

## The effect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties

Özge ÇELİK, Çimen ATAK

Department of Molecular Biology and Genetics, Faculty of Science and Letters, İstanbul Kültür University,  
34156 Ataköy, İstanbul - TURKEY

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**Abstract:** The aim of this study was to compare the salinity tolerances of 2 oriental tobacco varieties (İzmir Özbaş and Akhisar 97). Salinity stress experiments were performed under both in vitro and in vivo conditions. Seedlings of each variety were subjected to 0, 50, 100, 150, 200, 250, 300, and 350 mM NaCl. Photosynthetic pigment levels, lipid peroxidation rate, total protein content, antioxidant enzyme activities, and proline concentrations were determined for seedlings treated with salt for 14 days. The Akhisar 97 variety was found to be more sensitive to salinity stress than the İzmir Özbaş variety. Although proline is thought to accumulate in salt-tolerant plants, we found a negative correlation between salinity tolerance and proline accumulation in the plants. According to biochemical analyses, there were no differences in SOD, APX, GPX, or CAT activity levels between the 2 varieties, either in vivo or in vitro. However, differences in glutathione reductase (GR) activity between control plants and plants under NaCl stress were statistically significant in both varieties, both in vitro and in vivo. Our results support the hypothesis that GR is a key element in the evaluation of salinity tolerance of tobacco varieties.

**Key words:** Salinity tolerance, *Nicotiana tabacum* L., antioxidative enzymes, proline, glutathione reductase

### Tuz stresinin iki Türk tütün çeşidinin antioksidatif enzimleri ve prolin içeriğine etkisi

**Özet:** Bu çalışmada amaç, iki Türk tütün çeşidinin (İzmir Özbaş and Akhisar 97) tuz toleranslarının karşılaştırılmasıdır. Tuz stresi deneyleri hem in vitro hem de in vivo koşullarda gerçekleştirilmiştir. Her iki çeşide ait fideler 0, 50, 100, 150, 200, 250, 300 and 350 mM NaCl'e maruz bırakılmışlardır. 14 gün süreyle tuz stresine maruz bırakılan fidelerde fotosentetik pigment miktarları, lipid peroksidasyon oranları, total protein miktarları, antioksidatif enzim aktiviteleri ve prolin konsantrasyonları saptanmıştır. Akhisar 97 çeşidi tuzluluğa İzmir Özbaş'a göre daha hassas olarak bulunmuştur. Prolinin tuz toleranslı bitkilerde biriktiğinin düşünülmesine rağmen, tuz toleransı ve bitkilerde prolin birikimi arasında negatif korelasyon bulunmuştur. Biyokimyasal analiz sonuçlarına göre, hem in vitro ve in vivo deneylerde her iki çeşide ait süperoksit dismutaz, askorbat peroksidaz, guaiakol peroksidaz ve katalaz aktivitelerinde anlamlı farklılıklar bulunmamıştır. Ancak, her iki deneme koşulunda da kontrol ve tuz stresine bırakılan bitkiler arasında glutatyon redüktaz (GR) enzim aktivitelerinde istatistiksel açıdan anlamlı farklılıklar bulunmuştur. Elde edilen bulgular, tütün çeşitlerinin tuz toleransının değerlendirilmesinde GR'nin anahtar bir enzim olduğunu göstermektedir.

**Anahtar sözcükler:** Tuz toleransı, *Nicotiana tabacum* L., antioksidatif enzimler, prolin, glutatyon redüktaz

## Introduction

Environmental stresses strongly influence plant growth and development. Salinity is one of the most important of these stresses and can limit crop yield (1). High salt stress disrupts the homeostatic balance of water potential and ion distribution within a plant. Under high salinity, sodium toxicity may cause a range of disorders affecting germination, development, photosynthesis, protein synthesis, lipid metabolism, leaf chlorosis, and senescence (2-4). Salt tolerance is the ability to survive in soils with high soluble saline contents (5).

The general response of many plants to increased salinity is to accumulate high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in their vacuoles or to distribute these ions to different parts of the plant to activate metabolic functions. In this way, the plants protect their cytoplasmic water potential (2).

Plants require biochemical and molecular strategies to survive the problem of salinity. Biochemical strategies used to enhance salt tolerance in plants include the control of ion transfer from roots to leaves, the distribution of ions into cellular compartments, the synthesis of osmotic regulators, changes in photosynthesis and cell membranes, and the induction of antioxidative enzymes and certain plant hormones (6). Other salt regulatory mechanisms of plants are salt secretion, selective accumulation, and excretion.

Oxidative mechanisms in plants include the production of reactive oxygen species (ROS) (superoxide radicals ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radicals ( $\text{OH}^\cdot$ )) (7). Under physiological steady-state conditions, there is a balance between the production and scavenging of ROS (8). However, this homeostasis can be disturbed by a number of adverse environmental factors. Plants protect themselves from oxidative damage due to ROS through both enzymatic and nonenzymatic defense mechanisms (9). Enzymatic ROS-scavenging mechanisms in plants include production of superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT), and glutathione reductase (GR). The extent of oxidative stress experienced in a cell is determined by the levels of superoxide,  $\text{H}_2\text{O}_2$ , and hydroxyl radicals. Additionally, a balance among SOD, APX, and CAT

activities is crucial for suppressing toxic ROS levels within cells (10,11).

GR activity regulates the redox potential of cells and is important for the physiological needs of cells under oxidative stress. The role of GR is to protect the cell against oxidative stress effects by maintaining a high reduced glutathione-to-oxidized glutathione (GSH/GSSG) ratio (12,13).

One way that plants adjust to high salt concentrations is by increasing their tissue osmotic potential. This results in the accumulation of inorganic and organic solutes. The cellular response to short- and long-term salt stress by plants involves the synthesis and accumulation of osmoprotective compounds (14,15), which are small nontoxic molecules. Osmotically active compounds increase the osmotic potential of the cell (16).

Proline is the major compound that protects cells by stabilizing proteins and cellular membranes (17-19). In plants, proline is synthesized via 2 pathways: glutamate and ornithine. Under osmotic stress, the glutamate pathway is the main source of proline production. Proline oxidase is the main regulatory enzyme responsible for the accumulation of osmolytes; it converts proline into glutamate. It is not clear whether proline accumulation is a stress effect or one of the causes of stress tolerance.

The aim of this study was to compare the responses of 2 oriental tobacco (*Nicotiana tabacum* L.) varieties from Turkey, cvs. Akhisar 97 and İzmir Özbaş, to salinity. We cultivated plants both in vitro and in vivo. The plant growth parameters, antioxidant enzyme activities, lipid peroxidation activity levels, and proline accumulation were determined. The salt tolerances of the 2 varieties were compared according to the results of biochemical analyses.

## Materials and methods

### Plant material

Germination experiments were carried out under laboratory conditions to evaluate differences in the salinity stress tolerance of the tobacco seeds. Akhisar 97 and İzmir Özbaş (*Nicotiana tabacum* L.) oriental tobacco seeds were supplied by the Aegean Research Institute (İzmir, Turkey).

### In vitro salt treatment

Tobacco seeds were surface sterilized for 1 min in 70% (v/v) ethanol and then soaked in 20% (v/v) commercial bleach (commercial bleach contains about 5% (v/v) sodium hypochlorite) for 10 min. Seeds were rinsed 3 times in sterile distilled water. Sterile tobacco seeds were germinated on half-strength Murashige and Skoog ( $\frac{1}{2}$  MS) medium. One-month-old seedlings were transferred to fresh medium containing 0, 50, 100, 150, 200, 250, 300, or 350 mM NaCl in  $\frac{1}{2}$  MS medium. The experiment was performed in a growth chamber at 27 °C and under a 16-h/8-h light/dark photoperiod. After 14 days, the leaves of seedlings were used to determine the activities of antioxidant enzymes. In this study, every treatment had 3 replicates, and each replicate included 10 seedlings.

### In vivo salt treatment

Tobacco seeds are very small; therefore, seeds were germinated on  $\frac{1}{2}$  MS medium for 1 month and then transferred to perlite. The seeds were irrigated with quarter-strength Hoagland solution. Salt treatments with 2-month-old seedlings grown in perlite were started by adding the same concentrations of NaCl as used in the in vitro experiment. The seedlings were grown under controlled conditions (16-h/8-h light/dark regime at 27 °C) for 14 days. The leaves were harvested and used for biochemical analyses. Every treatment had 3 replicates, and each replicate had 15 seedlings.

