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RESPONSE OF ANTIOXIDANT SYSTEMS TO SHORT-TERM NaCl STRESS IN GRAPEVINE ROOTSTOCK-1616C AND *VITIS VINIFERA* L. CV. RAZAKI

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The present study examined free-radical scavenging enzyme activity and the levels of lipid peroxide, ascorbic acid, nitric oxide and glutathione in 1616C rootstock and the Razaki cultivar of *Vitis vinifera* L. under treatment with different concentrations of salt. At day 7, in leaves of both 1616C rootstock and cv. Razaki treated with 12 mM NaCl there were significant increases in glutathione peroxidase and catalase activity, and in the levels of thiobarbituric acid reactive substance and reduced glutathione, measured on a protein basis and fresh weight basis. Superoxide dismutase activity increased under NaCl treatment at day 7 in both samples versus the controls. In the Razaki cultivar, glutathione peroxidase activity was at maximum at day 7 under 12 mM NaCl treatment. Catalase activity was very low, and increased with increasing NaCl concentration in the Razaki cultivar and 1616C rootstock at day 7. In 1616C rootstock the nitrite level was lower than the controls within 4 days.

Key words: *Vitis vinifera* L., 1616C rootstock, antioxidant enzymes, glutathione, lipid peroxidation, ascorbic acid, nitric oxide.

INTRODUCTION

Reactive oxygen species (ROS) cause oxidative stress and are generated by a wide variety of factors in plants. During the course of normal metabolism, ROS are generated by photosynthesis; photooxidative damage can occur when ROS production exceeds antioxidant capacity (Foyer et al., 1994). Plants generate ROS when high intensity of light is combined with other environmental stresses such as salt (NaCl), drought, or nutrient deprivation. They can accumulate in response to biotic and abiotic stress and can initiate reaction cascades that result in the production of toxic products such as lipid peroxides and hydroxyl radicals, causing cell dysfunction and death (Alscher et al., 1997; Mittler, 2002). Salt stress is one of the major factors affecting plant function, inducing oxidative stress and limiting crop production (Price and Hendry, 1989). Plant cells have developed a comprehensive array of antioxidant defenses to prevent the formation of ROS or to limit their damaging effects. These include enzymes to decompose peroxides, proteins to sequester transition metals, and a range of compounds to scavenge free radicals. The most important enzymatic antioxidant is superoxide dismutase (SOD; EC 1.15.1.1); it catalyzes dismutation of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), which is then deactivated to water by catalase (CAT; EC 1.11.1.6) and/or glutathione peroxidase (GSPOD; EC 1.11.1.9). Glutathione, vitamins A, C and E and carotene provide antioxidant defenses through their ability to exist in reversible oxidized and reduced forms. Glutathione maintains protein thiol groups in the reduced state and also acts as a protective physiological antioxidant in biological

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systems (Glass and Stark, 1997). It plays a role in preventing oxidation of phenolic compounds in grape juice (Okuda and Yokotsuka, 1999). Ascorbic acid is a highly abundant metabolite and one of the most powerful natural antioxidants. As an antioxidant, it scavenges neutrophil oxidants, H_2O_2, and the hydroxyl radical (Elmadfa and Koenig, 1996). Ascorbic acid may protect membranes from free radical damage by regenerating tocopherol from the tocopheroxyl radical that is formed upon inhibition of lipid peroxidation by vitamin E in the aqueous phase (Perez et al., 2002).

Nitric oxide (NO) is a bioactive molecule that is produced from L-Arg by nitric oxide synthase (NOS) in various mammalian cells. Although the plant NOS gene, cDNA or protein has not been isolated yet, some studies have addressed NOS activity and NO in plants (Sen and Cheema, 1995). In both animals and plants, NO can be considered either protective or toxic depending on the concentration and the tissue where it is acting (Wink et al., 1999; Beligni and Lamattina, 2001). It has been reported that oxidative stress may reduce the regeneration potential of protoplasts (de Marco and Roubelakis-Angelakis, 1996) and that the ROS generating systems are different in grapevine (Papadakis and Roubelakis-Angelakis, 1999). ROS derived from oxygen are believed to affect the length of live and to be involved in senescence processes (Kanazawa et al., 2000).

