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High Salt Induced Oxidative Damage and Antioxidant Response Differs in *Nicotiana tabacum* L. and *Nicotiana rustica* L. Cultivars

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Abstract

We investigated effects of 10-day irrigation with 0.2M, 0.4M, and 0.6M NaCl on two tobacco variants cultivated in Turkey; Samsun (*Nicotiana tabacum*) and Hasankeyf (*Nicotiana rustica*). Salt treatments caused significant reduction in length, leafing, dry, and fresh weights together with a significant decline in relative water contents. Pigment levels decreased together with alterations in chlorophyll a/b and chlorophyll/carotenoid ratios. Anthocyanin levels and proline amount in roots increased. Malondialdehyde (MDA) and conductivity results indicated 0.6M NaCl induced damage in roots and leaves of Samsun. Since ascorbate peroxidase (APX) activity was higher in comparison to catalase (CAT), and increased whereas CAT activity decreased in response to salt treatment, APX seems to be more important in tobacco. Leaves and roots of cv. Samsun seems to be more effected by salt than Hasankeyf, whereas roots of Hasankeyf seems to have better adaptive responses against salt stress.

Keywords: Tobacco; NaCl stress; lipid peroxidation; antioxidant enzyme activity

INTRODUCTION

High salt concentration is a worldwide problem causing decrease in crop yields. One of the major limitations on agricultural development in many countries is the high salinity of the groundwater used in irrigation. In addition, extensive areas of irrigated land have been and are increasingly becoming degraded by salinization and water logging resulting from over-irrigation and other forms of poor agricultural management [1]. Salinity affects approximately 20% of the world's cultivated land and nearly half of all irrigated land [2]. According to Food and Agriculture Organization of the United Nations (FAO) an estimated 1,500,000 ha of arable land suffers from yield limitations because of salt and boron problems in Turkey.

Salinity inhibition of plant growth is the result of low osmotic potential of soil (water stress), nutritional imbalance, specific ion effect (salt stress), or a combination of these factors [3]. Plant species have developed different mechanisms to cope with these effects. The reduction of cellular osmotic potential by solute accumulation is one of the primary responses of plants for osmotic adjustment to be prevented from deleterious effects of salinity. This can be accompanied via accumulation of inorganic ions (Na^+, Cl^-, and K^+) and/or compatible organic solutes (soluble carbohydrates, amino acids, proline, betaines, etc) [4]. Environmental stress causes oxidative damage in nucleic acid, protein and lipid molecules of plant tissues by disturbance of balance between the production of reactive oxygen species (ROS), and the quenching activity of the antioxidants [5]. Various antioxidant molecules and enzymes contribute to antioxidant defense mechanism of plants. Carotenoids, ascorbate, glutathione, and tocopherols are the primary antioxidant components. Some enzymes of the antioxidant defense of plants include superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidases, and the enzymes of the ascorbate-glutathione cycle, ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.15.1.1) [6].

The genus *Nicotiana* consists of 64 species. Two of them, namely *N.tabacum* and *N. rustica* are commercially cultivated around the world. Many cultivated varieties of tobacco, *N.tabacum* and *N. rustica*, exist depending on the location and climatic condition. A large number of tobacco

varieties have been developed for different end uses through pure line selection from local land-races, mutation breeding, and hybridization involving local selections and exotic introductions followed by pedigree selection [7]. According to FAO, Turkey's tobacco production constitutes 4% of global production of 7,000,000 tones, placing Turkey the fifth in tobacco production. Despite the importance of increasing soil salinity in the cultivated areas and economic importance of tobacco plants, effects of salt on these plants have had little attention. One of the reasons for lack of data on the issue is that soil salinity has been considered a minor problem in tobacco growing regions [8]. However, soil salinity has been a growing concern in these areas [9]. When tobacco plants were grown in salt-affected soil and irrigated with saline waters, yield and quality of tobacco leaves were demonstrated to decrease under greenhouse conditions [10] and tobacco plants have been defined as an intermediate salt tolerant crop [9]. The aim of the present study was to investigate effects of 10-day saline irrigation (0.2M, 0.4M and 0.6M) on physiological parameters, pigment amount, proline levels, lipid peroxidation, and CAT and APX activities of two tobacco varieties cultivated in Turkey; Samsun (*Nicotiana tabacum* L.) and Hasankeyf (*Nicotiana rustica* L.).