To evaluate the results of the in vivo salinity experiment, a 0-to-5 growth scale was used to determine the detrimental effects of NaCl on the morphology of treated seedlings under in vivo conditions. We selected 10 seedlings randomly from every treatment for each of the 2 tobacco varieties and scored the seedlings based on their degree of defects (20). The main criteria used for scoring the plants and the scale points are given below.

- 0: No effect of NaCl on the plant
- 1: Decreased growth, local chlorosis, and breakage of the leaves
- 2: Chlorosis in the leaves and 25% necrotic sites
- 3: 25%-50% necrotic sites in the leaves and patching off

4: 50%-75% necrotic sites and dead tissue in the leaves

5: 75%-100% necrotic sites or plant death

### Chlorophyll and carotenoid content determination

Pigments were extracted from the leaves of seedlings treated with salt for 14 days. The extraction of leaf pigments was performed with 80% acetone, and the absorbance at 663 and 645 nm was measured with an Amersham spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). The chlorophyll a, chlorophyll b, and total chlorophyll quantities were calculated according to the method of Arnon (21). Total carotenoid content was measured at 470 nm. The pigment concentrations were expressed as  $\mu\text{g g}^{-1}$  fresh weight (FW).

### Enzyme extractions and assays

All experiments were performed at 4 °C. Leaf samples (0.5 g each) were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.8) for the protein and enzyme extractions. The buffer contained 1 mM disodium EDTA and 2% (w/v) polyvinylpolypyrrolidone (PVPP). Supernatants collected after centrifugation at  $13,000 \times g$  for 40 min were used to determine the protein contents and enzyme activities. Bovine serum albumin was used as the standard to determine the total soluble protein content according to the methods of Bradford (22).

APX (EC 1.11.1.11) activity was measured according to the methods of Nakano and Asada (23). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM hydrogen peroxide, and 0.1 mL of enzyme extract in a total volume of 1 mL. The concentration of oxidized ascorbate was calculated by the decrease in absorbance at 290 nm. The absorption coefficient was  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ . One unit of APX was defined as  $1 \text{ mmol mL}^{-1}$  ascorbate oxidized  $\text{min}^{-1}$  (24).

GPX (EC 1.11.1.7) activity was measured according to the method of Scebba et al. (25) at 25 °C. Guaiacol was used as the hydrogen donor, and the assay was based on monitoring the  $\text{H}_2\text{O}_2$  dissolution rate by peroxidase (POX). The 2-mL reaction mixture contained 11 mM  $\text{H}_2\text{O}_2$  in 65 mM phosphate buffer (pH 6.0), 2.25 mM guaiacol, and 50  $\mu\text{L}$  of the enzyme extract. The POX activity was estimated by the increase in absorbance of oxiguaiacol at 470 nm and was expressed as  $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$ .

GR (EC 1.6.4.2) activity was measured according to the method of Foyer and Halliwell (26) by monitoring the glutathione-dependent oxidation of NADPH. The complete reaction mixture contained 100 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.05 mM NADPH, 3 mM GSSG, and 50  $\mu$ L of enzyme extract in a final volume of 1 mL. The decrease in absorbance at 340 nm during 1 min was recorded. The extinction coefficient used for NADPH was 6.2 mM<sup>-1</sup> cm<sup>-1</sup>, and 1 unit of GR was defined as 1 mmol mL<sup>-1</sup> GSSG reduced min<sup>-1</sup>.

SOD (EC 1.15.1.1) activity was estimated due to its ability to inhibit the photochemical reduction of nitroblue tetrazolium salt (NBT). The SOD activity was measured at 560 nm according to the method of Beauchamp and Fridovich (27), and the assays were carried out at 25 °C. The final assay volume of 3 mL contained 33  $\mu$ M NBT, 10 mM L-methionine, 0.66 mM disodium EDTA, and 0.0033 mM riboflavin in 0.05 mM sodium phosphate buffer (pH 7.8). After riboflavin was added, the test tubes were incubated in the light for 15 min. One unit of SOD was defined as the amount of enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50%. The SOD activity of each extract was expressed as U protein mg<sup>-1</sup> FW.

CAT (EC 1.11.1.6) activity was measured according to the method of Bergmeyer (28). The reaction mixture contained 0.05 M sodium phosphate buffer (pH 7.0) with 0.1 mM EDTA and 3% H<sub>2</sub>O<sub>2</sub>. The decrease in the absorption was followed for 3 min at 240 nm, and 1 mmol H<sub>2</sub>O<sub>2</sub> mL<sup>-1</sup> min<sup>-1</sup> was defined as 1 U of CAT activity.

### Lipid peroxidation

Malondialdehyde is the product of lipid peroxidation. The level of this molecule in plant cells increases under stress conditions. Lipid peroxidation is a major parameter to identify the degree of stress to which a plant has been exposed, by determining cell membrane stability (29).

Frozen leaf samples were ground in liquid nitrogen, and 1 mL of extraction medium containing 0.5% (w/v) thiobarbituric acid and 20% (w/v) trichloric acid was added. The mixture was heated for 30 min at 95 °C, samples were cooled to 4 °C, and the reaction was stopped. After centrifugation for 10

min at 3000  $\times$  g, the absorbances of the supernatants were determined at 532 and 600 nm. The extinction coefficient for MDA is 155 mM<sup>-1</sup> cm<sup>-1</sup>. The results were expressed as  $\mu$ mol MDA g<sup>-1</sup> FW (30).

### Determination of free proline

The free proline content was determined according to Bates et al. (31). Frozen leaf tissue (0.5 g) was homogenized with 10 mL of 3% sulfosalicylic acid at 4 °C. The extract was filtered with Whatman No. 2 filter paper. In a test tube, 2 mL of filtrate, 2 mL of acid-ninhydrin, and 2 mL of glacial acetic acid were mixed and incubated at 100 °C for 1 h. The reaction was terminated on ice, and the reaction mixture was then extracted with 4 mL of toluene. The chromophore-containing toluene was separated from the hydrated phase. The absorbance at 520 nm was spectrophotometrically determined with toluene as the blank. The proline concentration was calculated based on a standard curve and was expressed as  $\mu$ mol proline g<sup>-1</sup> FW.

### Statistical analysis

All results are presented as the mean values  $\pm$  standard errors. The statistical significances of differences between mean values were assessed by analysis of variance and Duncan's multiple range tests.  $P < 0.05$  was considered significant. The differences between the results of the biochemical analyses of the *in vitro* and *in vivo* experiments were evaluated using a regression analysis.

## Results

### *In vitro* salt tolerance studies

The shoot development, fresh weights, and root lengths of both varieties were measured after 14 days of salt stress. The shoot and root development results are given in Figure 1. In the İzmir Özbaş variety, shoot length decreased in plantlets grown in salinities of 200 mM or higher compared to plants not grown under salt stress; these decreases were statistically significant ( $P < 0.05$ ). The Akhisar 97 seedlings grown in NaCl concentrations greater than 150 mM showed a statistically significant decrease in shoot length compared to seedlings grown in the absence of NaCl. Shoots of the Akhisar 97 variety showed a greater reduction in development in the

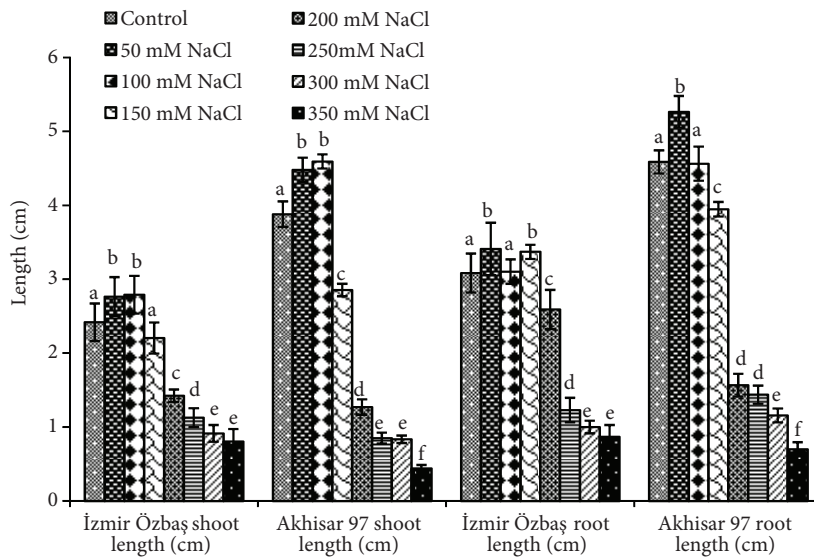


Figure 1. Seedling shoot and root lengths on day 14 in İzmir Özbaşı and Akhisar 97 (*Nicotiana tabacum* L.) tobacco varieties subjected to in vitro salinity at different concentrations (cm  $\pm$  SD).