The present study evaluates antioxidative defense systems in 1616C rootstock and the Razaki cultivar of *Vitis vinifera* L. during exposure to 3, 8 and 12 mM salt concentrations by assaying the activity of superoxide dismutase, glutathione peroxidase and catalase, and the level of thiobarbituric acid reactive substances (TBARS), ascorbic acid, nitric oxide and glutathione in leaves.

MATERIALS AND METHODS

PLANT MATERIAL

This experiment was carried out between March and August 2002 under controlled conditions in the greenhouse at the Department of Horticulture, Faculty of Agriculture, University of Ankara. Four-bud cuttings from 1616C rootstock and Razaki cv. were used. Buds were removed from the base of the plant and planted in plastic pots filled with peat, perlite and air-dried soil (ratio 2:1:2). Salt treatment began when homogen shoot length reached 70 cm (day 0): the plants were watered with 3 mM, 8 mM and 12 mM NaCl concentration. Tissue samples were collected at days 2, 4 and 7 from all stressed plants, frozen in liquid nitrogen, and kept at -80°C until use.

ENZYME EXTRACTION

All enzyme extraction operations were performed at 4°C. Homogenization medium consisted of 1 M Tris-HCl buffer (pH 8.0) containing 5 mM leupeptin, 1 mM PMSF, 500 mM EDTA, 100 mM $MgCl_2$, 20% (v/v) Triton X-100, 14 μM β-mercaptoethanol and 30% (w/v) insoluble PVP. All chemicals, enzymes and other reagents were of analytical grade or purest quality, purchased from Sigma, Merck and Aldrich. The procedure for enzyme extraction from leaf of 1616C rootstock and Razaki cv. followed several steps:

(1) Grinding of 5–10 fresh samples to fine powder in liquid nitrogen;

(2) Homogenization in 15–30 cm^3 ice-cold homogenization medium (3 × 30 sec with 5 min cooling interval at 4,000 × g) with a PRO 200 homogenizer (U.S.A.);

(3) Straining of homogenate through three layers of miracloth;

(4) Centrifuging of filtrate at 14,000 g × 25 min with a Sigma 3K30 centrifuge (U.S.A.);

(5) Measurement of protein content in supernatant by the method of Lowry et al. (1951).

MEASUREMENT OF ENZYMATIC ANTIOXIDANTS

Catalase (CAT), glutathione peroxidase (GSPOD) and superoxide dismutase (SOD) activity and the levels of ascorbic acid and thiobarbituric acid (TBARS) reactive substances were measured as described by Yildirim and Büyükbingöl (2002). The reaction mixture for CAT (Aebi, 1987) consisted of 50 mM phosphate buffer (pH 7.0), 30 mM H_2O_2 and the test sample. The rate of decomposition of H_2O_2 was determined from the absorbance changes at 240 nm with a Shimadzu UV1601 spectrophotometer (Japan). The enzyme activity for samples is expressed as k/sec/mg protein, where k is the first-order rate constant.

GSPOD activity was assayed according to Lawrence and Burk (1976) with slight modifications. The assay mixture consisted of 50 mM phosphate buffer (pH 7.0), 0.15 mM NADPH, 1.5 U glutathione reductase (GR), 4.0 mM glutathione (GSH), 3.0 mM H_2O_2 and the test sample. The reaction was started by the

addition of H_2O_2, and the conversion of NADPH to $NADP^+$ was monitored by continuous recording of the absorbance change of the system at 340 nm. GSPOD activity was expressed as mM NADPH oxidized to $NADP^+$ per min per mg protein, using a molar extinction coefficient of 6.22×10^6 for NADPH at 340 nm.

A simple assay system for superoxide dismutase was based on the inhibitory effects of SOD on the spontaneous oxidation of quercetin (Kostyuk and Potapovich, 1989). The oxidation rate of quercetin was determined by observing the absorbance changes at 406 nm. One U is the amount of SOD required to inhibit the initial rate of quercetin oxidation by 50%. The reaction mixture for SOD consisted of 16 mM phosphate buffer (pH 9.2), 0.890 mM TEMED, 0.0890 mM EDTA, 0.3 mM quercetin and the test sample. The reaction was started by adding quercetin to the mixture; after rapid mixing, the decrease in absorbance at 406 nm was followed for 20 min.