MATERIALS AND METHODS

Growth conditions and salt treatment

Two locally cultivated variants of tobacco; Samsun (*Nicotiana tabacum* L.) and Hasankeyf (*Nicotiana rustica* L.) were used in experiments. Seeds were sown into cell plants containing seedling substrate and grown for 50-55 days in defined greenhouse conditions (at 25-28ºC with a relative humidity of 60-65%). Plants were transferred to soil in individual pots containing an animal based soil fertilizer (soil:fertilizer; 2:1) and grown for another 20-25 days and 10-15 cm plants with 6-8 leaves were chosen for salt treatments. For salt treatment groups, 0.2M, 0.4M and 0.6M NaCl solution was added to cups under pots in every 2 days for 10 days. Control groups were irrigated with distilled water in same time intervals. At least two experimental setups contained at least four plants and two samplings for leaf and root tissues were performed for analysis of each plant.

Assessment of physical development under salt stress

After 10 days of irrigation, for the evaluation of physical development, length, leaf number, fresh, dry and turgor weights of plants were assessed. For the assessment of increase in length and leaf number prior to salt treatment plant length and leaf number were noted. At the end salt treatment, length and leaf number of plants were measured and alterations in length and leaf number were expressed as percent change according to initial measurements. For weight analysis plants were washed with distilled water and plant weight was measured (WW). Then, plants were floated in distilled water for 24 hours and turgid plant weight was measured (TW). Plants were dried at 60°C for 2 days and dry weights were measured (DW). Relative water content (RWC) was also calculated according to formula [11]:

$$RWC\ (\%) = [(WW-DW)\ .(TW-DW)^{-1}] \times 100$$

Determination of pigment amount

For the determination of chlorophyll a and b, total chlorophyll (a+b) and total carotenoid (xanthophylls and carotenes; x+c), small pieces of leaves (0.04 g) were put in pure acetone and incubated at 4°C for 4 days. Absorbance was measured at 470, 644.8 and 661.6 nm, and concentration of a and b, x+c in leaves were calculated according to Lichtenthaler and Buschmann [12]. Anthocyanin content of leaves and roots were determined by crashing tissue samples in 1 mL of 79% (v/v) methyl alcohol and 1% HCl (v/v) solution. Samples were incubated at 4°C for 4 days and absorbance of the sample solution was measured at 530 and 657 nm ($\varepsilon \approx 34{,}300\ M^{-1}.cm^{-1}$) [13,14].

Determination of proline levels

Proline amount in leaves and roots were determined according to modified method [11] described by Bates *et al*. [15]. In brief, samples (0.2 g) were homogenized with mortar and pestle in liquid nitrogen, and the homogenates were suspended in 3% (v/v) sulphosalicylic acid. Samples were centrifuged at 18500 g for 10 min, at 4ºC and 0.1 mL of supernatant was transferred into a solution of 0.2 mL acid ninhydrin, 0.2 mL 96% (v/v) acetic acid and 0.1 mL 3% (v/v) sulphosalicylic acid. After a 96ºC, 1 h incubation, 1 mL of toluene was added and samples were centrifuged at 18500 g, 5 min. Upper phase was transferred into quartz cuvvettes and absorbance was recorded at 520 nm against toluene. Proline amount was calculated by using proline standard curve.

Determination of lipid peroxidation

Lipid peroxidation was assayed by determining malondialdehyde (MDA) amount, a product of lipid peroxidation [16]. In brief, 0.2 g leaf and root samples were homogenized with mortar and pestle in liquid nitrogen, and the homogenates were suspended in 5% (w/v) tricholoro acetic acid (TCA). Samples were centrifuged at 13500 g for 15 min and supernatant was mixed with 1:1 volume of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA. Following a 96ºC, 25 min incubation, samples were cooled to room temperature, and centrifuged at 9400 g for 5 min. Absorbance of the supernatant was recorded at 532 and 600 nm against TBA in TCA. After subtracting the non-specific absorbance at 600 nm, MDA concentration was calculated by its extinction coefficient of 155 $mM^{-1}.cm^{-1}$.

Electrolyte leakage test

Electrolyte leakage was determined by measurement of electrolyte leaked from leaves and roots according to method described by Nanjo *et al*. [17]. For the measurement of the conductance of leaves, 2 leaves per plant and for the measurement of the conductance of roots total root tissue were immersed into 0.4M mannitol solution and incubated for 3 h at room temperature. Electrical conductance (C1) was measured by Adwa ECO 401 EC Meter. After boiling of sample for 15 min, electrical conductance (C2) was measured again for the determination of total ion concentration by complete membrane disintegration. Percent ion leakage was calculated according to formula $(C1.C2^{-1})*100$.