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

presence of 350 mM NaCl than shoots of the İzmir Özbaşı variety. When grown in the presence of 200 mM NaCl, the shoot lengths of the İzmir Özbaşı and Akhisar 97 plantlets showed 40.91% and 67.27% decreases, respectively, compared to control plantlets grown in the absence of NaCl.

Root length variations among plants grown under control conditions and in the presence of  $\geq 150$  mM NaCl were significant for the Akhisar 97 variety ( $P < 0.05$ ); roots from plants grown in the presence of NaCl were shorter than those of control plants. In contrast, plantlets of the İzmir Özbaşı variety only showed decreased root length when grown in the presence of  $\geq 200$  mM NaCl. Compared to control plants, at this concentration of NaCl, root length in the İzmir Özbaşı variety decreased by 16.09%; the Akhisar 97 variety showed a 65.79% decrease.

The fresh weights of the İzmir Özbaşı tobacco plants grown in the presence of 300 mM NaCl were significantly lower than those of control plants ( $P < 0.05$ ). In contrast, the Akhisar 97 variety showed decreased fresh weights compared to controls at NaCl concentrations greater than 200 mM ( $P < 0.05$ ) (Figure 2).

The growth profiles of both tobacco varieties were evaluated after 14 days of NaCl treatment. Plant growth in each tobacco species was increasingly inhibited with rising salt concentrations; growth of the Akhisar 97 variety was inhibited at a lower NaCl concentration than growth of the İzmir Özbaşı variety (Figures 3 and 4).

### Chlorophyll and carotenoid contents

Chlorophyll concentrations in plants from each variety were measured. Chlorophyll a, chlorophyll b, and total chlorophyll concentrations all decreased with increasing NaCl concentrations. Chlorophyll levels in the Akhisar 97 variety were lower than control values when plants were grown in the presence of 200 mM NaCl; however, in the İzmir Özbaşı variety, chlorophyll levels were lower than those in control plants when plants were grown in the presence of 250 mM NaCl. In the Akhisar 97 variety, carotenoid concentrations in plants grown in the presence of  $\geq 200$  mM NaCl were lower than those in control plants ( $P < 0.05$ ), whereas in the İzmir Özbaşı variety, similar decreases were observed at  $\geq 150$  mM NaCl ( $P < 0.05$ ) (Table 1).

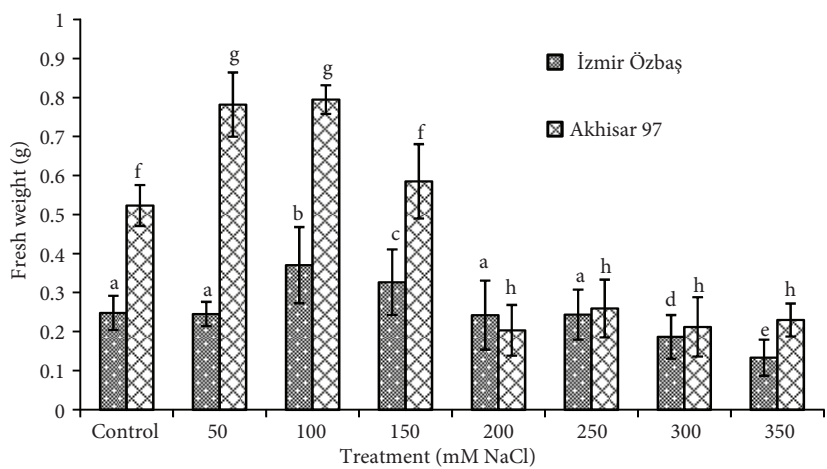


Figure 2. Seedling fresh weights on day 14 in İzmir Özbaş and Akhisar 97 (*N. tabacum* L.) tobacco varieties subjected to in vitro salinity at different concentrations ( $g \pm SD$ ).

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.



Figure 3. Growth of İzmir Özbaş (*N. tabacum* L.) tobacco varieties on day 14 of in vitro salinity stress at different concentrations.



Figure 4. Growth of Akhisar 97 (*N. tabacum* L.) tobacco varieties on day 14 of in vitro salinity stress at different concentrations.

Table 1. Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid concentrations on day 14 in tobacco plantlets subjected to in vitro salinity stress at different concentrations of NaCl ( $\pm$ SD).

| Akhisar 97 variety  | Chlorophyll a                 | Chlorophyll b                 | Total chlorophyll             | Carotenoid concentration       |
|---------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|
| Control             | 40.67 $\pm$ 1.23 <sup>a</sup> | 7.66 $\pm$ 1.20 <sup>a</sup>  | 48.33 $\pm$ 0.86 <sup>a</sup> | 13.015 $\pm$ 0.84 <sup>a</sup> |
| 50 mM NaCl          | 44.96 $\pm$ 0.45 <sup>b</sup> | 7.14 $\pm$ 1.20 <sup>a</sup>  | 52.10 $\pm$ 1.30 <sup>b</sup> | 14.166 $\pm$ 0.48 <sup>b</sup> |
| 100 mM NaCl         | 45.81 $\pm$ 2.29 <sup>c</sup> | 20.92 $\pm$ 0.98 <sup>b</sup> | 66.74 $\pm$ 0.75 <sup>c</sup> | 15.476 $\pm$ 0.64 <sup>c</sup> |
| 150 mM NaCl         | 41.16 $\pm$ 1.32 <sup>a</sup> | 12.59 $\pm$ 0.87 <sup>c</sup> | 53.75 $\pm$ 0.96 <sup>d</sup> | 13.155 $\pm$ 0.42 <sup>a</sup> |
| 200 mM NaCl         | 28.50 $\pm$ 1.13 <sup>d</sup> | 7.74 $\pm$ 0.76 <sup>a</sup>  | 36.24 $\pm$ 0.57 <sup>e</sup> | 10.203 $\pm$ 0.64 <sup>d</sup> |
| 250 mM NaCl         | 19.39 $\pm$ 1.26 <sup>e</sup> | 1.45 $\pm$ 1.20 <sup>d</sup>  | 20.84 $\pm$ 0.65 <sup>f</sup> | 8.316 $\pm$ 0.24 <sup>e</sup>  |
| 300 mM NaCl         | 15.65 $\pm$ 0.98 <sup>f</sup> | 1.56 $\pm$ 0.54 <sup>d</sup>  | 17.22 $\pm$ 0.36 <sup>g</sup> | 6.334 $\pm$ 0.45 <sup>f</sup>  |
| 350 mM NaCl         | 9.39 $\pm$ 1.11 <sup>g</sup>  | 0.45 $\pm$ 0.36 <sup>e</sup>  | 9.84 $\pm$ 0.72 <sup>h</sup>  | 5.774 $\pm$ 0.16 <sup>g</sup>  |
| İzmir Özbaş variety | Chlorophyll a                 | Chlorophyll b                 | Total chlorophyll             | Carotenoid concentration       |
| Control             | 28.27 $\pm$ 1.00 <sup>a</sup> | 15.14 $\pm$ 0.96 <sup>a</sup> | 43.42 $\pm$ 0.78 <sup>a</sup> | 10.92 $\pm$ 0.65 <sup>a</sup>  |
| 50 mM NaCl          | 38.26 $\pm$ 1.30 <sup>b</sup> | 17.35 $\pm$ 0.63 <sup>b</sup> | 55.61 $\pm$ 0.54 <sup>b</sup> | 11.21 $\pm$ 0.32 <sup>a</sup>  |
| 100 mM NaCl         | 59.70 $\pm$ 1.20 <sup>c</sup> | 32.87 $\pm$ 0.85 <sup>c</sup> | 92.57 $\pm$ 1.20 <sup>c</sup> | 12.83 $\pm$ 0.64 <sup>b</sup>  |
| 150 mM NaCl         | 51.75 $\pm$ 0.99 <sup>d</sup> | 22.31 $\pm$ 0.86 <sup>d</sup> | 74.06 $\pm$ 1.06 <sup>d</sup> | 9.19 $\pm$ 0.52 <sup>c</sup>   |
| 200 mM NaCl         | 40.99 $\pm$ 0.87 <sup>e</sup> | 16.13 $\pm$ 1.20 <sup>e</sup> | 57.13 $\pm$ 0.95 <sup>b</sup> | 8.38 $\pm$ 0.16 <sup>d</sup>   |
| 250 mM NaCl         | 17.20 $\pm$ 0.95 <sup>f</sup> | 6.99 $\pm$ 1.05 <sup>f</sup>  | 24.19 $\pm$ 0.56 <sup>e</sup> | 5.35 $\pm$ 0.24 <sup>e</sup>   |
| 300 mM NaCl         | 10.48 $\pm$ 0.56 <sup>g</sup> | 5.86 $\pm$ 0.76 <sup>g</sup>  | 16.34 $\pm$ 0.73 <sup>f</sup> | 4.75 $\pm$ 0.17 <sup>f</sup>   |
| 350 mM NaCl         | 7.37 $\pm$ 0.45 <sup>h</sup>  | 3.92 $\pm$ 0.56 <sup>h</sup>  | 11.28 $\pm$ 0.93 <sup>g</sup> | 4.15 $\pm$ 0.15 <sup>g</sup>   |

