypes had been identified as highly desirable in earlier studies through an assessment of various parameters such as significant rooting potential, unaffected levels of chlorophyll, and accumulations of osmoprotectants (i.e., proline, glycine betaine, and polyols and inorganic nitrogenous compounds) compared with other mulberry genotypes (Shaik Mohamed Anas and Vivekanandan, 1991, 1994a, 1999). They had also shown differing levels of salt tolerance when grown under increasing concentrations of NaCl, as a test of their inherent capacity.

MATERIALS AND METHODS

Cuttings of both Tr4 and Tr10 mulberry genotypes were collected from the mulberry garden at the Bharathidasan University. Samples were 15 cm long, with four to five live buds each (Krishnaswamy, 1978). For

*Corresponding author; fax +971-2-6442565
e-mail dar01@emirates.net.ae

each treatment, 25 cuttings were inserted into three replicate plastic culture trays (28 × 9 cm), then each tray was covered with a perforated thermacole pith for the particular NaCl concentration to be tested. The basal, i.e., 'chisel'-cut, end of each cutting was dipped into the experimental solution. We used the standard culturing solution of Hewitt (1966). NaCl was added immediately to give final concentrations of 10, 20, or 30 mM. The solution pH was then adjusted to 7.0 with 0.1 N NaOH. Electrical conductivities of the solutions were 2.70, 3.90, and 4.60 mS cm^{-1}, respectively. The base nutrient solution served as the control (0.52 mS cm^{-1}). Culture solutions were changed at 3-day intervals. The cuttings were raised in the laboratory at 30 ± 1°C and a relative humidity of about 60 ± 5%, under natural white light with an intensity of up to 600 µE m^{-2} s^{-1}.

When the saplings were 60 days old, they were carefully removed from the culture trays. Both leaves and roots were washed with distilled water and used for the enzyme analysis. Nitrate and nitrite reductases were assayed according to the methods of Hageman and Hucklesby (1971) and Finka et al. (1977), respectively. The freshly harvested and washed leaves were cut into 1-cm discs and the roots were broken into small, 100-mg bits. These samples were then vacuum-filtered for 3 min in 5.0 mL of 0.1 M phosphate buffer (pH 7.5) that contained 0.1 M potassium nitrate and 5% n-propanol. After infiltration, the vials were incubated in the dark for 30 min at 30°C. For the blank, a 1.0-mL sample was taken from the 0.1 M phosphate buffer after the vacuum infiltration ('0' time). The concentration of nitrate released to the incubation medium was determined by adding 1.0 mL of 1% (w/v) sulphanilamide in 3 N hydrochloric acid and 1.0 mL of 0.02% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride to a 1.0-mL sample from the incubation medium. After 10 min the optical density was measured at 550 nm in a UV-vis spectrophotometer, with potassium nitrite as the standard. Activity was expressed as µ mole nitrate released h^{-1} g^{-1} fw.

We isolated and purified intact chloroplasts for the nitrite-reductase assay using the method of Gnanam and Kulandaivelu (1969). 100 mg of fresh leaves were ground with a semi-frozen grinding medium that contained 20 mM Tris HCl buffer (pH 7.5), 330 mM sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, and 2 mM sodium isocarbate. The homogenate was filtered through eight layers of muslin cloth, then centrifuged by accelerating to approximately 600 rpm and returning to rest in 90 s, using a refrigerated centrifuge. After centrifugation, the supernatant was discarded and the pellet surface washed with the grinding medium, which was then decanted. Small quantities of resuspension medium (1 mL per tube) containing 330 mM sorbitol, 2 mM EDTA, 5 mM $MgCl_2$, and 10 mM NaCl in 20 mM Tris HCl buffer (pH 7.5) were added to resuspend the chloroplasts. The suspension was then stored in an ice bucket. The assay mixture contained 0.3 mL of intact chloroplasts as a crude enzyme source and 1.5 mL of the following reaction mixture: 6.25 mL of 0.5 M Tris HCl buffer (pH 7.5), 4.32 mg of sodium nitrate, 6.01 mg of methylviologen, and 25 mL of distilled water. The blank contained everything but the enzyme extract.