Determination of APX and CAT activities

For determination of APX activity [18], samples (0.5 g) were homogenized with mortar and pestle in liquid nitrogen,

and the homogenates were suspended in 50 mM Tris-HCl buffer containing 2% (w/v) polyvinylpoly pyrolidone, 2mM ascorbate and 1mM EDTA. After centrifugation at 13500 g for 20 min, at 4ºC, total protein amount in supernatants were determined according to Bradford method [19]. 100 µg of total protein was added to assay solution (50 mM potassium phosphate buffer (pH 6.6) with 2.5 mM ascorbate) and reaction was initiated by addition of 10 mM H_2O_2. Decrease in the absorbance of ascorbate was recorded at 290 nm for 3 min against assay solution ($\varepsilon = 2.8\ mM^{-1}.cm^{-1}$).

For determination of CAT activity [20], samples (0.5 g) were homogenized with mortar and pestle in liquid nitrogen, and the homogenates were suspended in suspension buffer. After centrifugation at 13500 g for 20 min, at 4ºC, total protein amount in supernatants were determined according to Bradford method. 100 µg of total protein was added to 50 mM potassium phosphate buffer (pH 7.0) and reaction was initiated by addition of 100 mM H_2O_2. Decrease in the absorbance H_2O_2 of was recorded at 240 nm for 3 min against assay solution ($\varepsilon = 39.4\ mM^{-1}.cm^{-1}$).

Statistical analysis

All data are expressed as mean ± standard error of the means (SEM). Mean difference between control and salt treated groups of two cultivars were statistically evaluated by one-way ANOVA analysis at the 0.05 level and post hoc Tukey analysis were carried out to find groups whose mean differences were significant. In addition, differences between two tobacco cultivars in treatments were statistically evaluated by independent-samples t-test at the 0.05 level.

RESULTS

Physiological changes under salt stress

Salt treatment caused reduction in physiological parameters of both tobacco cultivars (Fig. 1, 2 and 3). According to figure 2, 0.4 and 0.6M salt treated plants had significantly decreased stem length and leaf number when compared to untreated controls. In addition, fresh and dry weights of plants in three salt treatment groups significantly reduced causing a decrease in relative water content of the plants as well (Fig. 3). WW, DW and RWC values gradually decreased with increasing salt concentrations. In 0.2M treatment group, WW values (11.38±0.59 g and 6.7±0.21 g for Hasankeyf and Samsun, respectively) reduced nearly to half values of that of the untreated group (19.6±1.84 g and 12.33±0.7 g for Hasankeyf and Samsun, respectively) in both cultivars while decrease in DW values were relatively less significant. This decline seemed to be correlated to water loss in these plants which was also correlated to reduction in RWC values.

Effect of salt treatment on pigment amount

(Table 1) summarizes the effects of salt treatment on chlorophyll, carotenoid, and anthocyanin levels of plants. Chlorophyll a and b content in leaves of 0.6M salt treated plants (a: 123.83±23.73 and 380.53±45.94 $\mu g.g^{-1}FW$; b: 73.69±9.74 and 182.79±26.06 $\mu g.g^{-1}FW$ for cv. Hasankeyf and Samsun, respectively) significantly decreased when compared to control group (a: 278.01±212.87 and 566.4±8.59 $\mu g.g^{-1}FW$; b: 137.83±5.58 and 373.84±22.7 $\mu g.g^{-1}FW$ for Hasankeyf and Samsun, respectively) also causing a significant reduction in total chlorophyll content. Chlorophyll a and b, and total chlorophyll content of leaves of Samsun were significantly higher than that of Hasankeyf in all treatment and control groups. Chlorophyll a to b ratio increased in leaves of Samsun due to 0.6M salt treatment. In concordance, this ratio was relatively higher in leaves of 0.6M salt treated cv. Samsun ($p<0.024$), in contrast to its relatively low level than value of cv. Hasankeyf in the control group ($p<0.036$). Total carotenoid amount decreased in leaves of 0.6M salt treated Hasankeyf plants while remained unchanged in cv. Samsun leaves. Total carotenoid amounts in leaves of Hasankeyf were lower than Samsun in both control and 0.6M treatment groups ($p<0.03$ and $p<0.025$, respectively). Total chlorophyll to carotenoid ratio in leaves of cv. Samsun significantly decreased in 0.6M treatment group whereas the levels remained unchanged in leaves of cv. Hasankeyf. The ratios were higher in control, 0.2M, and 0.4M treatment groups of cv. Samsun when compared to ratios in cv. Hasankeyf leaves. Anthocyanin levels in leaves of both cultivars significantly increased in 0.6M salt treatment groups (178.3±21.31 and 282.52±22.69 $nmole.g^{-1}FW$; for cv. Hasankeyf and Samsun, respectively) when compared to untreatment group (49.19±11.6 and 75.07±13.23 $nmole.g^{-1}FW$; for cv. Hasankeyf and Samsun, respectively). Similarly, anthocyanin content of the 0.6M salt treated roots of cv. Hasankeyf and Samsun (325.29±17.00 and 384.34±21.57 $nmole.g^{-1}FW$, respectively) significantly increased when compared to root content of untreated plants (275.62±13.09 and 236.66±2.91 $nmole.g^{-1}FW$; for Hasankeyf and Samsun, respectively).