\*All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replicates.

### Lipid peroxidation

Salinity-induced increases in lipid peroxidation, as estimated through malondialdehyde (MDA) production, were observed in the leaves of both tobacco varieties. Lipid peroxidation rates in both varieties increased in the presence of high concentrations of NaCl, although the effects were greater in Akhisar 97. Statistically significant increases in lipid peroxidation rates were observed at 100 mM NaCl for the İzmir Özbaş variety and at 50 mM for the Akhisar 97 variety (Figure 5). Peroxidation rates increased by 52.73% and 90.38% for the İzmir Özbaş and Akhisar 97 varieties, respectively, compared to control plants at 350 mM NaCl.

### Protein content

Total protein content results are given in Figure 6. These results show that leaves from both varieties subjected to NaCl concentrations of up to 200 mM

had increased protein content relative to leaves from control plants. When treated with 250 mM NaCl, leaves of İzmir Özbaş seedlings showed total protein concentration increases of 38.97% compared to controls, whereas a 27.90% decrease in protein content was observed in leaves from the Akhisar 97 variety. When treated with 350 mM NaCl, 51.51% and 58.58% decreases were observed in the İzmir Özbaş and Akhisar 97 varieties, respectively, compared to control plants.

### Antioxidant enzyme activities

Antioxidative enzymes are the first response mechanism against environmental stresses. As such, their activity profiles are important in the evaluation of tolerance mechanisms.

APX and GPX (Figures 7 and 8), SOD and CAT (Figures 9 and 10), and GR (Figures 11 and 12) activities were determined for both cultivars. The

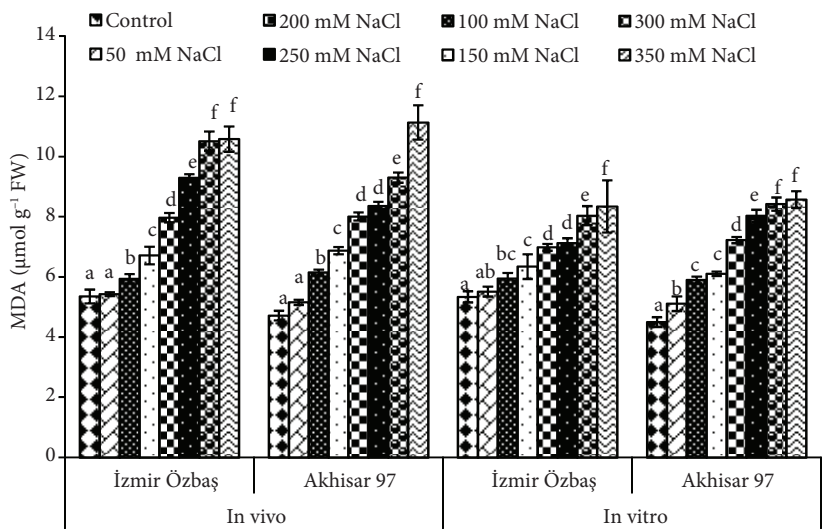


Figure 5. Seedling lipid peroxidation rates on day 14 in İzmir Özbaşı and Akhisar 97 (*N. tabacum* L.) tobacco varieties subjected to in vitro and in vivo salinity at different concentrations ( $\mu\text{mol g}^{-1}\text{FW} \pm \text{SD}$ ).

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

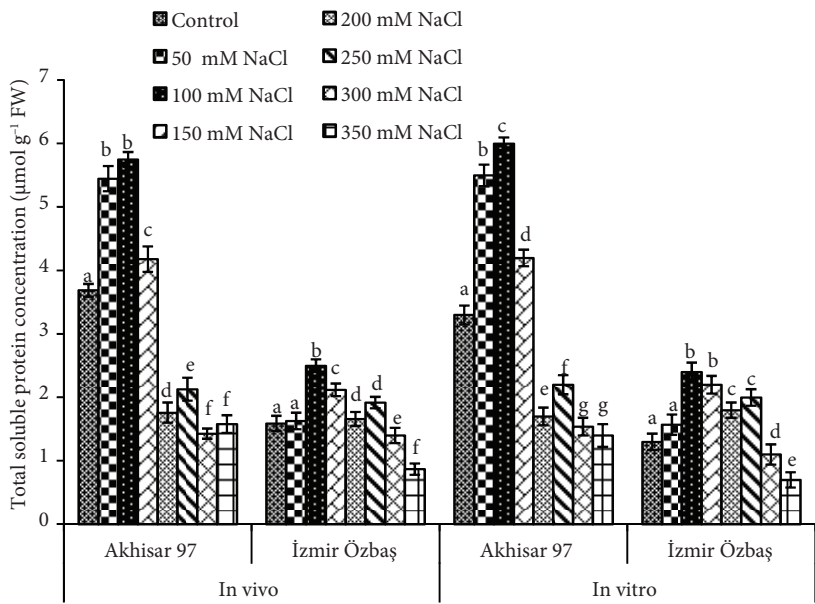


Figure 6. Seedling total soluble protein concentrations on day 14 in İzmir Özbaşı and Akhisar 97 (*N. tabacum* L.) tobacco varieties subjected to in vitro and in vivo salinity at different concentrations ( $\mu\text{g}/\mu\text{L} \pm \text{SD}$ ).

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

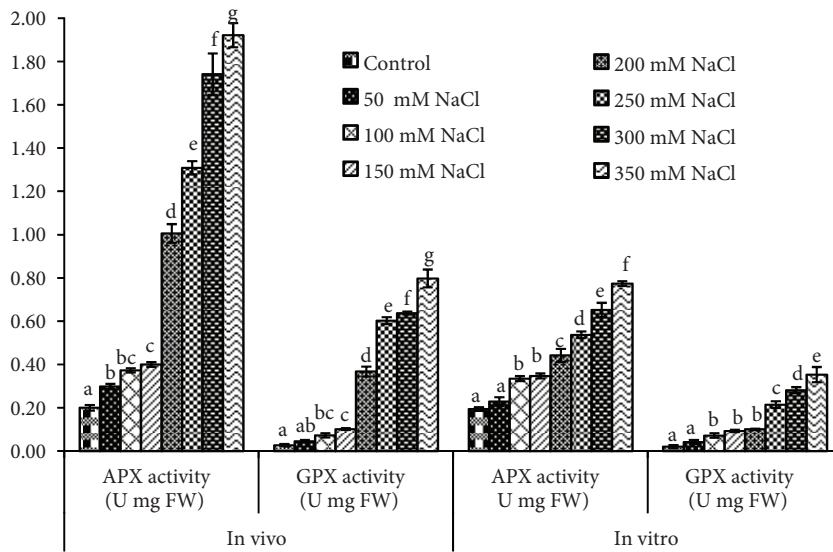


Figure 7. Seedling ascorbate peroxidase and guaiacol peroxidase activities on day 14 in İzmir Özbaş (*N. tabacum* L.) tobacco variety subjected to in vitro and in vivo salinity at different concentrations.