The reaction was initiated by the addition of 0.2 mL of a freshly prepared sodium dithionite solution that contained 25 mg of $Na_2S_2O_4$ in 1.0 mL of a 25-mg mL^{-1} $NaHCO_3$ solution. This reaction mixture turned blue because methylviologen acts as a donor for the reduction of NO_2 to NH_2, as catalyzed by nitrite reductase (Finka et al., 1977). The blue-colored mixture was then incubated for 10 min. Afterward, the reaction was stopped by vigorously shaking the reaction mixture in a vortex until the color disappeared. From this an aliquot of 20 µL was taken and added to 1 mL of distilled water, to which 1 mL of 1% (w/v) sulphanilamide in 3 N HCl and 1.0 mL of 0.02% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride had been added. The absorbance of the pink color that developed was read at 540 nm, and the amount of nitrite that disappeared was calculated from the standard graph. Enzymatic activity was expressed as µ moles nitrite that disappeared h^{-1} g^{-1} fw.

Protease activity was estimated by using the method of Penner and Ashon (1967). Leaves and roots were ground separately with 20 mL of ice-cold 0.1 M phosphate buffer (pH 6.5). The homogenate was filtered and centrifuged at 3000 rpm for 15 min, using a refrigerated centrifuge. The supernatant was used as the source of crude enzyme. To a 2.0-mL sample, 1.0 mL of 0.1 M phosphate buffer (pH 6.5) and 1.0 mL of Bovine Serum Albumin (BSA) (10 mg mL^{-1}) were added. The reaction mixture was incubated at room temperature for 2 h, and the reaction was then terminated by the addition of 3 mL of 5% trichloroacetic acid (TCA). The centrifuged supernatant was used for estimating the amount of soluble peptides, according to the method of Lowry et al. (1951). Enzymatic activity was expressed as µg peptides released min^{-1} mg^{-1} protein.

Peroxidase was assayed by the method of Malik and Singh (1980). Leaves and roots were ground sep-

arately with 0.1 M phosphate buffer (pH 6.5). The homogenate was centrifuged at 5000 rpm at 4°C for 10 min and the clear supernatant was used for the assay. Briefly, 0.5 mL of the sample, 3.2 mL of 0.1 M phosphate buffer (pH 6.5), and 0.1 mL of an orthodianisidine solution (1 mg mL^{-1} in methanol) were placed in a clean, dry cuvette and inserted into a UV-vis spectrophotometer for measuring at 430 nm. Then, 0.2 mL of H_2O_2 (0.2 M) was added to the reaction mixture and the increase in absorbance was recorded every 30 s, for up to 3 min. The linear phase of the enzyme activity was taken for data calculation Values were plotted and the change in absorbance per unit time per mg protein was calculated.. One unit was defined as an increase of 0.01 OD min^{-1}.

Acid and alkaline phosphatases were assayed following the methods of Ikawa et al. (1964) and Torriani (1967), respectively. Leaves and roots were ground separately with 0.1 M acetate buffer (pH 5.0) for acid phosphatase or with 0.1 M Tris HCl buffer (pH 8.2) for alkaline phosphatase, as the sources of the crude enzyme. For each 1.0-mL sample, 1.0 mL of substrate (6.6 mM nitrophenyl phosphate [NPP] in 0.6 M acetate buffer; pH 5.0) was added, then the mixture was incubated at 30°C for 15 min. The reaction was terminated by the addition of 2.0 mL of 0.2 N NaOH. For the control, the reaction was terminated at '0' time. Absorbance was read at 410 nm, and the number of μ moles of substrate hydrolyzed per unit time was calculated using EM 410 of 1.62×10^4 for p-nitrophenol. For alkaline phosphatase, 1.0 mL of substrate (6.6 mM nitrophenyl phosphate [NPP] in 0.6 M Tris HCl buffer; pH 8.2) was added to 1.0 mL of the sample, then incubated at 30°C for 15 min. The reaction was terminated by the addition of 2.0 mL of 0.2 M sodium hydrogen phosphate. Again, the reaction for the control was terminated at '0' time. Absorbance and the number of μ moles of substrate per unit time were determined as before.

Assays of α and β amylases were carried out according to the method of Dure (1960). Leaves and roots were ground separately with distilled water and centrifuged at 3000 rpm at 5°C for 10 min. The supernatant was saved and kept at 70°C for 5 min to inactivate β-amylase. To 1.0 mL of the enzyme extract, 1.0 mL of 0.1 M citrate buffer (pH 5.0) and 0.5 mL of 2% soluble starch were added, then held at 30°C for 10 min. Afterward, 2.0 mL of dinitrosalicylic acid reagent was added and kept in a boiling-water bath for 5 min. After cooling, the volume was made up to 10 mL with distilled water. Absorbance was read at 540 nm; maltose was used as the standard. For β amylase, the supernatant was used as the crude enzyme extract. For each 1.0-mL sample, 1.0 mL of 0.1 M citrate buffer (pH 3.4) was added, then the mixture was held for 5 min to inactivate a amylase. Afterward, 2.0 mL of 2% soluble starch was added and kept at 30°C for 10 min. A reagent of 2.0 mL dinitrosalicylic acid was added, then kept in a boiling-water bath for 5 min. After cooling, the volume was made up to 10 mL with double distilled water. Absorbance was read at 540 nm; maltose was used as the standard. Enzymatic activities were expressed as μg maltose released h^{-1} mg^{-1} protein.