Effect of salt treatment on proline amount

Proline levels in leaves and roots of salt treated plants were higher when compared to control (Fig. 4). Proline amounts were relatively higher in roots than leaves. 0.4M salt treatment caused 3.4 and 5.8 fold significant increase in roots of cv.Hasankeyf. Similarly, proline levels significantly increased in roots of salt treated Samsun plants (4, 4.2 and 7.4 folds for 0.2, 0.4 and 0.6M NaCl, respectively). Roots of 0.6M salt treated Hasankeyf plants had significantly higher amounts of proline when compared to that of Samsun. Leaves of untreated Hasankeyf plants had significantly higher proline than leaves of untreated Samsun plants (3.7 fold). However, proline amount in Samsun leaves significantly increased due to 0.4 and 0.6M salt treatment (6.9 and 12 fold, respectively) whereas there was not any significant change in leaves of Hasankeyf plants. Concordantly, proline amount in leaves of 0.6M salt treated Samsun plants was significantly higher than Hasankeyf leaves.

Effect of salt treatment on MDA levels

According to MDA levels (Fig. 5), lipid peroxidation significantly increased in roots of cv.Samsun (1.6 fold) due to 0.6M salt treatment. Similarly, 0.6M salt treatment caused a significant increase (1.6 fold) in MDA amount of leaves of cv.Samsun. Lipid peroxidation was higher in leaves of two cultivars when compared to roots in all treatment groups. MDA levels in roots of cv. Hasankeyf were significantly lower in control and all treatment groups when compared to that of cv. Samsun which indicates higher oxidative damage root membranes of cv. Samsun.

Effect of salt treatment on leaf and root membrane permeability

According to electrolyte leakage test (Fig. 6), 0.6M salt treatment caused 2 fold increases in root membrane permeability of Samsun cultivar when compared to control. On the other hand, salt treatment did not affect leaf membrane permeability of tobacco cultivars.

Effect of salt treatment on enzyme activities

According to Table 2, APX activity was higher in roots of Hasankeyf plants than leaves. There was not any increase in leaf APX activity of cv. Hasankeyf due to salt treatment. However, 0.4M and 0.6M salt treatment caused significant increase in APX activity of roots (1.3 and 1.7 fold, respectively). Leaves of cv.Samsun had significantly higher APX activity in control and treatment groups. Conversely, APX activity was significantly lower in cv. Samsun roots when compared to cv. Hasankeyf in control and treatment groups. In leaves and roots of cv. Samsun APX activity significantly increased due to 0.6M salt treatment (2.3 and 2.5 fold, respectively).

CAT activity was higher in leaves than roots of both cultivars (Table 2). There was not any significant alteration CAT activity levels of leaves of cv. Hasankeyf whereas there was a significant decline in leaves of cv. Samsun. On the other hand, it decreased in roots of cv. Hasankeyf due to 0.6M salt treatment (0.4 fold). CAT activity in roots of untreated cv. Hasankeyf was significantly higher than cv.Samsun.

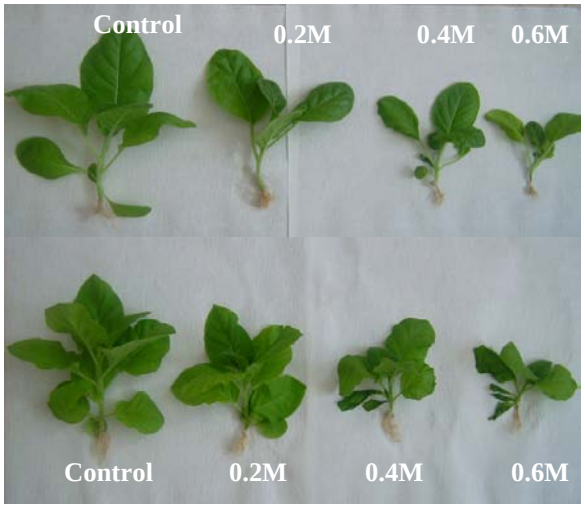

Fig. 1 Representative photographs of salt treated a) *N. rustica* (Hasankeyf), and b) *N. tabacum* (Samsun).