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

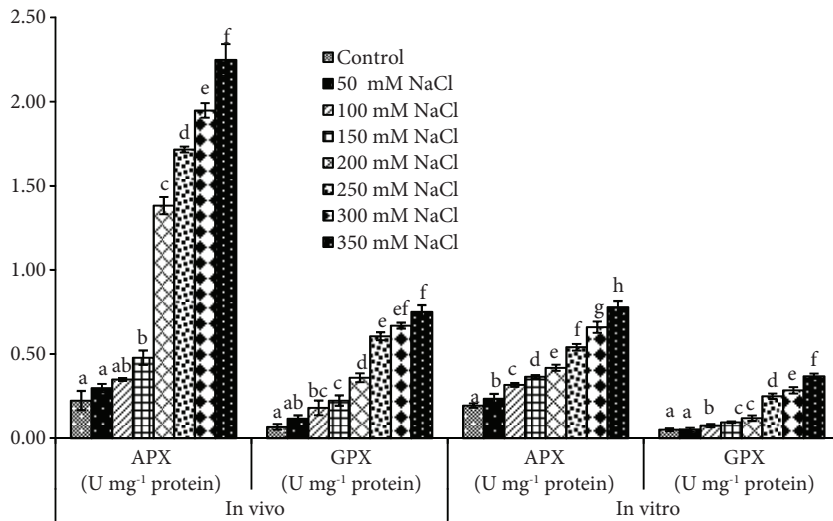


Figure 8. Seedling ascorbate peroxidase and guaiacol peroxidase activities on day 14 in Akhisar 97 (*N. tabacum* L.) tobacco variety subjected to in vitro and in vivo salinity at different concentrations.

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

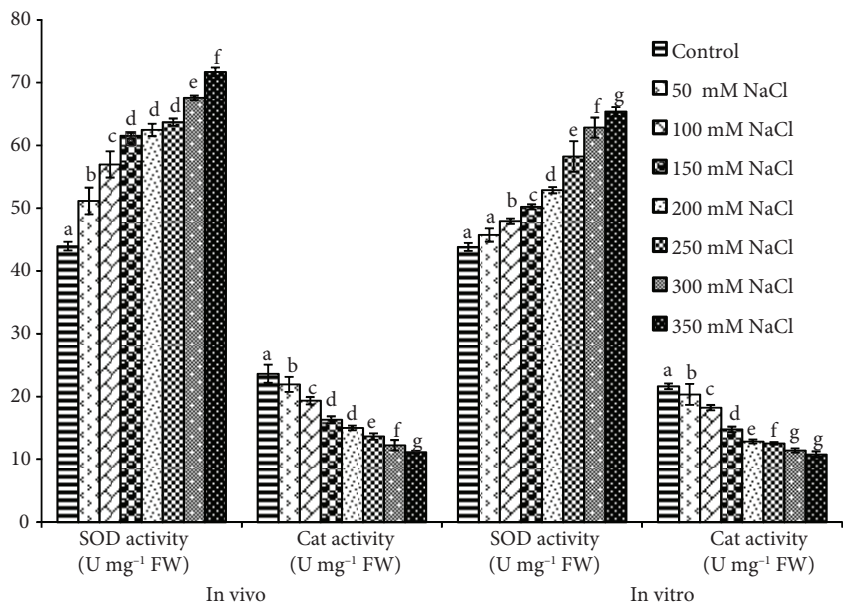


Figure 9. Seedling superoxide dismutase and catalase activities on day 14 in İzmir Özbaş (*N. tabacum* L.) tobacco variety subjected to in vitro and in vivo salinity at different concentrations.

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

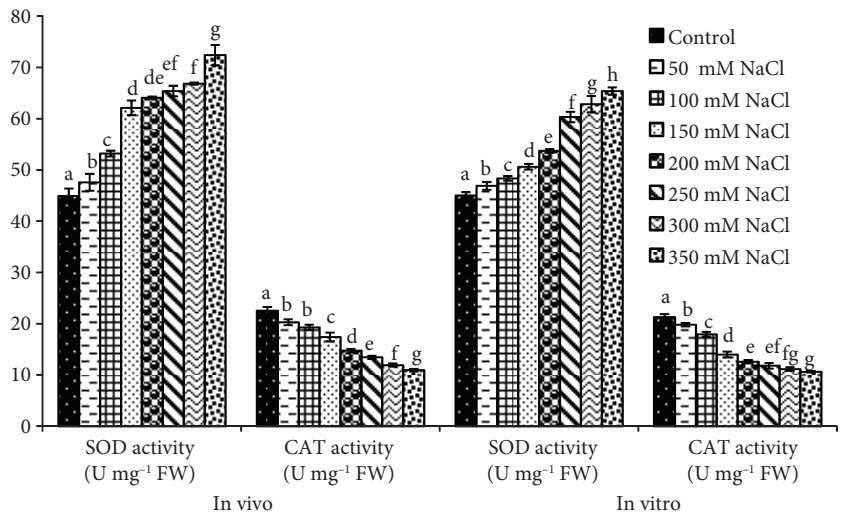


Figure 10. Seedling superoxide dismutase and catalase activities on day 14 in Akhisar 97 (*N. tabacum* L.) tobacco variety subjected to in vitro and in vivo salinity at different concentrations.

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

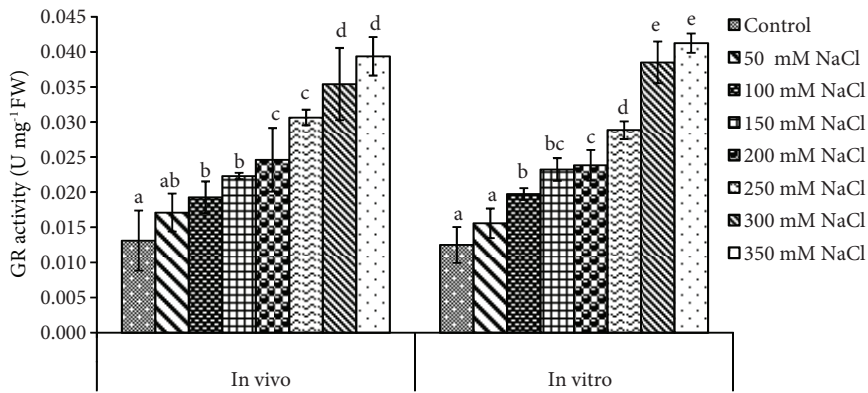


Figure 11. Seedling glutathione reductase enzyme activity on day 14 in İzmir Özbaş (*N. tabacum* L.) tobacco variety subjected to in vitro and in vivo salinity at different concentrations.

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

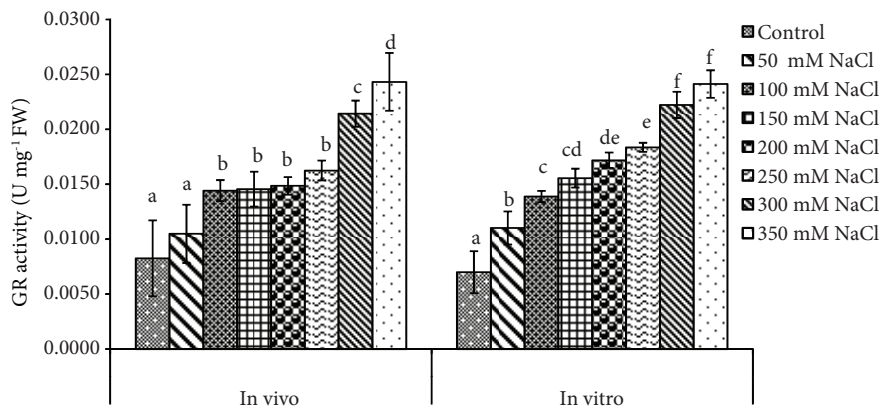


Figure 12. Seedling glutathione reductase enzyme activity on day 14 in Akhisar 97 (*N. tabacum* L.) tobacco variety subjected to in vitro and in vivo salinity at different concentrations.