MEASUREMENT OF NONENZYMATIC ANTIOXIDANTS

Tissue ascorbic acid (AA) content was measured in deproteinized homogenates by the method of Roe and Kuether (1967) in which the colored complex formed was measured spectrophotometrically.

Glutathione (GSH) content was determined by the procedure of Owens and Belcher (1965) with slight modifications. Freshly prepared sample was freed from proteins by mixing with an equal volume of 3% (w/v) metaphosphoric acid and 30% (w/v) NaCl solution. Deproteinized sample was centrifuged at $3{,}500 \times g$ for 10 min at 4°C. This spectrophotometric procedure measures the change in absorbance at 412 nm when glutathione reduces 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB).

Nitric oxide, as an unstable molecule, reacts with oxygen and biological molecules to form several end products (e.g., nitrite, nitrate and S-nitrosothiols) that can be determined by different methods (Braman and Hendrix, 1989; Mesaros, 1999). The most preferred method is based on the Griess reaction, easily applied in the laboratory. A leaf sample extracted as described above (0.5 ml) was incubated with nitrate reductase (EC 1.6.6.2) from *Aspergillus* sp. (50 mU/100 μl of sample) with NADPH (final concentration 80 μmol/l) diluted in 20 mmol/l Tris buffer (pH 7.6) for 30 min at room temperature for nitrate reduction (Guevara et al., 1998). The control sample was analyzed daily against an exogenous standard (sodium nitrate, 50 μmol/l). After the reduction, 5% (w/v) $ZnSO_4$ was added for deproteinization. Then this mixture was centrifuged at $5000 \times g$ for 10 min. The sample's nitrite levels were measured by the Griess reaction (Green et al., 1982). Absorbance at 540 nm was measured and compared to a standard curve constructed using known nitrite concentrations.

Substances that react with thiobarbituric acid were measured as described by Uchiyama and Mihara (1978). TBARS react with products of lipid peroxidation, mainly malondialdehyde, producing a colored compound that can be measured at 535 nm, providing indirect evidence of the process of lipid peroxidation. The reaction mixture consisted of 1% phosphoric acid 0.6% (w/v) thiobarbituric acid, and the test sample and was incubated at 95°C for 45 min. Samples were then cooled on ice, n-butanol was added and the mixture was centrifuged at $5000 \times g$ for 5 min. The n-butanol layer was used for spectrophotometric measurement at 535 nm using a molar extinction coefficient of 1.56×10^4. The results were expressed as nmol TBARS per mg protein.

The protein content of the samples was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

STATISTICAL ANALYSIS

All experiments were performed in at least 3 replicates and the values were expressed as means SD. The data were analyzed using Duncan's multiple range test; $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

EFFECT OF NaCl STRESS ON ENZYMATIC ANTIOXIDANTS

SOD activity increased significantly in leaves of 1616C rootstock and Razaki cultivar at day 7 of treatment with 3, 8 and 12 mM salt (Fig. 1). In Razaki cv. the control activity of SOD was lower than the activity recorded for the 1616C rootstock; following salt treatment, however, SOD activity increased greatly in the cultivar. Salt treatment decreased SOD activity at day 2 in 1616C rootstock leaves. SOD activity increased significantly in the samples at day 7 under all salt concentrations. Similar results were observed for Razaki cv. leaves at days 4 and 7 of treatment; SOD activity was also high at day 2 under all salt concentrations.