Catalase was assayed using the method of Clairborne (1985). Leaves and roots were ground separately at 4°C with polyvinylpolypyrrolidone (25% by weight) and four volumes of buffer consisting of 50 mM KH_2PO_4/K_2HPO_4 and 0.1 mM EDTA (pH 7.0). The supernatant was used as the source of crude enzyme. Catalase was measured by a modified spectrophotometric procedure (based on the rate of decomposition of H_2O_2) by following the decline in absorbance at 240 nm (Clairborne, 1985). The assay was performed in a 3.0-mL quartz cuvette containing 200 μL enzyme extract, 50 mM potassium phosphate buffer (pH 7.0), and 37.5 mM H_2O_2. Rate of H_2O_2 decomposition was determined during the linear phase of the reaction (20-60 s), and enzymatic activity was expressed as units min^{-1} mg^{-1} protein (Beers and Sizer, 1952). One unit of activity of catalase was equal to one μ mole of H_2O_2 decomposed min^{-1} at 25°C.

$$\text{Units mg}^{-1}\text{ protein} = \frac{\text{Absorbance min}^{-1} \times 1000}{43.6^* \times \text{mg protein mL}^{-1}\text{ reaction mixture}}$$

*43.6 = molar absorbency index for hydrogen peroxide at 240 nm in a 1-cm cuvette.

Superoxide dismutase was assayed with the method of Beauchamp and Fridovich (1971). Intact chloroplasts that were prepared following the method of Gnanam and Kulandaivelu (1969) were used as a crude enzyme source. The reaction mixture in a final volume of 3 mL contained 2.5 mL of 50 mM phosphate buffer (pH 7.8), 0.1 mL of 30 mM methionine, 0.1 mL of 2.25 mM nitroblue tetrazolium, 0.1 mL of 3 mM EDTA, 100 μL enzyme extract, and 0.1 mL of 90 μM riboflavin. The riboflavin was added last, and the tubes were then shaken and placed 30 cm below a light bank of four 15-W fluorescent lamps (PAR: 300 μE m^{-2} s^{-1}). The reaction was started by switching on the light, then allowing it to run for one hour. (The reaction was linear during this period.) Afterward, the

reaction was stopped by switching off the light and covering the tubes with black cloth. Absorbance of the reaction mixture was read at 560 nm. A non-irradiated reaction mixture, which did not develop a blue color, served as the control. The volume of the enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit. Maximum color was observed in the reaction mixture that lacked enzyme. Activity was expressed as the number of units min^{-1} mg^{-1} protein.

All data collected during the study period were subjected to statistical analysis (Standard Deviation and F-test) according to the standard procedures described by Zar (1974).

RESULTS

The effects of NaCl treatments on assays for hydrolases and phosphatases are presented in Tables 1 and 2, and Tables 3 and 4, respectively. Neither nitrate nor nitrite reductase activities were affected by salt treatment in either the roots or leaves of genotype Tr4. Peroxidase activities were higher, by two and

Table 1. Effect of NaCl salinity (0 to 30 mM) on the activities of nitrate reductase, nitrite reductase, peroxidase, catalase, superoxide dismutase, protease, amylases, and phosphatases in the leaves of mulberry genotype Tr4. The 60-day-old saplings were grown under hydroponic conditions at room temperature (30 ± 1°C) with a relative humidity of 60 ± 5%. Data are the means of three different experiments ± SD; those in parenthesis indicate % control values.