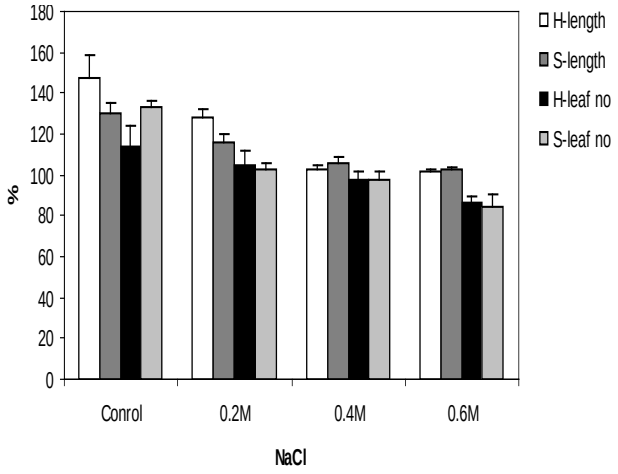

Fig. 2 Effect of NaCl treatment on growth of tobacco (H; Hasankeyf, S; Samsun). a, b, c and d represent significant difference between control and treatment groups (a: $p<0.001$; b: $p<0.001$; c: $p<0.001$; d: $p<0.001$) according to one-way ANOVA analysis.

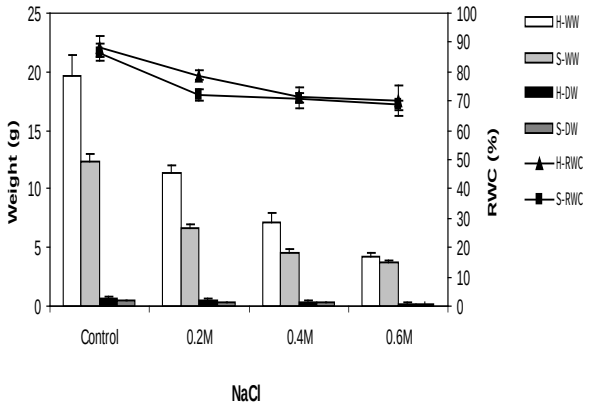

Fig. 3 Effect of NaCl treatment on fresh weight (WW), dry weight (DW) and relative water content (RWC) of two tobacco cultivars (H; Hasankeyf, S; Samsun). a, b, c, and d represent significant difference between control and treatment groups (a: $p<0.001$; b: $p<0.001$; c: $p<0.001$; d: $p<0.001$; * $p<0.014$; ** $p<0.001$) according to one-way ANOVA analysis.

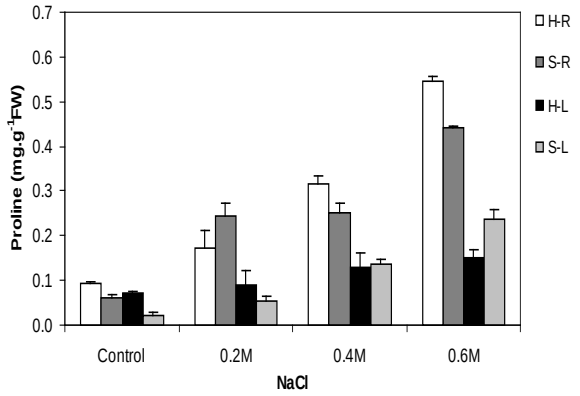

Fig. 4 Effect of NaCl treatment on proline levels. a, b and c represent significant difference between control and treatment groups (a: $p<0.001$; b: $p<0.001$; c: $p<0.005$) according to one-way ANOVA analysis; p values represent significant differences among tobacco cultivars according to independent-samples t-test.

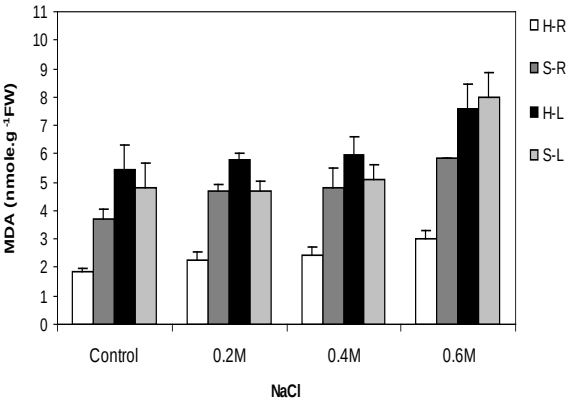

Fig. 5 Effect of NaCl treatment on MDA levels. R and L represent root and leaf, respectively; a and b represent significant difference between control and treatment groups (a: $p<0.035$; b: $p<0.025$) according to one-way ANOVA analysis; p values represent significant differences among tobacco cultivars according to independent-samples t-test.