GSPOD activity decreased significantly in leaves of 1616C rootstock after 2 days of treatment at all

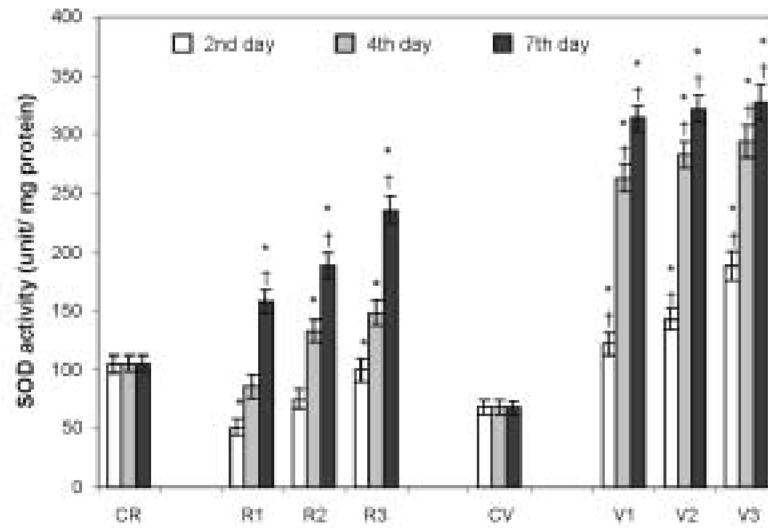

Fig. 1. Changes in superoxide dismutase activity in NaCl-treated 1616C rootstock (R) and Razaki cultivar (V). 1 – treated with 3 mM NaCl; 2 – treated with 8 mM NaCl; 3 – treated with 12 mM NaCl; CR – control rootstock; CV – control Razaki cultivar. Results are expressed as means ± SD. (n = 2–4 replicates). * – $p < 0.05$ versus respective control group. † – $p < 0.05$ versus respective day 2 group. Other details given in Materials and Methods.

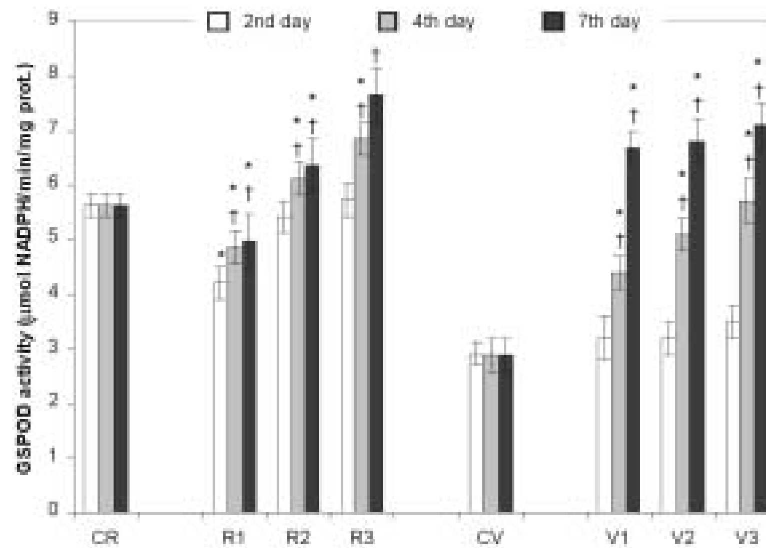

Fig. 2. Changes in glutathione peroxidase activity in NaCl-treated 1616C rootstock (R) and Razaki cultivar (V). 1 – treated with 3 mM NaCl; 2 – treated with 8 mM NaCl; 3 – treated with 12 mM NaCl; CR - control rootstock; CV – control Razaki cultivar. Results are expressed as means ± SD. (n = 2–4 replicates). * – $p < 0.05$ versus respective control group. † – $p < 0.05$ versus respective day 2 group. Other details given in Materials and Methods.

concentrations of NaCl, but then increased with the number of days of treatment (Fig. 2). Enzyme activity in Razaki cv. and 1616C rootstock leaves remained almost unchanged at day 2. In Razaki cv. under salt treatment, unlike in the control plants, GSPOD activity continued to increase sharply.