Enzymes	Strength of NaCl (mM)			
	0	10	20	30
Nitrate reductase (μm NO_2 released h^{-1} g^{-1} fw)	7.91±0.32	7.46±0.31 (94.31)	7.44±0.33 (94.06)	7.43±0.30 (93.93)
Nitrite reductase (μm NO_2 disappeared h^{-1} g^{-1} fw)	4.69±0.31	4.67±0.31 (99.57)	4.45±0.33 (94.88)	4.15±0.30 (88.49)
Peroxidase (Units min^{-1} mg^{-1} protein)	1.62±0.12	2.41±0.23 (148.76)	2.70±0.21 (166.67)	4.70±0.35 (290.12)
Catalase (Units min^{-1} mg^{-1} protein)	16.38±1.50	14.57±1.90 (88.95)	12.28±1.20 (74.97)	11.12±1.00 (67.89)
Superoxide dismutase (Units min^{-1} mg^{-1} protein)	30.24±2.50	35.10±3.10 (116.07)	39.47±3.50 (130.52)	40.07±2.90 (132.51)
Protease (μg peptides released h^{-1} mg^{-1} protein)	779.65±14.10	835.20±18.00 (107.12)	932.75±25.80 (119.64)	981.00±26.50 (125.82)
α-amylase (μg maltose released h^{-1} mg^{-1} protein)	130.78±5.70	140.94±6.40 (107.77)	175.89±3.80 (134.49)	185.30±8.90 (141.69)
β-amylase (μg maltose released h^{-1} mg^{-1} protein)	152.60±6.00	167.10±8.30 (109.50)	181.35±9.90 (118.84)	270.00±12.80 (176.93)
Acid phosphatase (μm nitrophenol released h^{-1} mg^{-1} protein)	104.53±5.40	115.92±5.50 (110.90)	129.87±5.60 (124.24)	141.45±5.10 (135.32)
Alkaline phosphatase (μm nitrophenol released h^{-1} mg^{-1} protein)	192.40±6.40	199.64±6.50 (103.76)	199.00±5.90 (103.43)	170.77±5.80 (88.76)

Strength of NaCl = $F_{0.05}$ = 2.92
Enzymes = $F_{0.05}$ = 2.16
The calculated values of F are less than the table value at the 5% level of significance.

Table 2. Effect of NaCl salinity (0 to 30 mM) on the activities of nitrate reductase, nitrite reductase, peroxidase, catalase, superoxide dismutase, protease, amylases, and phosphatases in the roots of mulberry genotype Tr4. The 60-day-old saplings were grown under hydroponic conditions at room temperature (30 ± 1°C) with a relative humidity of 60 ± 5%. Data are the means of three different experiments ± SD; those in parenthesis indicate % control values.

Enzymes	Strength of NaCl (mM)			
	0	10	20	30
Nitrate reductase ($\mu m\ NO_2$ released $h^{-1}\ g^{-1}$ fw)	4.97±0.31	4.39±0.30 (88.33)	4.00±0.31 (80.48)	4.00±0.31 (80.48)
Nitrite reductase ($\mu m\ NO_2$ disappeared $h^{-1}\ g^{-1}$ fw)	4.94±0.34	4.89±0.35 (98.99)	4.45±0.30 (90.08)	4.35±0.30 (88.06)
Peroxidase (Units $min^{-1}\ mg^{-1}$ protein)	1.19±0.10	1.45±0.13 (121.85)	2.14±0.14 (179.83)	2.49±0.20 (209.24)
Catalase (Units $min^{-1}\ mg^{-1}$ protein)	39.32±2.90	30.30±2.90 (76.28)	24.55±2.10 (61.81)	24.11±2.20 (60.69)
Superoxide dismutase (Units $min^{-1}\ mg^{-1}$ protein)	33.32±2.90	44.67±3.10 (134.06)	57.46±3.00 (172.45)	61.77±2.70 (185.38)
Protease (μg peptides released $h^{-1}\ mg^{-1}$ protein)	446.92±12.20	476.56±10.70 (106.63)	567.52±15.70 (127.09)	584.49±11.40 (130.78)
α-amylase (μg maltose released $h^{-1}\ mg^{-1}$ protein)	126.05±4.60	122.04±4.20 (96.82)	110.55±4.0 (87.70)	102.12±3.90 (81.01)
β-amylase (μg maltose released $h^{-1}\ mg^{-1}$ protein)	115.75±5.70	107.75±5.10 (93.09)	98.23±5.20 (84.86)	89.35±5.00 (77.19)
Acid phosphatase (μm nitrophenol released $h^{-1}\ mg^{-1}$ protein)	45.15±3.20	61.50±3.00 (136.21)	66.93±3.10 (148.24)	74.86±4.20 (165.80)
Alkaline phosphatase (μm nitrophenol released $h^{-1}\ mg^{-1}$ protein)	171.19±7.40	181.61±7.00 (106.08)	187.81±7.20 (109.71)	194.23±7.10 (113.46)

Strength of NaCl = $F_{0.05}$ = 2.92
Enzymes = $F_{0.05}$ = 2.16
The calculated values of F are less than the table value at the 5% level of significance.

three-fold, for leaves and roots, respectively, when salt concentrations were increased, compared with the control (Tables 1 and 2). Catalase activity decreased by more than 30% in both leaves and roots at 30 mM NaCl.