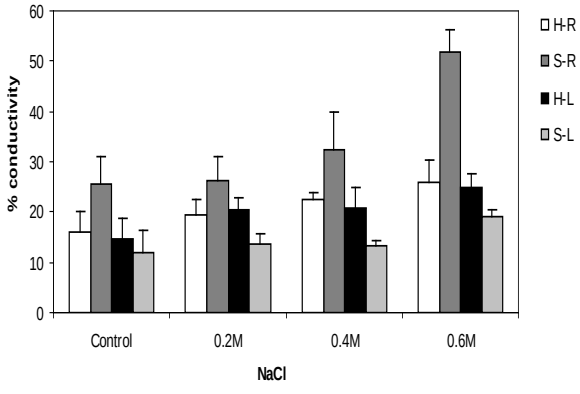

Fig. 6 Effect of NaCl treatment on membrane permeability. a and b represent significant difference between control and treatment groups ($p<0.001$) according to one-way ANOVA analysis.

DISCUSSION

Salinity, due to either salt content of soil or saline water irrigation, decreases the crop quality and yield. At physiological level, salt stress adversely affects various growth and development parameters of plants. At cellular level, salt stress enhances ROS production, which in turn results in alterations in antioxidant defense of plants. The extent of these physiological and biochemical changes may vary depending on the salt tolerance/sensitivity of the cultivars.

In the present study, two variants of tobacco locally cultivated in Turkey, Hasankeyf and Samsun, were studied for their physiological and biochemical responses under increasing NaCl stress. Stem lengths and leafing of plants (Fig. 1 and 2) demonstrated that 0.4M and 0.6M salt treatment caused a significant decline in growth of tobacco plants. Similarly, a gradual decline in weight and dry weights of plants were observed in correlation to increasing salt concentration (Fig. 3). Concordantly, relative water content of the salt treated plants decreased which explains more dramatic reduction in WW than DW values. Increased osmotic pressure caused significant water loss in plant tissues. Values obtained from two cultivars for the above parameters did not significantly differ.

Analysis of the chlorophyll content of the leaves demonstrated that high salt concentration caused reduction in chlorophyll a, b and total chlorophyll amount in leaves of two cultivars (Table 1). Previous studies also demonstrated that salinity could affect chlorophyll concentration of leaves through inhibition of synthesis of chlorophyll or an acceleration of its degradation [21]. In addition to chlorophyll content, a significant decrease of photosynthetic quantum and net CO_2 uptake yield were also observed with two sorghum varieties at high salinity (200mM<) in previous studies [22]. The weight ratio of chlorophylls a and b to total carotenoids is an indicator of the greenness of plants. Lower values for the ratio (a+b)/(x+c) are an indicator of senescence, stress, and damage to the plant and the photosynthetic apparatus, which is expressed by a faster breakdown of chlorophylls than carotenoids [12]. Reduction in the ratio was observed in leaves of 0.6M salt treated Samsun plants.

Anthocyanin biosynthesis may vary among different developmental stages and may be induced by environmental factors such as visible and UVB radiation, cold temperatures and water stress. For example anthocyanin accumulation has been shown to be induced by sucrose [23]. The subsequent production and localization of anthocyanins in root, stem and leaf tissues may allow the plant to develop resistance to a number of environmental stresses [reviewed in 24]. The accumulation of water soluble solutes in plant tissues helps plants to maintain its turgor in high levels of external ion concentrations and uptake water which is the basis of osmoprotection in salt tolerant plants. In concordance to previous studies, anthocyanin accumulation in leaf and root of tobacco plants seems to be an osmoprotective response against water stress exerted by high salt concentration.

Table 1. Effect of NaCl treatment on chlorophyll a and b, total chlorophyll (a+b), a/b ratio, total carotenoid (x+c), total chlorophyll to carotenoid ratio (a+b / x+c) and anthocyanin levels of tobacco cultivars (H; Hasankeyf, S; Samsun)

