



CaO nanoparticles improve salt stress tolerance in diploid and tetraploid alfalfa (*Medicago sativa* L.) by modulating miRNA expression and physiological responses

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Abstract

This study investigated the effects of salt stress (NaCl) and CaO nanoparticles (CaO NP), as well as their combinations, on the expression levels of miRNAs in diploid (Bilensoy) and tetraploid (Alsancak) alfalfa varieties, while also assessing physiological and biochemical parameters. MiR159a, miR166a and miR393a have been downregulated by all applications in the leaves of both varieties. In the roots, following all treatments, only miR393a was downregulated in the diploid Bilensoy whereas in Alsancak, miR166a and miR393a were downregulated. MiR156a was the only miRNA consistently upregulated in both leaves and roots in all treatments in both varieties. Similarly, miR393a was found to be downregulated in all treatments, with the exception of one. The morphology of leaves, roots and stems that changed in both varieties with salt stress showed a tendency to improve with NP and combined applications. In both alfalfa varieties, the content of H₂O₂, soluble sugar, MDA and proline and the activities of POD and SOD increased in both leaves and roots with salt stress. However, combined applications showed varying trends, particularly notable decreases in some cases, in leaves and roots. These effects were more pronounced in Alsancak variety, particularly in the enzymatic activities observed in the roots. Photosynthetic pigments (chlorophyll and carotenoid), which decreased in the leaves of both varieties with salt stress compared to the control, also increased with combined applications. The supportive miRNA gene expressions, along with the observed morphological and physiological responses, highlighted the healing effect of CaO NPs in mitigating the impacts of salt stress in both alfalfa varieties.

Key message

CaO NPS are effective materials in improving salt stress in alfalfa. Tetraploid alfalfa is more tolerant to salt stress than diploid one.

Keywords Alfalfa · miRNA · Antioxidant · Salt stress · CaO nanoparticle

Introduction

Medicago sativa L. (alfalfa) is a forage plant belonging to the Fabaceae family (Hrbackova et al. 2020) and among the most important and oldest forage crops in the world (Ahmed et al. 2019). Alfalfa belongs to the *Medicago sativa-falcata* complex, which includes species with the potential to hybridize in nature. Members of this complex have been reduced to both diploid and tetraploid subspecies over time (Şakiroğlu and İlhan 2020). Turkey is among the important origin gene centers of alfalfa. Therefore, as a result of breeding studies, diploid and tetraploid new commercial varieties have been developed. Diploid Bilensoy and tetraploid

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Alsancak varieties are among the valuable commercial varieties that can be used in this context and whose ploidy levels have been confirmed by flow cytometry analysis (Yazıcılar et al. 2021).

Salt stress is among critical environmental factors for alfalfa and reduces the yield by negatively affecting the vegetative-generative cycles such as germination and flowering (Bhattarai et al. 2020). It causes the closure of stomata and, resulting a decrease in photosynthesis (Shi-Chu et al. 2019). Although alfalfa is considered to be moderately salt tolerant, salinity remains an important abiotic factor that significantly limits its productivity. Salt stress significantly effect the growth and yield of alfalfa, primarily through ion and osmotic toxicity, which ultimately leads to decreased nutritional quality and biomass (Cen et al. 2020; Peel 2023). High salinity disrupt water absorption by creating physiological stress that manifests as reduced root and shoot growth, lower leaf area, and decreased forage yield (Wang et al. 2017; Anderson 2023; Qiu et al. 2023). Moreover, salt stress induces oxidative stress, leading to increased reactive oxygen species (ROS) production, which further exacerbates plant damage (Li et al. 2022; Liu 2024). Decreases in crop growth and productivity due to soil salinity have been reported in forage legumes, including alfalfa (Al-Farsi et al. 2020). Salt-tolerant alfalfa cultivars have been developed through traditional breeding approaches. However, it is critical to evaluate the response of alfalfa to salt stress in physiological and genetic processes and to increase salt tolerance (Bhattarai et al. 2020).