CAT activity in 1616C rootstock and Razaki cv. grown with different concentrations of salt increased

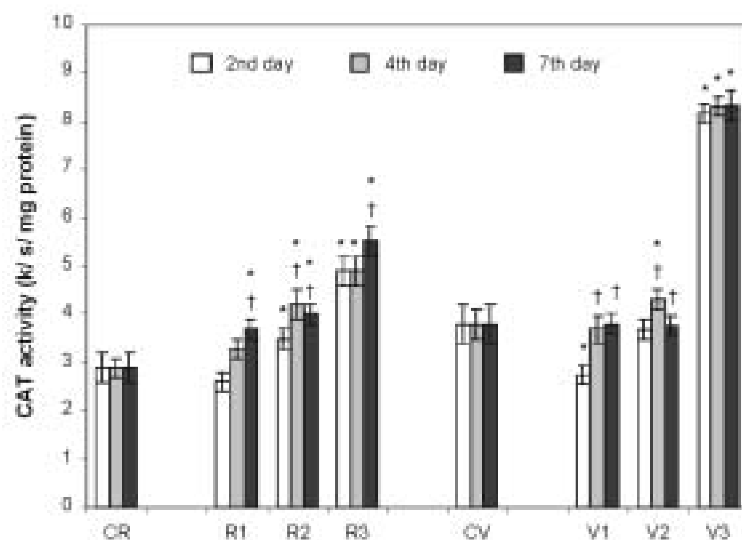

Fig. 3. Changes in catalase activity in NaCl-treated 1616C rootstock (R) and Razaki cultivar (V). 1 – treated with 3 mM NaCl; 2 – treated with 8 mM NaCl; 3 – treated with 12 mM NaCl; CR – control rootstock; CV – control Razaki cultivar. Results are expressed as means ± SD. (n = 2–4 replicates). * – $p < 0.05$ versus respective control group. † – $p < 0.05$ versus respective day 2 group. Other details given in Materials and Methods.

significantly after 4 days of treatment (Fig. 3). The rate of increase was significantly higher in Razaki cv. than in 1616C rootstock after 7 days of treatment with 12 mM salt. CAT activity in both 1616C rootstock and Razaki cv. control leaves remained almost unchanged.

EFFECT OF NaCl STRESS ON NONENZYMATIC SCAVENGER COMPONENTS

The TBARS level increased significantly in leaves of 1616C rootstock and Razaki cv. treated with 3, 8 and 12 mM NaCl (Tabs. 1, 2). In 1616C rootstock leaves, TBARS content was ~1.8 times higher than in control leaves, and in Razaki cv. it was ~2.4 times higher than in the controls after 7 days of treatment. The TBARS levels of control leaves remained almost unchanged.

Ascorbic acid (AA) levels decreased in 1616C and Razaki cv. leaves after the second day of treatment with 3 and 8 mM salt (Tabs. 1, 2). There was a significant decrease ($p < 0.05$) in the level of AA under 12 mM salt stress after 7 days of treatment in both the rootstock and cultivar. AA increased in rootstock treated with 3, 8, and 12 mM salt at day 2. In Razaki cv. the AA level increased at day 2 under the same treatments.

Nitrite levels were not changed significantly on day 2 of 3, 8 and 12 mM salt treatment in 1616C rootstock samples (Tab. 1). After 7 days of the treatment the nitrite level was significantly decreased ($p < 0.05$) in rootstock. In Razaki cv., nitrite content was unchanged at day 2 for all salt treatments (Tab. 2). In general, salt stress decreased the nitrite level in 1616C rootstock and Razaki cv.

Glutathione content increased significantly more in rootstock leaves than in Razaki cultivar treated with different concentrations of NaCl (Tabs. 1, 2). In freshly harvested samples, glutathione levels were significantly higher ($p < 0.05$) than the respective controls at the measurement intervals. Glutathione levels of the 1616C and cultivar control groups did not differ significantly ($p > 0.05$). Nor were there any significant differences in GSH levels between salt concentrations at day 7 of treatment in the rootstock and Razaki cultivar ($p > 0.05$). In 1616C rootstock leaves, GSH content was ~16, ~14.6 and ~12.5 times higher than in control leaves. In Razaki cv. it was ~12.5 times higher under treatment with 3 mM salt, and ~11.4 times higher than their respective controls under 8 and 12 mM NaCl treatment at day 7. This shows that GSH content significantly increased under salt stress; moreover, there was a negative correlation between GSH level and salt concentration and day of treatment.