In contrast, the activity of superoxide dismutase exhibited an increasing trend that was more pronounced in roots than in leaves. Similarly, protease activity was marginally higher in both leaves and roots. The activities of β- and α-amylases were about 40-70% higher in the leaves, (whereas the roots had a marginal decrease, about 20%, when treated with 30 mM NaCl. Acid phosphatase increased from marginal to significant levels in both leaves and roots, but levels of alkaline phosphatase showed no such changes (Tables 1 and 2).

In the leaves of genotype Tr10, individual levels of nitrate and nitrite reductases as well as peroxidase did not differ significantly at various NaCl concentrations (Table 3). Superoxide dismutase increased marginally. In contrast, protease and α and β amylases exhibited increasing trends for all treatments. Catalase and the acid and alkaline phosphatases had declining trends. Levels of nitrate reductase, nitrite reductase, and alka-

Table 3. Effect of NaCl salinity (0 to 30 mM) on the activities of nitrate reductase, nitrite reductase, peroxidase, catalase, superoxide dismutase, protease, amylases, and phosphatases in the leaves of mulberry genotype Tr10. The 60-day-old saplings were grown under hydroponic conditions at room temperature (30 ± 1°C) with a relative humidity of 60 ± 5%. Data are the means of three different experiments ± SD; those in parenthesis indicate % control values.

Enzymes	Strength of NaCl (mM)			
	0	10	20	30
Nitrate reductase ($\mu m\ NO_2$ released $h^{-1}\ g^{-1}$ fw)	8.98±0.68	8.45±0.63 (94.09)	8.36±0.64 (93.09)	8.24±0.63 (91.76)
Nitrite reductase ($\mu m\ NO_2$ disappeared $h^{-1}\ g^{-1}$ fw)	4.74±0.37	4.47±0.35 (94.30)	4.65±0.30 (98.10)	4.65±0.32 (98.10)
Peroxidase (Units $min^{-1}\ mg^{-1}$ protein)	2.20±0.19	2.31±0.22 (105.00)	2.85±0.21 (129.54)	2.98±0.25 (135.45)
Catalase (Units $min^{-1}\ mg^{-1}$ protein)	15.34±1.50	14.40±1.20 (93.87)	13.22±1.00 (86.18)	12.97±1.20 (84.55)
Superoxide dismutase (Units $min^{-1}\ mg^{-1}$ protein)	30.72±2.30	38.74±2.70 (126.11)	39.15±2.90 (127.44)	41.14±3.10 (133.92)
Protease (μg peptides released $h^{-1}\ mg^{-1}$ protein)	653.90±11.20	756.90±10.30 (115.75)	799.50±11.70 (122.27)	872.00±11.90 (133.35)
α-amylase (μg maltose released $h^{-1}\ mg^{-1}$ protein)	145.87±5.80	156.60±5.40 (107.35)	170.56±5.70 (116.93)	179.85±4.90 (123.29)
β-amylase (μg maltose released $h^{-1}\ mg^{-1}$ protein)	152.60±4.90	161.53±5.20 (105.85)	181.35±4.20 (118.84)	204.00±5.00 (133.68)
Acid phosphatase (μm nitrophenol released $h^{-1}\ mg^{-1}$ protein)	125.92±5.40	105.31±4.90 (83.63)	98.17±4.80 (77.96)	88.00±5.10 (69.88)
Alkaline phosphatase (μm nitrophenol released $h^{-1}\ mg^{-1}$ protein)	224.25±6.40	223.12±6.20 (99.50)	221.37±6.00 (98.71)	188.00±6.10 (83.83)

Strength of NaCl = $F_{0.05}$ = 2.92
Enzymes = $F_{0.05}$ = 2.16
The calculated values of F are less than the table value at the 5% level of significance.

line phosphatase in the roots did not vary among the NaCl treatments (Table 4). Trends for the activities of peroxidase, protease, α and β amylases, and superoxide dismutase increased while those for acid phosphatase and catalase decreased.