Nowadays, researchers are pursuing innovative strategies to manage salt and other stress factors. Nanoparticle (NP) technology is one of the popular strategies used to increase product efficiency (Assad et al. 2022). NPs are materials with diameters between 1 and 100 nm in at least one dimension. Various NPs applied as nano-agrochemicals have been developed to increase agricultural productivity (Rajput et al. 2021). Metal oxides such as CaO are widely used in various fields and are among the category of NPs with the highest annual production worldwide (Nazir et al. 2022). CaO NPs are inexpensive, fairly simple, non-corrosive, economical and easy to use. In particular, CaO is also used as a destructive adsorbent for toxic chemicals (Kamboj et al. 2020). In plants, CaO NPs promote root hair growth. Because adequate calcium concentrations increase ammonium, potassium and phosphorus absorption, stimulate photosynthesis and increase the size of plant parts, resulting in an increase in plant biomass. It also improves the economy of production by making nitrogen use more efficient and reduces nitrogen pollution of the environment. Additionally, these NPs increase seedling growth and reduce toxicity levels. CaO NPs also modulate biochemical parameters such as total carbohydrates, total protein, photosynthetic pigments

and POD enzyme activities in plants during the physiological response process (Gandhi et al. 2021).

Many studies show that plant miRNAs play an important role in plant response to various stresses (Chaudhary et al. 2021). During abiotic stress, miRNAs are up- or down-regulated, suggesting that they are involved in the regulation of stress by targeting various genes or TFs (Singh et al. 2021). These RNAs are 20–24 nucleotide long, non-coding endogenous regulatory RNA sequences and regulate many biological processes through gene silencing in the gene expression process (Kumar et al. 2021a, b). They are involved in all processes in plant growth and development timing, tissue-organ differentiation, and stress-related phenomena. Many miRNAs have been studied in alfalfa plants under salt stress. Among these, the expression of mtr-miR156 miRNA was reduced in response to stress-induced changes, demonstrating the importance of miRNA arrangements in response to salt/alkaline stress in plants (Cao et al. 2018). However, it has also been reported that there may be a significant increase in the expression of miR156 in alfalfa with salt stress. This increase results in a decrease in plant growth during stress and a decrease in leaf area. An increase in miR156 levels causes a decrease in the expression of the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) gene family. The decrease in the expression of the SPL gene family causes the regulation of plant growth and development. This may help plants respond appropriately to salt stress (Arshad et al. 2017). MiR159 functions in stress responses, allowing the regulation and targeting of transcription factors involved in signal transduction and regulation of gene expression. This miRNA targets MYB (V-MYB AVIAN MYELOBLASTOSIS VIRAL ONCOGENE HOMOLOG) and TCP (TEOSINTE BRANCHED1, CYCLOIDEA and PROLIFERATING CELL FACTORS) transcription factors responsible for flowering time, anther and leaf development during the short day photoperiod (Liu et al. 2008). The putative target gene of miR166 is GAI (GIBBERELLIC-ACID INSENSITIVE) (MTR_3G065980), known as a GRAS (Gibberellic acid insensitive (GAI), Repressor of GAI (RGA) and Scarecrow (SCR) family transcription factor, which is downregulated by salt stress in wild-type alfalfa, while it is upregulated by salt stress in mutant alfalfa lines. This transcription factor plays a role in the salt and drought stress response (Golldack et al. 2014). MiR393 is regulated in different ways by stress conditions. It regulates by targeting F-box proteins and bHLH (basic HELIX-LOOP-HELIX) transcription factors, which are responsible for bacterial disease resistance, which play an important role in the abiotic stress process (Liu et al. 2008). Of these, the expression of miRNAs miR166 and miR393 increases with salt stress in wild alfalfa leaves, while the expression of miR159 decreases. On the other hand, it has

been observed that the expression of miR159 and miR166 increased in mutant alfalfa leaves (Ma et al. 2020).

While previous studies have documented the general regulatory roles of specific miRNAs, such as miR156, miR159, miR166, and miR393, under salt stress, there is limited information on how these miRNAs interact with physiological and biochemical responses, particularly in different ploidy levels of alfalfa. Additionally, the potential of advanced technologies, such as nanoparticle-based approaches, to modulate these miRNA expressions and enhance stress tolerance remains largely unexplored. This study aims to address this gap by investigating the effects of salt stress and CaO NPs on the miRNA expression, physiological parameters, and biochemical responses in diploid (Bilensoy) and tetraploid (Alsancak) alfalfa varieties. By integrating molecular and physiological insights, the study seeks to elucidate how CaO NPs can enhance salt stress tolerance, offering a novel perspective and practical implications for sustainable agricultural practices.