	NaCl							
	Control		0.2M		0.4M		0.6M	
cv.	**H**	**S**	**H**	**S**	**H**	**S**	**H**	**S**
Leaf								
a (µg.g^{-1}FW±SEM)	278.01 ±12.87$^{a\ 1}$	566.40 ±8.59$^{g\ 1}$	272.73 ±15.79^{7}	568.63 ±9.74^{7}	274.72 ±11.28^{11}	556.15 ±20.24^{11}	123.83 ±23.73$^{a\ 16}$	380.53 ±45.94$^{g\ 16}$
b (µg.g^{-1}FW±SEM)	137.83 ±5.58$^{b\ 2}$	373.84 ±22.70$^{h\ 2}$	138.84 ±4.11^{8}	363.95 ±10.10^{8}	136.08 ±14.46^{12}	313.86 ±23.02^{12}	73.69 ±9.74$^{b\ 17}$	182.79 ±26.06$^{h\ 17}$
a+ b (µg.g^{-1}FW±SEM)	412.30 ±13.04$^{c\ 3}$	962.01 ±17.68^{gi3}	411.57 ±19.89^{9}	932.59 ±0.37^{9}	415.19 ±6.94^{13}	759.76 ±19.29^{13}	197.52 ±33.44$^{c\ 18}$	563.33 ±71.72^{gi18}
a/b (Ratio±SEM)	2.08 ±0.14^{4}	1.43 ±0.01$^{j\ 4}$	1.92 ±0.06	1.57 ±0.07	1.92 ±0.28	1.78 ±0.09	1.65 ±0.11^{19}	2.10 ±0.06$^{j\ 19}$
x+c (µg.g^{-1}FW±SEM)	95.98 ±3.32$^{d\ 5}$	124.61 ±6.02^{5}	92.42 ±10.59	126.67 ±9.10	81.42 ±3.42^{14}	129.49 ±3.52^{14}	60.0 ±14.37$^{d\ 20}$	117.77 ±7.81^{20}
(a+b)/(x+c) (Ratio±SEM)	4.30 ±0.01^{6}	8.33 ±0.40^{k6}	4.12 ±0.52^{10}	7.87 ±0.50^{10}	3.89 ±0.5^{15}	5.73 ±0.28^{15}	3.49 ±0.68	4.76 ±0.45^{k}
Anthocyanin (nmole.g^{-1}FW±SEM)	46.19 ±11.60^{e}	75.07 ±13.23^{l}	103.52 ±27.38	126.89 ±10.83	128.95 ±14.30	208.77 ±50.56	178.30 ±21.31^{e}	282.52 ±33.69^{l}
Root								
Anthocyanin (nmole.g^{-1}FW±SEM)	275.62 ±13.09^{f}	236.66 ±2.91^{m}	289.48 ±6.46	280.33 ±26.16	319.86 ±35.21	362.29 ±15.32	325.29 ±17.00^{f}	384.34 ±21.57^{m}

Superscript letters represent significant difference between control and treatment groups according to one-way ANOVA analysis; a (p<0.001), b (p<0.002), c (p<0.001), d, (p<0.048), e (p<0.014), f (p<0.020), g (p<0.012), h (p<0.002), i (p<0.003), j (p<0.004), k (p<0.004), l (p<0.008), m (p<0.018).Superscript numbers represent significant difference in between tobacco cultivars among treatments according to independent-samples t-test; 1 (p<0.001), 2 (p<0.001), 3 (p<0.001), 4 (p<0.036), 5 (p<0.030), 6 (p<0.002), 7 (p<0.001), 8 (p<0.001), 9 (p<0.001), 10 (p<0.019), 11 (p<0.001), 12 (p<0.004), 13 (p<0.001), 14 (p<0.001), 15 (p<0.032), 16 (p<0.008), 17 (p<0.017), 18 (p<0.010), 19 (p<0.024), 20 (p<0.025).

Table 2. Effect of NaCl treatment on ascorbate peroxidase (APX) and catalase (CAT) activities of tobacco cultivars (H; Hasankeyf, S; Samsun)

		APX activity (µmol ASC.min^{-1}.mg^{-1} protein ± SEM)		**CAT activity (nmol H_2O_2.min^{-1}.mg^{-1} protein ± SEM)**	
NaCl	**cv.**	**Leaf**	**Root**	**Leaf**	**Root**
Control	**H**	2.55±0.07^{1}	16.25±1.12$^{b\ 5}$	18.81±1.60^{9}	763.63±90.52$^{e\ 10}$
	S	6.82±1.61$^{a\ 1}$	6.31±1.12$^{c\ 5}$	38.33±0.97$^{d\ 9}$	467.10±84.33^{10}
0.2M	**H**	2.93±0.34^{2}	16.61±0.65^{6}	15.02±4.23	338.61±54.75
	S	13.51±0.05^{2}	9.64±1.97^{6}	10.50±1.07	379.93±99.31
0.4M	**H**	3.76±0.74^{3}	21.70±1.28$^{b\ 7}$	14.26±2.04	515.25±106.91
	S	13.79±0.13^{3}	13.83±2.45^{7}	9.36±2.03	361.82±85.52
0.6M	**H**	5.79±0.94^{4}	26.92±2.21$^{b\ 8}$	15.29±1.08	304.35±70.42^{e}
	S	15.81±2.06$^{a\ 4}$	15.48±2.05$^{c\ 8}$	10.00±1.6^{d}	313.71±10.27

Superscript letters represent significant difference between control and treatment groups (a: p<0.024; b: p<0.015; c: p<0.035; e: p<0.012; d: p<0.022) according to one-way ANOVA analysis.
Superscript numbers represent significant difference in between tobacco cultivars among treatments (1: p<0.006; 2: p<0.012; 3: p<0.009; 4: p<0.047; 5: p<0.013; 6: p<0.028; 7: p<0.019; 8: p<0.015; 9: p<0.014; 10: p<0.022) according to independent-samples t-test.