DISCUSSION

Nitrate reductase (NR) is a key enzyme in assimilation and growth of higher plants. Its activity can be affected by drought (Paliwal and Ilangovan, 1990; Sreenivasulu Reddy et al., 1990) and salinity (Dias and Costa, 1983; Krishna Rao and Gnanam, 1990; Mohammed Gufran Khan, 1994). However, for both the leaves and roots of genotypes Tr4 and Tr10, activity was unaffected, even at concentrations of up to 30 mM NaCl. Activity was about two-fold higher in leaves than in roots. In some halophytes, the enzyme has been found to be unaffected by low to medium salt concentrations (Austenfeld, 1974). The unperturbed level of NR activity in both the leaves and roots of our two genotypes may also have been due to unaffected levels of nitrate (Shaik Mohamed Anas,

Table 4. Effect of NaCl salinity (0 to 30 mM) on the activities of nitrate reductase, nitrite reductase, peroxidase, catalase, superoxide dismutase, protease, amylases, and phosphatases in the roots of mulberry genotype Tr10. The 60-day-old saplings were grown under hydroponic conditions at room temperature (30 ± 1°C) with a relative humidity of 60 ± 5%. Data are the means of three different experiments ± SD; those in parenthesis indicate % control values.

Enzymes	Strength of NaCl (mM)			
	0	10	20	30
Nitrate reductase ($\mu m\ NO_2$ released $h^{-1}\ g^{-1}$ fw)	4.98±0.33	4.98±0.31 (100.00)	4.94±0.32 (99.20)	4.46±0.31 (89.56)
Nitrite reductase ($\mu m\ NO_2$ disappeared $h^{-1}\ g^{-1}$ fw)	4.94±0.34	4.89±0.32 (98.99)	4.89±0.33 (98.99)	4.89±0.33 (98.99)
Peroxidase (Units $min^{-1}\ mg^{-1}$ protein)	2.81±0.21	3.46±0.29 (123.13)	3.80±0.27 (135.23)	4.68±0.31 (166.55)
Catalase (Units $min^{-1}\ mg^{-1}$ protein)	42.05±2.90	33.39±2.50 (79.40)	30.86±2.10 (73.39)	23.36±2.00 (55.55)
Superoxide dismutase (Units $min^{-1}\ mg^{-1}$ protein)	37.42±2.70	39.29±2.70 (104.99)	40.06±2.40 (107.05)	46.27±2.10 (123.65)
Protease (μg peptides released $h^{-1}\ mg^{-1}$ protein)	488.17±10.30	551.76±11.40 (113.03)	604.45±9.80 (123.82)	672.00±10.20 (137.66)
α-amylase (μg maltose released $h^{-1}\ mg^{-1}$ protein)	122.47±5.70	143.87±3.90 (117.47)	162.08±4.20 (132.34)	206.40±4.20 (168.53)
β-amylase (μg maltose released $h^{-1}\ mg^{-1}$ protein)	94.18±3.70	102.46±3.90 (108.79)	118.58±3.00 (125.91)	143.60±3.10 (152.47)
Acid phosphatase (μm nitrophenol released $h^{-1}\ mg^{-1}$ protein)	44.16±2.90	40.34±3.00 (91.35)	38.50±2.80 (87.18)	37.50±3.10 (84.92)
Alkaline phosphatase (μm nitrophenol released $h^{-1}\ mg^{-1}$ protein)	220.80±5.40	228.48±5.20 (103.48)	244.47±5.30 (110.72)	226.00±5.10 (102.35)

Strength of NaCl = $F_{0.05}$ = 2.92
Enzymes = $F_{0.05}$ = 2.16
The calculated values of F are less than the table value at the 5% level of significance.

1996).

Similarly, nitrite reductase (NiR) activity was unaffected by NaCl salinity in the leaves and roots of both of our genotypes. Enzymes such as catalase, peroxidase, and superoxide dismutase have a role in protecting the plant from any kind of stress (Fridovich, 1975; Kalir and Poljakoff-Mayber, 1981; Edreva et al., 1989; Vivekanandan and Saralabai, 1994). When plants or cells are grown under saline conditions, oxygen toxicity may be induced by free radicals (Kalir and Poljakoff-Mayber, 1981). The levels of peroxidase and superoxide dismutase increased quite significantly in the leaves as well as the roots of our genotypes. These increased activities may demonstrate the stress-tolerant nature of the genotypes against NaCl toxicity. Peroxidase is a ubiquitous enzyme that is distributed in all plant tissues. It is physiologically important because of its association with various catalytic functions. The increased activity of peroxidase isindicative of the ability of the genotype to break down toxic substances such as peroxide and phenols under stress (Subhashini and Reddy, 1990).

The results described above gain indirect support from observations by Saralabai and Vivekanandan

(1995). They found higher levels of superoxide dismutase and peroxidase in the electrostatic precipitator (ESP)-dusted leaves of several leguminous crops. Those leaves then became resistant to the dust emanating from the nearby cement factory. In contrast, catalase activity was reduced as the level of salinity increased. Our leaves had higher levels of NR activity than did the roots. Protease activity also increased in both roots and leaves as the concentration of NaCl was increased. These observations are consistent with those of Shaik Mohamed Anas and Vivekanandan (1994b), for whom protein levels rose with a concomitant increase in free amino acids in genotypes Tr4 and Tr10.