TABLE 1. Changes in reduced glutathione (nmol g^{-1}fresh weight), ascorbic acid (mg g^{-1}fresh weight), nitrite (mM mg^{-1}protein) and TBARS (nmol mg^{-1} protein) levels of control and NaCl-treated 1616C Rootstock

	MATERIAL	TBARS	AA	NITRITE	GSH
2nd day	1616C Rootstock Control	9.4 ± 0.6	0.145 ± 0.005	39.6 ± 0.8	0.028 ± 0.002
	1616C Rootstock with 3 mM NaCl	9.4 ± 0.8†	0.191 ± 0.004*†	39.8 ± 1.1†	0.118 ± 0.004*†
	1616C Rootstock with 8 mM NaCl	12.4 ± 1.1*†	0.180 ± 0.005*†	40.1 ± 1.3†	0.100 ± 0.004*†
	1616C Rootstock with 12 mM NaCl	12.3 ± 0.9*†	0.170 ± 0.006*†	40.4 ± 1.2†	0.092 ± 0.005*†
4th day	1616C Rootstock Control	9.4 ± 0.7	0.145 ± 0.007	39.6 ± 0.9	0.028 ± 0.002
	1616C Rootstock with 3 mM NaCl	14.3 ± 0.9*†	0.169 ± 0.005*	28.1 ± 1.2*†	0.093 ± 0.004*†
	1616C Rootstock with 8 mM NaCl	14.1 ± 1.0*†	0.164 ± 0.005*†	29.1 ± 1.3*	0.076 ± 0.004*†
	1616C Rootstock with 12 mM NaCl	13.3 ± 1.0*†	0.143 ± 0.006*†	30.5 ± 1.3*	0.065 ± 0.005*†
7th day	1616C Rootstock Control	9.4 ± 0.7	0.145 ± 0.005	39.6 ± 0.9	0.028 ± 0.002
	1616C Rootstock with 3 mM NaCl	15.5 ± 1.0*	0.161 ± 0.005*	24.2 ± 1.4*	0.045 ± 0.003*
	1616C Rootstock with 8 mM NaCl	16.6 ± 1.1*	0.135 ± 0.005	28.4 ± 1.4	0.041 ± 0.005*
	1616C Rootstock with 12 mM NaCl	18.4 ± 0.9*	0.104 ± 0.006*	29.1 ± 1.4*	0.035 ± 0.005*

Results are means ± SD. (n = 2 to 4 independent experiments). p values are shown as *$p < 0.05$ versus respective control groups. †$p < 0.05$ versus respective seventh day. Other details are given in Materials and Methods.

TABLE 2. Changes in reduced glutathione (nmol g^{-1}fresh weight), ascorbic acid (mg g^{-1}fresh weight), nitrite (mM mg^{-1}protein) and TBARS (nmol mg^{-1} protein) levels of control and NaCl-treated Razaki cultivar

	MATERIAL	TBARS	AA	NITRITE	GSH
2nd day	Razaki cultivars Control	6.6 ± 0.6	0.187 ± 0.006	42.7 ± 0.8	0.029 ± 0.002
	Razaki cultivars with 3 mM NaCl	9.81 ± 0.8*†	0.203 ± 0.004*†	40.0 ± 1.2*	0.071 ± 0.004*†
	Razaki cultivars with 8 mM NaCl	9.37 ± 0.7*†	0.202 ± 0.005*†	40.1 ± 1.3*	0.068 ± 0.003*†
	Razaki cultivars with 12 mM NaCl	11.37 ± 0.9*†	0.195 ± 0.005*†	41.6 ± 1.4	0.067 ± 0.003*†
4th day	Razaki cultivars Control	6.6 ± 0.7	0.187 ± 0.007	42.7 ± 0.7	0.029 ± 0.003
	Razaki cultivars with 3 mM NaCl	9.59 ± 0.6*†	0.183 ± 0.005	42.2 ± 1.2	0.057 ± 0.006*†
	Razaki cultivars with 8 mM NaCl	14.22 ± 1.0*†	0.174 ± 0.006*†	40.2 ± 1.3*†	0.051 ± 0.005*†
	Razaki cultivars with 12 mM NaCl	14.10 ± 1.0*†	0.164 ± 0.005*†	38.0 ± 1.4*†	0.044 ± 0.005*†
7th day	Razaki cultivars Control	6.6 ± 0.7	0.187 ± 0.006	42.7 ± 0.8	0.029 ± 0.003
	Razaki cultivars with 3 mM NaCl	15.20 ± 0.7*	0.180 ± 0.004	41.8 ± 1.3	0.035 ± 0.005*
	Razaki cultivars with 8 mM NaCl	15.30 ± 1.1*	0.159 ± 0.005*	38.2 ± 1.4*	0.032 ± 0.005
	Razaki cultivars with 12 mM NaCl	17.24 ± 0.9*	0.076 ± 0.006*	34.7 ± 1.4*	0.032 ± 0.005