Cytosolic proline helps to maintain osmotic adjustment contributing to membrane stability and reducing the disruptive effect of NaCl on cell membrane as a free radical scavenger [25]. Increase in root proline amount was more than that of Samsun in 0.6M treatment group of Hasankeyf. So proline accumulation in roots may be more important osmoprotective mechanism in low salt concentrations in Samsun plants and in higher concentration in Hasankeyf. Significantly increased proline amount observed in Samsun leaves due to 0.4M salt treatment was more or less same in leaves of Hasankeyf. So, the basal level in leaves of Hasankeyf may provide sufficient protection against NaCl.

More toxic ion effects are expected in root tissue than leaves due to uptake of ions from roots. However, if the ions are transported to leaves, effects of salt become apparent in leaves as well. A low rate of Na+ and Cl- transport to leaves, and the ability to compartmentalize these ions in vacuoles to prevent their build up in cytoplasm or cell walls is one of the mechanisms of salt tolerance [26]. In terms of proline accumulation, more ions might have transported to leaves of Samsun (probably more than that of Hasankeyf) and this might have caused proline accumulation in leaves of 0.6M salt treated Samsun plants, which is also supported by increased lipid peroxidation levels in Samsun leaves in this treatment group despite the unchanged levels in Hasankeyf leaves (Fig. 5). Similarly, lipid peroxidation, which is an indication of oxidative damage, was significantly increased in roots 0.6M salt treated Samsun plants whereas levels remained unchanged in roots of Hasankeyf plants. Increased ion conductivity is an indication of disruption of membrane integrity which is the result of oxidative damage to membrane structure. In concordance with the MDA levels, conductivity in roots of cv. Samsun increased due to 0.6M salt treatment (Fig. 6). However, leaves in 0.6M salt treated group of cv. Samsun seems to have preserved membrane integrity through increased lipid peroxidation.

Since the extent of oxidative stress in a cell is determined by the amounts of superoxide, hydrogen peroxide and hydroxyl radicals, the balance of SOD, APX, and CAT, activities is important for suppression of toxic ROS levels in a cell. Apel and Hirt [27] proposed that changing the balance of scavenging enzymes would induce compensatory mechanisms, such CAT activity was reduced, and other scavenging enzymes such as APX and GPX were upregulated. Likewise, in the present study, APX activity increased while CAT activity decreased in roots of cv. Hasankeyf due to 0.6M salt treatment (Table 2). Similarly, in leaves of Samsun plants 0.6M salt treatment caused an increase in APX activity and a decrease in CAT activity. Firstly, APX has a higher affinity for H_2O_2 (µM range) than CAT (mM range). Secondly, ascorbate-glutathione cycle is found in all compartments of the cells whereas CAT is only present in peroxisomes [28]. Conclusively, APX plays a more crucial role in controlling the ROS level although, CAT is indispensible when high levels of ROS are produced [29]. In concordance, APX activity was remarkably higher than CAT activity (i.e units were expressed as µmol $ASC.min^{-1}.mg^{-1}$ protein and nmol $H_2O_2.min^{-1}.mg^{-1}$ protein, respectively) in all tobacco groups studied. Leaves of cv. Samsun had significantly higher APX activity in control and treatment groups when compared to that of cv. Hasankeyf. Conversely, APX activity was significantly lower in cv. Samsun roots when compared to cv. Hasankeyf in control and treatment groups. Results of the APX and CAT activities indicated that APX was the responsible enzyme, for salt mediated ROS in tobacco cultivars, and two cultivars had different enzyme activity levels in control and treatment groups depending on the tissue.

Leaf and root tissues of plants have different responses to salt stress. In this study, physiological and biochemical responses of two cultivated varieties of tobacco (*Nicotiana tabacum* cv. Samsun and *Nicotiana rustica* cv. Hasankeyf) to increasing NaCl concentrations were analyzed in both root and leaf tissues. Considering chlorophyll a/b ratio, proline, MDA and conductivity levels, leaves and roots of cv. Samsun seems to be more effected by salt treatments than cv. Hasankeyf whereas roots of cv. Hasankeyf seems to have better adaptive responses against salt stress as supported by anthocyanin, proline and APX levels. Determination of salt tolerance of cultivated varieties of crops enables cultivation of more salt-tolerant cultivars in more saline soils, and thereby enhancement of the crop yields. Such determinations about defense mechanisms may also lead to the development of more salt tolerant varieties by conventional breeding practices such as hybridization and grafting.

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