Materials and methods

Plant material

Two commercial alfalfa (*Medicago sativa* L.) varieties (diploid Bilensoy and tetraploid Alsancak) were used as plant materials for salt (NaCl) and NP (CaO nanoparticles) application. The seeds of these varieties were obtained from INTFA agricultural shopping center and Aegean Agricultural Research Institute of the Ministry of Agriculture and Forestry of the Republic of Turkey. The seeds were first washed with tap water. It was incubated in 70% ethanol for 10 min on a magnetic stirrer. Sterilized for 20 min with 20% NaOCl. Then it was washed 3 times with autoclaved water.

Synthesis of CaO nanoparticles

CaO nanoparticles (NPs) were obtained ready-made from Atatürk University (Erzurum), Faculty of Science,

Department of Chemistry. The synthesis of CaO NPs was performed by the Nadaroglu group using a green synthesis method with 0.1 mM CaCl₂ solution and pomegranate (*Punica granatum* L.) extract. The size, structure, and surface properties of the nanoparticles were characterized using SEM, XRD and FTIR analyses (Nadaroglu et al. 2017) (Supplementary Figs. 1, 2 and 3).

Salt stress and CaO nanoparticle application

Plant growth medium (MS) including agar in Petri dishes was prepared by combining the necessary all microelements and macroelements (pH 5.7). For salt stress, 50 mM and 100 mM NaCl were added to the medium. The mixtures were autoclaved, cooled, and then supplemented with vitamins. The media used were prepared to contain 0.5 and 1.5 ppm CaO NP. An experimental design was prepared that included separate applications of 50 and 100 mM NaCl, and 0.5 and 1.5 ppm CaO NPs, and their combinations, in addition to a control group. Distilled water devoid of both components was used as the control. The diameter of the Petri dishes used for the analyses was 90 mm. Sufficient space was left between each sown seed to ensure optimum growth conditions. A total of 8 Petri dishes were used for each concentration in our study. For the analyses, twenty sterilized seeds were planted in each petri dish and the harvesting process was carried out three weeks later. The seeds sowed in MS media were grown at an illumination intensity of 1500 lx for 15 days at 25 ± 1 °C and a photo-period of 16 h light/8 hours dark. The application groups and dosages were designed based on preliminary studies and previous reference studies (Yazıcılar et al. 2021; Yazıcılar and Bezirganoğlu 2023) and they are given in Table 1 below.

RNA isolation

RNA was isolated using the leaves and roots of both alfalfa varieties. Firstly, the centrifuge was set to 4 °C. 0.3 g of sample was crushed in liquid nitrogen. It was homogenized by adding 1 mL of Trizol. It was kept at 25 °C for 5 min. 200 µL of chloroform was added and shaken. It was kept at 25 °C for 3 min. It was centrifuged at 12,000 rpm for 15 min at +4 °C. The supernatant was taken. 250 µL of isopropanol was added and turned upside down. It was waited for 10 min at 25 °C. It was centrifuged at 12,000 rpm for 10 min. The supernatant was removed. 1 mL of 70% ethanol, kept at -20 °C, was added to the pellet and turned upside down. It was centrifuged at 7500 rpm for 5 min. The supernatant was removed. It took a while for the alcohol to go away. Pellets were dissolved with 40 µL of ddH₂O₂ (ultra pure water) (Ma et al. 2020).

Table 1 Salt stress and CaO NP dosages applied in the study

Treatment	Treatment No	Concentrations	Petri Number
Control	1	0 mM NaCl+0 ppm CaO	8
Salt stress (NaCl)	2	50 mM NaCl	8
	3	100 Mm NaCl	8
CaO NP	4	0.5 ppm CaO	8
	5	1.5 ppm CaO	8
Combinations	6	50 mM NaCl+0.5 ppm CaO	8
	7	50 mM NaCl+1.5 ppm CaO	8
	8	100 mM NaCl+0.5 ppm CaO	8
	9	100 mM NaCl+1.5 ppm CaO	8