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

activities of all of these enzymes were increased on day 14 of NaCl treatment. Indeed, activity levels were statistically higher than activities recorded in control plants at day 14 ( $P < 0.05$ ), and levels increased with increasing salt concentrations in both tobacco varieties. SOD and APX activities also increased with increasing NaCl concentrations in both Akhisar 97 and İzmir Özbaş varieties. GPX activity showed a strong increase at 250 mM in both varieties compared to GPX activity in control plants. GR activity for İzmir Özbaş control plants was higher than that for

Akhisar 97 control plants. Increases in GR activity in the tobacco leaves of plants from both varieties subjected to salinity gradually increased with rising NaCl concentrations ( $P < 0.05$ ).

The CAT activity in each tobacco variety under different NaCl concentrations was evaluated. Statistically significant decreases in CAT activity were observed in both varieties compared to controls at all NaCl concentrations ( $P < 0.05$ ); increasing NaCl concentrations resulted in increasingly lower CAT activity.

## In vivo salt tolerance studies

### Evaluation of growth scale

Plantlets subjected to NaCl stress for 14 days were evaluated for growth. In the Akhisar 97 tobacco variety, the detrimental effects of  $\geq 200$  mM NaCl on plant growth were observed and were statistically significant compared to the control ( $P < 0.05$ ). In the İzmir Özbaşı variety, the detrimental effects of  $\geq 250$  mM NaCl on plant growth were observed and were statistically significant compared to the control ( $P < 0.05$ ).

### Chlorophyll and carotenoid contents

In the Akhisar 97 variety, levels of chlorophyll a, chlorophyll b, and total chlorophyll were significantly increased in plants treated with 100 mM NaCl compared to the control. Chlorophyll a and b concentrations remained constant at NaCl levels of up to 150 mM. NaCl concentrations higher than 150 mM caused statistically significant decreases in

chlorophyll a, chlorophyll b, and total chlorophyll levels. In the İzmir Özbaşı variety, the chlorophyll a and total chlorophyll concentrations both increased with increasing NaCl concentrations up to 200 mM NaCl. Chlorophyll a, chlorophyll b, and total chlorophyll all decreased in plants grown in NaCl concentrations of 250 mM or higher when compared with levels recorded in control plants (Table 2). The carotenoid concentration in the Akhisar 97 tobacco variety was significantly increased (compared to controls) in plants grown in NaCl concentrations of up to 100 mM; however, they were then decreased at NaCl concentrations greater than 150 mM. In the İzmir Özbaşı variety, an increase in carotenoid concentration was observed in plants grown in the presence of 100 mM NaCl, but the difference was not significant. Additionally, there was no significant difference in carotenoid concentration in plants grown in the presence of 200 mM NaCl when compared to levels recorded in control plants (Table 2).

Table 2. Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid concentrations on day 14 in tobacco plantlets subjected to in vivo salinity stress at different concentrations of NaCl ( $\pm$ SD).

| Akhisar 97 variety   | Chlorophyll a                 | Chlorophyll b                 | Total chlorophyll             | Carotenoid concentration      |
|----------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Control              | 33.37 $\pm$ 1.13 <sup>a</sup> | 9.34 $\pm$ 1.20 <sup>a</sup>  | 42.71 $\pm$ 0.56 <sup>a</sup> | 15.06 $\pm$ 0.64 <sup>a</sup> |
| 50 mM NaCl           | 34.68 $\pm$ 0.54 <sup>a</sup> | 11.36 $\pm$ 1.50 <sup>b</sup> | 46.04 $\pm$ 1.36 <sup>b</sup> | 17.17 $\pm$ 0.52 <sup>b</sup> |
| 100 mM NaCl          | 36.54 $\pm$ 2.34 <sup>b</sup> | 14.35 $\pm$ 0.87 <sup>c</sup> | 50.89 $\pm$ 0.79 <sup>c</sup> | 18.48 $\pm$ 0.64 <sup>c</sup> |
| 150 mM NaCl          | 32.66 $\pm$ 1.32 <sup>a</sup> | 9.67 $\pm$ 0.69 <sup>a</sup>  | 42.33 $\pm$ 0.67 <sup>a</sup> | 14.15 $\pm$ 0.42 <sup>d</sup> |
| 200 mM NaCl          | 24.34 $\pm$ 0.98 <sup>c</sup> | 7.13 $\pm$ 1.45 <sup>d</sup>  | 31.47 $\pm$ 1.01 <sup>d</sup> | 11.20 $\pm$ 0.65 <sup>e</sup> |
| 250 mM NaCl          | 22.1 $\pm$ 1.20 <sup>d</sup>  | 7.47 $\pm$ 0.97 <sup>d</sup>  | 29.57 $\pm$ 1.34 <sup>e</sup> | 10.32 $\pm$ 0.22 <sup>f</sup> |
| 300 mM NaCl          | 17.39 $\pm$ 0.89 <sup>e</sup> | 6.25 $\pm$ 0.65 <sup>e</sup>  | 23.64 $\pm$ 0.64 <sup>f</sup> | 8.33 $\pm$ 0.36 <sup>g</sup>  |
| 350 mM NaCl          | 14.67 $\pm$ 1.01 <sup>f</sup> | 5.16 $\pm$ 0.34 <sup>f</sup>  | 19.83 $\pm$ 0.58 <sup>g</sup> | 6.77 $\pm$ 0.18 <sup>h</sup>  |
| İzmir Özbaşı variety | Chlorophyll a                 | Chlorophyll b                 | Total chlorophyll             | Carotenoid concentration      |
| Control              | 27.38 $\pm$ 1.05 <sup>a</sup> | 14.35 $\pm$ 1.02 <sup>a</sup> | 41.73 $\pm$ 0.67 <sup>a</sup> | 10.92 $\pm$ 0.27 <sup>a</sup> |
| 50 mM NaCl           | 33.25 $\pm$ 1.26 <sup>b</sup> | 18.64 $\pm$ 0.91 <sup>b</sup> | 51.89 $\pm$ 1.04 <sup>b</sup> | 11.21 $\pm$ 0.84 <sup>b</sup> |
| 100 mM NaCl          | 36.88 $\pm$ 0.97 <sup>c</sup> | 17.69 $\pm$ 0.38 <sup>c</sup> | 54.57 $\pm$ 0.86 <sup>c</sup> | 12.83 $\pm$ 0.76 <sup>c</sup> |
| 150 mM NaCl          | 38.64 $\pm$ 0.97 <sup>d</sup> | 20.31 $\pm$ 0.48 <sup>d</sup> | 58.95 $\pm$ 1.02 <sup>d</sup> | 11.19 $\pm$ 0.24 <sup>b</sup> |
| 200 mM NaCl          | 35.21 $\pm$ 0.85 <sup>e</sup> | 12.37 $\pm$ 0.67 <sup>e</sup> | 47.58 $\pm$ 0.95 <sup>e</sup> | 10.61 $\pm$ 0.65 <sup>a</sup> |
| 250 mM NaCl          | 20.39 $\pm$ 1.06 <sup>f</sup> | 8.14 $\pm$ 1.00 <sup>f</sup>  | 28.53 $\pm$ 0.74 <sup>f</sup> | 7.70 $\pm$ 0.34 <sup>d</sup>  |
| 300 mM NaCl          | 17.32 $\pm$ 0.67 <sup>g</sup> | 6.39 $\pm$ 1.03 <sup>g</sup>  | 23.71 $\pm$ 0.68 <sup>g</sup> | 5.11 $\pm$ 0.19 <sup>e</sup>  |
| 350 mM NaCl          | 15.02 $\pm$ 0.48 <sup>h</sup> | 5.09 $\pm$ 0.84 <sup>h</sup>  | 20.11 $\pm$ 0.90 <sup>h</sup> | 3.77 $\pm$ 0.35 <sup>f</sup>  |

\*All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replicates.