Both α and β amylases had higher levels of activity as salinity increased; this was stronger in the leaves than in the roots. In fact, the activities of the α and β amylases in the roots of Tr4 were marginally reduced. Higher levels of amylases, particularly in leaves, have been well associated with increased total soluble sugars observed in the leaves of these two genotypes (Shaik Mohamed Anas and Vivekanandan, 1994b). Increased amylase activity usually is correlated with salt tolerance in cotton (*Gossypium* sp.) and soybean (*Glycine max*) (Krishnamurthy et al., 1987).

Both alkaline and acid phosphatases exhibited significant increases in the roots and leaves of Tr4, whereas no such trend was found in Tr10. In general, the activity of alkaline phosphatase was higher than that of acid phosphatase in both genotypes. Higher levels of phosphatases might be helpful in maintaining greater metabolic status of the cell by increasing the turnover rate of phosphate and, thereby, providing more energy for metabolism (Dubey and Sharma, 1989).

In summary, the activities of peroxidase and superoxide dismutase increased quite significantly in the leaves and roots of both genotypes. The unaffected levels of activity for both nitrate and nitrite reductases were viewed as an indicator of the stress-tolerant nature of the two genotypes. Increased enzymatic activities may be a sign of the tolerance possessed by these two genotypes against NaCl toxicity. Our results are similar to those from studies in which levels of amino acids, ascorbic acid, and β-carotene, as well as rooting and sprouting percentages, were increased (sometimes significantly) at a concentration of 30 mM NaCl, compared with resistant genotypes (Shaik Mohamed Anas and Vivekanandan, 1994a). In addition, earlier studies with our two genotypes had shown that levels of osmoprotectants, such as glycine betaine, polyols, and proline, increased in both leaves and roots when compared with resistant genotypes (Shaik Mohamed Anas and Vivekanandan, 1999).

ACKNOWLEDGEMENTS

We greatly acknowledge the financial assistance provided by the Central Silk Board, Government of India, Bangalore, for carrying out this work.

Received September 1, 2000; accepted November 8, 2000.

LITERATURE CITED

Austenfeld FA (1974) Der einfluss des NaCl and anderer alkalisakze auf die nitratreduktar aktivitat von *Salicornia europea L.*, Z. Pflanzenphysiol 71: 288-296

Beauchamp C, Fridovich I (1971) Superoxide dismutase: Improved assay and an assay applicable to acrylamide gels. Anal Biochem 44: 276-287

Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195: 135-140

Clairborne A (1985) Catalase activity, *In* RA Greenwald, ed, Handbook of Methods for Oxygen Radical Research, CRC Press, Inc, Boca Raton, Fl pp 283-284

Dias MA, Costa MM (1983) Effect of low salt concentrations on nitrate reductase and peroxidase of sugar beet leaves. J Exp Bot 34: 537-543

Dorcus D (1995) Evaluation of mulberry genotypes for drought resistance in alkaline soil. Ph.D. thesis. Bharathidasan University, Tiruchirapalli, India

Dorcus D, Vivekanandan M (1991) Screening of mulberry genotypes for rainfed conditions. Sericologia 31: 233-241

Dubey RS, Sharma KN (1989) Acid and alkaline phosphatases in rice seedlings growing under salinity stress. Indian J Plant Physiol 32: 217-223

Dure LS (1960) Site of origin and extent of activity of amylases in maize germination. Plant Physiol 35: 925-934

Edreva AM, Georgieva ID, Cholakova NI (1989) Pathogenic and non-pathogenic stress effects on peroxidases in leaves of tobacco. Environ Exp Bot 29: 365-377

Finka RL, Warner RL, Muzit TJ (1977) Effect of herbicides on in vivo nitrate and nitrite reduction. Weed Sci 25: 18-22

Franco JA, Esteber C, Rodriguez C (1993) Effects of salinity on various growth stages of muskmelon cv. Revigel. J Hort Sci 68: 899-904

Fridovich I (1975) Superoxide dismutase. Annu Rev Biochem 4: 47-59

Gnanam A, Kulandaivelu G (1969) Photosynthetic studies with leaf cell suspensions from higher plants. Plant Physiol 44: 1451-1456

Hageman RH, Hucklesby D (1971) Methods in Enzymology, *In* Sanpietro, ed, Academic Press, New York, p 491