RNA concentration

Concentrations of RNAs isolated using Trizol were measured in nanodrop absorbance at 260 nm wavelength. Concentrations of RNAs were calculated as 1000 ng/RNA absorbance value.

cDNA synthesis

cDNA was synthesized using the Hydra cDNA synthesis kit. The stem-loop primers of 4 miRNAs were coupled in a single tube. The total volume was adjusted to be 20 µL, with 4 µL of 5X RT Buffer, 1 µL dNTP, 2 µL (x2) stem-loop, 1 µL cDNARTase, RNA and Nuclease-free H₂O as variable. Reaction steps: 25°C 10 min; 50°C 30 min; 85°C for 5 min; 4°C ∞. After cDNA synthesis was finished, it was diluted with 60 µL of ddH₂O.

Realtime PCR

SOLIS BIODYNE EvaGreen was used for RT-PCR. 2 µL of Supremix (5x HOT FIREPoIR EvaGreenR qPCR), 0.2 µLs of Forward and Reverse primers, 2 µL of cDNA and 5.6 µL of dH₂O were taken and the total volume was prepared to be 10 µL. The real-time PCR steps for the reference gene U6 are as follows: 2 µL of Supremix, (5x), 0.2 µL of Forward, Reverse and Universal primers, 2 µL of cDNA and 5.4 µL of dH₂O, and a total volume of 10 µL were prepared. The annealing temperature of the reaction varied according to the miRNA used and the plant tissues. PCR reactions of miRNA-393a-root, miRNA-166a-root, U6-root, miRNA-159a-leaf and U6-leaf sequences 95°C 15 min, 95°C 15 s, 58°C 30 s, 72°C 30 s was established. For miRNA156a-root and miRNA156a-leaf sequences, reaction conditions of 95

°C for 15 min, 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s were preferred. For miRNA-159a-root sequences, these conditions were 95°C 15 min, 95°C 15 s, 60°C 30 s, and 72°C 30 s. In the miRNA-393a-leaf sequence, the reaction was carried out at 95°C for 15 min, 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. In miRNA-166a-leaf sequence, reaction conditions of 95°C for 15 min, 95°C for 15 s, 55°C for 30 s and 72°C for 30 s were applied. All reactions were run for 40 cycles (The kit protocol was used). Primers were designed using the Blast database and were obtained from Sentebio-lab. The primers used are shown in Table 2.

Heatmap

Realtime PCR results were tested in three biological replicates for each sample and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The results were evaluated by making heatmap in heatmapgenerator5 tool.

Determination of hydrogen peroxide content

Plants samples (0.2 g) was homogenized with cold acetone and centrifuged for 10 min at 10,000 xg at +4 °C. Then, 0.15 mL of 5% Ti(SO₄)₂ and 0.3 mL of NH₄OH were added into 1.5 mL of the supernatant. After the precipitate formed, it was centrifuged at 10,000 xg for 10 min. The supernatant was removed. The pellet was dissolved in 3 mL of 2 M H₂SO₄. The absorbance at 415 nm was measured in the nanodrop. With the help of the previously prepared H₂O₂ standard graph, it was converted to the amount of H₂O₂ in nanograms. The results are given as the amount of H₂O₂ per g leaf and root (n grams/g tissue) (Mori et al. 1956; He et al. 2005).

Determination of soluble sugar content

A 0.1 g of plant sample was crushed in liquid nitrogen and then homogenized with 5 mL of 2.5 N HCl. Afterward, the mixture was centrifuged at 9000 g for 10 min. The supernatant was carefully transferred to another glass tube and 2 mL of 3,5-dinitrosalicylic acid (DNSA) reagent was added to the supernatant. The mixture was incubated for 20 min in a 90 °C water bath, then quickly transferred to an ice+water bath to cool. Na₂CO₃ was added to the cooled mixture. Subsequently, 100 µL was placed in 96-well plates and measured at 550 nm in triplicate according to the method described by Miller et al. (1959).