## Lipid peroxidation

Under increased NaCl concentrations, membrane lipids are damaged by ROS and lipid peroxidation. Oxidative damage to tissue lipids was estimated in this study by measuring MDA content (32). In the İzmir Özbaş variety, lipid peroxidation levels were  $5.36 \mu\text{mol g}^{-1}$  FW in the control group and were increased to  $7.98 \mu\text{mol g}^{-1}$  FW in plants grown in the presence of 200 mM NaCl. For the Akhisar 97 variety, lipid peroxidation levels were  $4.72 \mu\text{mol g}^{-1}$  FW in the control; however, in plants grown at <200 mM NaCl, this value increased to  $8.25 \mu\text{mol g}^{-1}$  FW. For the higher NaCl concentrations (>200 mM), these values were increased to 8.60, 9.29, and  $10.59 \mu\text{mol g}^{-1}$  FW in the İzmir Özbaş variety and 9.12, 9.59, and  $11.47 \mu\text{mol g}^{-1}$  FW in the Akhisar 97 variety (Figure 5).

The negative effects increased with increasing NaCl concentrations in Akhisar 97 plants. In addition, these lipid peroxidation data showed that the Akhisar 97 variety was more sensitive to NaCl than was the İzmir Özbaş variety.

## Protein content

Total protein concentration of the *in vivo* NaCl-treated plants was determined and is given in Figure 6. In the Akhisar 97 variety, plants grown in the presence of 100 mM NaCl showed higher total protein content than control plants ( $P < 0.05$ ). At concentrations of NaCl higher than 200 mM, decreases in the total soluble protein concentration were observed in the Akhisar 97 variety compared to the control. At 250 mM NaCl, the total protein content in the İzmir Özbaş variety increased compared to the control. At concentrations of NaCl greater than 250 mM, decreases in the total soluble protein concentration were observed in the İzmir Özbaş variety relative to the control.

## Antioxidant enzyme activities

The APX, GPX, SOD, CAT, and GR activities recorded for both varieties during the *in vivo* salinity experiments are given in Figures 7-12. In both tobacco varieties, SOD activities increased with increasing NaCl concentrations. At all NaCl concentrations, APX activities recorded in both varieties increased significantly compared to controls. A sharp increase in APX activity was found in plants grown in the presence of NaCl compared with plants grown under control conditions. Plants of the Akhisar 97 variety

showed higher GPX activity levels than did plants of the İzmir Özbaş variety. The GPX activity in both varieties increased significantly with increasing NaCl concentrations (Figures 7 and 8). The GR activity in both varieties gradually increased with increasing NaCl concentrations. For plants grown in the presence of 250 mM NaCl, the GR activity in İzmir Özbaş seedlings increased 2.34 times compared to control plants; the GR activity in Akhisar 97 seedlings increased only 1.96 times relative to the control plants (Figures 11 and 12). The activity of CAT, an important scavenger of  $\text{H}_2\text{O}_2$ , showed a gradual decrease in both varieties as NaCl concentrations increased; CAT levels were always significantly lower in plants treated with NaCl compared to control plants ( $P < 0.05$ ) (Figures 9 and 10).

## Evaluation of proline accumulation

### *In vitro* salt tolerance studies

Leaves from both the Akhisar 97 and İzmir Özbaş varieties subjected to different NaCl concentrations were assayed for accumulated proline content. The proline contents in plants from both varieties treated with 0-350 mM NaCl *in vitro* are shown in Figure 13. In general, proline accumulation increased gradually with increasing concentrations of NaCl in both varieties. The largest increase in proline content compared to control plants was observed in plants of both varieties treated with 350 mM NaCl ( $P < 0.05$ ). The increase in proline content in the İzmir Özbaş variety was 9.4-fold compared to controls; it was 28.4-fold compared to controls in the Akhisar 97 variety.

### *In vivo* salt tolerance studies

The proline accumulation results from *in vivo* studies are given in Figure 14. The accumulation of proline increased with increasing NaCl concentrations in both varieties, confirming the results of the *in vitro* salt treatments ( $P < 0.05$ ). Whereas plants of the Akhisar 97 variety exposed to NaCl showed greater proline accumulation by 23-fold compared to control plants, the İzmir Özbaş variety showed 8.19-fold greater proline accumulation compared to controls.

## Evaluation of *in vitro* and *in vivo* experimental results

There were no statistically significant differences between control plants and NaCl-treated plants in shoot and root lengths or fresh weights in either the

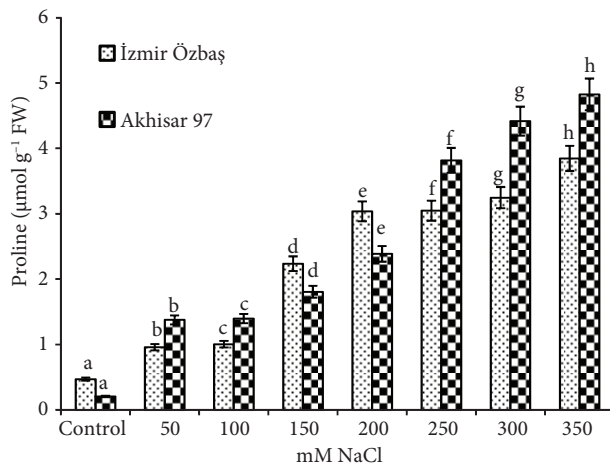


Figure 13. Proline contents of *N. tabacum* L. plantlets subjected to in vitro salinity stress at different concentrations ( $\mu\text{mol/g FW} \pm \text{SD}$ ).

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

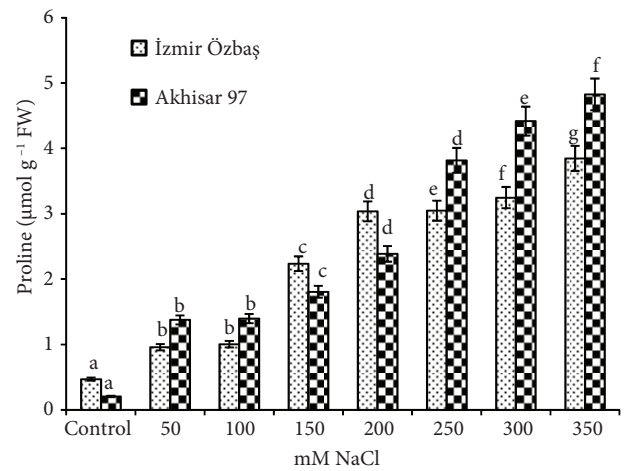


Figure 14. Proline contents of *N. tabacum* L. plantlets subjected to in vivo salinity stress at different concentrations ( $\mu\text{mol/g FW} \pm \text{SD}$ ).

\* All results were evaluated within their own varieties; means that are not shown with the same letter are significantly different by Duncan's multiple range tests ( $P < 0.05$ ); each mean represents 3 replications.

in vitro or in vivo studies. When we compared these results between the tobacco species, it was observed that the Akhisar 97 variety was more sensitive to salinity than was the İzmir Özbaşı variety. The biochemical results were statistically evaluated for differences between the in vitro and in vivo salinity stress experiments and for differences between varieties. There were no differences in the SOD, APX, GPX, or CAT activities observed between the varieties from either in vivo or in vitro studies; however, the differences in GR activity between control and NaCl-treated plants were statistically significant for both in vitro and in vivo salinity experiments.

## Discussion

Salinity plays a major role in limiting cellular processes; thus, dealing with this stress is very important for plant growth. Mechanisms used by plants to overcome the detrimental effects of abiotic stresses are very complicated because the growth and development mechanisms of the plants and the stress effector mechanisms need to be balanced.

The novel aspect of our study is the rigorous comparison between plants grown in vivo and plants grown in vitro under several NaCl concentrations.

In our study on growth parameters, the fresh weight, shoot length, and root length were increased in Akhisar 97 and İzmir Özbaşı tobacco plantlets subjected to up to 150 mM NaCl under in vitro and in vivo conditions compared with measurements from control plants. Although increases were observed in both experiments at levels of NaCl  $< 200$  mM, salinities  $> 200$  mM caused statistically significant decreases in these parameters ( $P < 0.05$ ) in the Akhisar 97 variety and the İzmir Özbaşı variety.