Hewitt EJ (1966) The composition of the nutrient solution, *In* Sand and Water Culture Methods Used in the Study of Plant Nutrition, Commonwealth Agricultural Bureaux, Farnham Royal Bucks, England, p 1990

Ikawa IJ, Nisizawa K, Mira NK (1964) Specifications of several acid phosphatases from plant sources. Nature 230-239

Johnson DW, Smith SE, Dobrenz AK (1992) Selection for increased forage yield in alfalfa at different NaCl levels. Euphytica 60: 27-35

Kalir A, Poljakoff-Mayber A (1981) Changes in activity of malate dehydrogenase, catalase, peroxidase and superoxide dismutase in leaves of *Halimore portulacoides* (L.) Allen exposed to high sodium chloride concentrations. Ann Bot 47: 75-85

Krishna Rao R, Gnanam A (1990) Inhibition of nitrate and nitrite reductase activities by salinity stress in *Sorghum vulgare*. Phytochemistry 29: 1047-1049

Krishnamurthy R, Anbazhagan M, Bhagwat KA (1987) Accumulation of free amino acids and distribution of Na, Cl and K in the rice varieties exposed to NaCl stress. Indian J Plant Physiol 30: 183-188

Krishnaswamy S (1978) Mulberry Cultivation in South India. CSR&TI Bulletin No. 1, Mysore, India, p 15-17

Lowry PH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Malik CP, Singh MB (1980) Plant Enzymology and Histoenzymology. Kalyani Publishers, New Delhi, p 53-54

Mohammed Gufran Khan (1994) Effect of salt stress on nitrate reductase activity in some leguminous crops. Indian J Plant Physiol 37: 185-187

Paliwal K, Ilangovan M (1990) Factors influencing in vivo determination of nitrate reductase (EC 1.6.6.1) activity in mulberry (*Morus alba L.*). Sericologia 30: 369-379

Penner D, Ashon FM (1967) Hormonal control of protease activity in squash cotyledons. Plant Physiol 47: 791-796

Ramanjulu S, Veeranjaneyalu K, Sudhakar C (1993) Physiological changes induced by NaCl in mulberry var. Mysore Local. Indian J Plant Physiol 36: 273-275

Ramanjulu S, Veeranjaneyalu K, Sudhakar C (1994) Short-term shifts in nitrogen metabolism in mulberry *Morus alba* under salt shock. Phytochemistry 37: 991-995

Saralabai VC, Vivekanandan M (1995) Effects of the application of cement kiln exhausts on leaf biochemistry in certain legume crops. Photosynthetica 31: 399-409

Shaik Mohamed Anas S (1996) Effects of sodium chloride salinity on mulberry. Ph.D. thesis. Bharathidasan University, Tiruchirapalli, India.

Shaik Mohamed Anas S, Vivekanandan M (1991) Are triploid mulberry varieties saline tolerant? National Sericultural Conference on Mulberry, International Sericultural Research and Training Institute, p 69

Shaik Mohamed Anas S, Vivekanandan M (1994a) Evaluation of salinity tolerance in mulberry varieties by exploiting rooting potential. Sericologia 34: 311-321

Shaik Mohamed Anas S, Vivekanandan M (1994b) Salinity responses of the triploid mulberry varieties. Indian J Seric 33: 92-94

Shaik Mohamed Anas S, Vivekanandan M (1999) Accumulation of certain organic metabolites in different mulberry genotypes during NaCl salinity. Sericologia 39: 441-446

Sreenivasulu Reddy P, Padmavathi M, Sudhakar C, Veeranjaneyalu K (1990) Photosynthetic CO_2 assimilation and carbohydrate status during water stress in cowpea. Indian J Exp Biol 28: 346-348

Subhashini K, Reddy GM (1990) Effect of salt stress on enzyme activities in callus cultures of tolerant and susceptible rice cultivars. Indian J Exp Biol 28: 277-279

Susheelamma BM, Jolly, MS, Giridhar K, Sengupta K (1990) Evaluation of germplasm genotypes for drought resistance in mulberry. Sericologia 30: 327-341

Torriani A (1967) Alkaline phosphatases from *Escherichia coli, In* GL Cantoni, Dabies, eds, Procedure in Nucleic Acid Research, Harper and Row, New York

Vivekanandan M, Saralabai VC (1994) SOD-An indicator of air pollution tolerance? *In* K Asada, T Yoshikawa, eds, Frontiers of Reactive Oxygen Species in Biology and Medicine, Excepta Medica, International Congress Series 1058, Elsevier Science BV, Amsterdam, p 553-554

Zar GA (1974) Biostatistical Analysis. Prentice Hall, India