Results are means ± SD. (n = 2 to 4 independent experiments). p values are shown as *$p < 0.05$ versus respective control groups. †$p < 0.05$ versus respective seventh day. Other details in Materials and Methods.

In the present study, significant increases were recorded in the activity of GSPOD and CAT, and in the levels of TBARS and GSH in leaves of both 1616C rootstock and Razaki cv. treated with 12 mM NaCl at day 7. The activity of SOD also increased under salt stress at day 2, but only in Razaki cv. did SOD activity remain high at day 7. Cells can adapt under oxidative stress, and there is now a thriving area of research investigating the mechanism by which oxidative stress alters gene expression. For example, it has been reported that the mRNA level of the *cat1* gene for glyoxysomal catalase increased during senescence of pumpkin cotyledons, but that the mRNA of the *cat2* gene for peroxisomal catalase disappeared during senescence (Esaka et al., 1997). If oxidative stress is too intense or prolonged, oxidative damage to all cell targets (DNA, lipids, proteins) can be aggravated. If the oxidative stress is particularly severe, it can produce cell death (Halliwell, 1997).

Increased SOD activity could be due to its induction by increased superoxide anion production. The O_2^- radical can be generated by plasma membrane NADPH oxidase, and it can then form H_2O_2 by SOD-mediated dismutation (Ogawa et al., 1997). GSPOD is one of the defense enzymes that act on peroxides to remove them. The results displayed an increase in CAT activity after 7 days of 12 mM NaCl treatment. The increase at day 7 suggests a response to oxidative stress due to an increase in endogenous

H_2O_2 production. In nature, catalase activity is too low to detect in grape (Okuda and Yokotsuka, 1999). In the present investigation, CAT activity was increased by salt treatment but remained very low. The increase in the activity of CAT and GSPOD in NaCl-treated samples may indicate an adaptive response to changing conditions in the environment or else a compensatory mechanism developed to deal with increased generation of free radicals. Shull et al. (1991) reported that GSPOD mRNA increased at higher concentrations of H_2O_2. An abundant supply of NADPH from activation of the pentose phosphate pathway (Simmonds and Simpson, 1972) may be related to the increased activity of GSPOD, especially in Razaki cv. leaves, under salt stress.

The results show that TBARS levels increased in leaves at days 2, 4 and 7 under different salt concentrations, suggesting an increase in ROS with time and with the concentration of NaCl. H_2O_2 can react with O_2^- to form hydroxyl radicals, resulting in increased lipid peroxidation and hence higher TBARS levels. Increased membrane permeability and lipid peroxidation was reported in senescent tobacco leaves (Dhindsa et al., 1981). Ascorbic acid biosynthesis and peroxidase activity are suggested to be induced by light, and the flavonoid-redox cycle may be an alternative H_2O_2 detoxification system in grapevine leaves (Perez et al., 2002). In the present study, the increased level of ascorbic acid after 7 days of treatment may be an effect of this alternative defense system. Treatment of rootstock and cultivar with 12 mM NaCl for 7 days resulted in 72% and 41% oxidation of AA. Glutathione plays an important role in protecting plants from oxidative and environmental stresses. The elevated GSH levels we observed mainly at day 2 of NaCl treatment at all concentrations could be a mechanism to protect leaves by preventing oxidation of sulfhydryl groups. This idea is supported by several reports showing that elevated levels of GSH are associated with increased oxidative stress tolerance (Alscher al., 1997).

In this study we found that oxidative stress occurred during short-term NaCl stress. A lethal level of ROS causes severe damage to the cell, whereas a moderate level of ROS mobilizes defenses against salt stress, as displayed in these results.

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