Tantawy et al. (33) found that increased stress caused all of the measured growth parameters in tomato plants (plant height, leaf area, total chlorophyll, and fresh weight) to respond negatively. Additionally, it has been reported that salinity causes decreases in germination (34), leaf area, relative growth rate (35), and coleoptile growth (36). Atak et al. (37) observed that the root and shoot lengths of triticale plantlets decreased with increasing NaCl concentrations.

In our study, we found that NaCl concentrations greater than 150 and 200 mM caused chlorosis for the Akhisar 97 and İzmir Özbaşı tobacco varieties, respectively, both in vivo and in vitro. The level of chlorosis increased with increasing NaCl concentrations. Carotenoid concentrations were

also determined in both tobacco varieties under both experimental conditions. NaCl concentrations of up to 200 mM caused increases in carotenoid concentrations, both in vivo and in vitro. In vivo, we found that concentrations of NaCl greater than 250 mM caused a statistically significant decrease in the carotenoid content of Akhisar 97 plants; however, for İzmir Özbaş plants, 250 mM NaCl was required to cause a similar decrease in the carotenoid concentration in vivo.

Chlorophyll content is a basic way to evaluate the effects of environmental stress (38). Photosynthesis is the main ROS-producing process in chloroplasts, and ROS can cause photoinhibitory and photooxidative damage (39,40). Chlorosis is a common response to salinity, and it causes the inhibition of photosynthesis. Thus, pigment degradation is a rapid indicator of plant response (41). Öncel and Keleş (5) treated 2 genotypes of *Triticum aestivum* with 200 mM NaCl for 5 days; they determined that chlorophyll a and b and total chlorophyll contents decreased, as did proline concentrations. Jampeetong and Brix (41) confirmed these findings. Cha-um and Kirdmanee (42) showed that chlorophyll a and b, total chlorophyll, and total carotenoid content were all reduced due to decreases in osmotic pressure.

Different plants can be subjected to different abiotic stresses during their lifetimes. For all plants, the basic cellular mechanisms that respond to these stresses are conserved. Such stressors can cause osmotic stress, oxidative stress, and differences in protein synthesis (43).

In this study, total soluble protein concentration was increased in plants grown in vivo under 50, 100, and 150 mM NaCl concentrations, due to the synthesis of proteins required to protect the plant against salt stress. However, in response to increasing salinity, harmful effects are increased, many metabolic pathways are inhibited, and many proteins are catabolized. The end result is decreased total protein content under high stress. We observed this effect as a decrease in protein concentration recorded in plants grown under high concentrations of NaCl (>200 mM NaCl), and these results were in agreement with those of Mazzucotelli et al. (44).

The salt stress tolerance of a plant may depend on the improved activities of its antioxidant enzymes. In this study, we evaluated the effects of in vitro and in vivo salt stresses in 2 tobacco varieties and measured the activities of SOD, CAT, APX, GPX, and GR, as well as the MDA content, of plants exposed to NaCl stress.

MDA can be used as a parameter for evaluating a plant's tolerance to environmental stresses (45). Variations in MDA content have been reported for different plant species under different conditions. Our findings showed that under salinity stress caused by different concentrations of NaCl, MDA levels in the Akhisar 97 and İzmir Özbaş varieties increased significantly compared to control plants, both in vivo and in vitro. Plants of the İzmir Özbaş variety had lower MDA levels than plants of the salt-sensitive Akhisar 97 variety.

In this study, increased salt concentrations led to significant changes in the levels of antioxidative enzymes in both tobacco cultivars. SOD activity in İzmir Özbaş and Akhisar 97 plants increased with increasing salinity. Similar responses have been observed in *B. maritima* and *B. vulgaris* 'Ansa' (46), cotton (47), maize (48), and cabbage (49). APX and POX activities showed the same trends as SOD activities. CAT is hypothesized to be the most important H<sub>2</sub>O<sub>2</sub>-scavenging enzyme in leaves (48). Higher CAT activity decreases the H<sub>2</sub>O<sub>2</sub> content in the cell, and thus increases the membrane stability (50). CAT activity levels were higher in plants of the İzmir Özbaş variety than in plants of the Akhisar 97 variety. Both in vivo and in vitro, the CAT activity levels in both cultivars were decreased more than 2-fold in the leaves of plants treated with 350 mM NaCl compared to control plants. GR is responsible for converting GSSG to GSH (13). Our results showed that GR activity levels in the leaves of salt-stressed plants were greater than those recorded in control plants. We observed a larger increase in GR activity in İzmir Özbaş than in Akhisar 97 under both experimental conditions. Our results are similar to those obtained by Posmyk et al. (49). They treated *Brassica juncea* with Cu<sup>2+</sup> stress and observed increases in APX, SOD, and POX activities, as well as decreases in CAT activity, in leaves from stressed plants.

Yazici et al. (15) subjected 2-month-old purslane seedlings to 0, 70, and 140 mM NaCl for 18 days. They reported that the enhanced activities of antioxidative enzymes and an accumulation of proline under saline conditions helped improve the salt tolerance of *Portulaca oleracea* L.

Glycine betaine (GB) and proline are the most common osmolytes produced in plants under salt stress. Tobacco plants do not produce GB under physiologic conditions (43); therefore, in our study, we evaluated proline accumulation. Shannon and Grieve (51) reported that no relationship was found between NaCl stress and proline accumulation in 7 different cultivars. Proline accumulation and stress tolerance correlation have been reported in different studies, and it has been observed that proline concentrations are higher in stress-tolerant plants than in stress-sensitive plants (10).

We found that the Akhisar 97 variety had a higher proline concentration than İzmir Özbaşı in control plants. We also found a difference between in vivo and in vitro experimental results for low concentrations of NaCl. Although the proline content of leaves subjected to 50 and 100 mM NaCl showed statistically significant differences in the in vitro salinity experiment compared with leaves from controls, there was no difference between the groups in the in vivo studies.

Patnaik and Debata (52) used palmarosa callus tissue generated from cultured nodal explants as their experimental material and varied concentrations of NaCl to select rapidly growing callus; NaCl-tolerant callus lines showed adaptive mechanisms including increased proline accumulation. Liu and van Staden (53) treated *Glycine max* (L.) Merr 'Acme' callus cultures with increasing concentrations of NaCl, up to 150 mM. They determined that 100 mM NaCl was appropriate for the selection of stress-resistant callus lines. They also showed that the concentrations of soluble carbohydrates and proline were higher in resistant lines than in sensitive lines. Proline is known to accumulate under drought and saline conditions (10,32,54). However, the opposite results have also been reported. Lutts et al. showed that salt-sensitive rice cultivars accumulated more proline

than did tolerant genotypes (43). Although a positive correlation between abiotic stress tolerance and free proline accumulation has been reported (18), a negative correlation between proline accumulation and salt tolerance has also been reported (19).

In our study, we found consistently higher GR activity levels in plants of the İzmir Özbaşı variety than in plants of the Akhisar 97 tobacco variety; this difference increased with increasing NaCl concentrations under both experimental conditions.

Changes in the GR state are important for monitoring the stress response. The glutathione redox pair is fundamental for providing homeostasis in cellular redox potential. Glutathione metabolic differences between salt-tolerant and salt-sensitive varieties have been reported. As a response to salt stress, increases in the GSH/GSSG ratio have been reported in salt-tolerant varieties (55,56).

In conclusion, the Akhisar 97 and İzmir Özbaşı Turkish oriental tobacco varieties showed different salt stress tolerances, both in vitro and in vivo. The Akhisar 97 tobacco variety was more sensitive to salt stress than was the İzmir Özbaşı variety, and damage to its cell membranes was higher than in İzmir Özbaşı. All of the antioxidative enzymes showed similar activity patterns under saline conditions in both varieties. Although Akhisar 97 plants were more sensitive than İzmir Özbaşı plants, they accumulated more proline than did İzmir Özbaşı plants. This finding demonstrated that there is a negative correlation between proline concentration and salt tolerance in the studied oriental tobacco varieties. The differences between the GR metabolisms of tobacco varieties are considered to be the key element in determining the salt stress tolerance of the 2 oriental tobacco varieties.

**Corresponding author:**

Özge ÇELİK

Department of Molecular Biology and Genetics,

Faculty of Science and Letters,

İstanbul Kültür University,

34156 Ataköy, İstanbul - TURKEY

E-mail: ocelik@iku.edu.tr

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