Table 2 Sequence information of primers used in qRT-PCR

miRNAs	Primers
miRNA-mtr-393a	5'ACCGGTCCAAAGGGATCGCA 3'
miRNA- mtr-166a	5'ACCGGTCCGACCAGGCT 3'
miRNA- mtr-156a	5'ACCGGGGTGACAGAAGAGAGAG 3'
miRNA- mtr-159a	5'ACTGGGTTTGGATTGAAGGGAGC3'
Reverse Primer (universal)	5'CCAGTGCAGGGTCCGAGGTA3'
Reference gene-U6-Forward	5'ATTGAACATACAGGA3'
Reference gene-U6-Reverse	5'CGATAAAATTGGACCATTCTCTCG3'
Stem-loop miRNA-mtr-393a	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATCAA3'
Stem-loop miRNA- mtr-166a	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAA3'
Stem-loop miRNA- mtr-156a	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTGCT 3'
Stem-loop miRNA- mtr-159a	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACAGC 3'

Determination of lipid peroxidation level

Lipid peroxidation (LPO) level was assessed by measuring malondialdehyde (MDA) content. Tissue samples (0.2 g) were initially homogenized in liquid nitrogen followed by homogenization in 5 mL of 0.1% trichloroacetic acid (TCA). The resulting homogenate was centrifuged at 15,000 rpm for 15 min at +4 °C. The supernatant was taken into another eppendorf tube. Then, it was prepared as 1 mL of 20% TCA+1 mL of 0.5% TBA and 1 mL of 0.1% TCA in the blank. A 1 mL aliquot of supernatants were added to 1 mL of 1% thiobarbituric acid (TBA) and incubated in a water bath at 95 °C for 30 min, followed by rapid cooling on ice. Absorbance measurements were made at 440 nm, 532 nm and 600 nm in 96 well-plates with 3 repetitions. The averages of absorbance values for each concentration were calculated by subtracting the blank absorbance value. The calculation was done according to the following formula (Hodges et al. 1999).

$$\text{MDA (nmol/mL)} = \frac{[(A_{532}-A_{600}) - [(A_{440}-A_{600}) \times (0.0571)]]}{157000} \times 10^6$$

Determination of proline content

A sample weighing 0.4 g was crushed in liquid nitrogen and then homogenized by adding 7.5 mL of 3% sulfosalicylic acid. Centrifugation was carried out for 10 min at 6000 g. Two ml of the supernatant was combined 1 mL of acetic acid and 1 mL of acid ninhydrin were added to it. It was prepared as 1 mL of acetic acid, 1 mL of acid ninhydrin and 2 mL of 3% sulfosalicylic acid in the blank. It was waited for 1 h in a 100 °C water bath. Taken in ice water. 4 mL of toluene was added. 100 µL was taken and placed in 96 well-plates. Absorbance values were measured at 520 nm. The blank absorbance value was subtracted from the absorbance values of each concentration (Bates et al. 1973). The proline concentration was determined with a calibration curve.

Determination of photosynthetic pigment content

First, leaves (0.5 g) were ground in liquid nitrogen and then homogenized with 10 mL of 80% cold acetone. Subsequently, 1.5 mL was taken into the falcon tube and centrifuged at 3000 rpm for 5 min. The supernatant was carefully transferred to another falcon tube. Absorbance values were calculated by measuring with wavelengths of 450 nm, 645 nm and 663 nm determined by Witham et al. (1971).

Determination of antioxidant enzyme activities

To extract antioxidant enzymes, 0.2 g of fresh tissue was first homogenized with liquid nitrogen. The homogenized sample was then mixed with 2 mL of a cold buffer solution (0.15 M KH_2PO_4 , pH 7.0) that included 1% polyvinylpyrrolidone (PVP) and 1 mM ethylenediaminetetraacetic acid (EDTA). The mixture was centrifuged at 12,000 rpm for 15 min at +4 °C. The supernatant obtained from this process was used as the substrate for enzyme activity assays, following the method described by Esim et al. (2013). For guaiacol peroxidase (POD; EC 1.11.1.7), the reaction mixture consisted of 3 mL containing 100 µL of 0.1 M KH_2PO_4 (pH 5.5), 5 mM guaiacol, and 10 µL of enzyme extract. The increase in absorbance was measured at 470 nm over 5 min at 1-minute intervals. One enzyme unit was defined as the amount of enzyme that increased the absorbance by 0.01 per minute at 25 °C (Esim et al. 2013). For Superoxide Dismutase (SOD; EC 1.15.1.1), the reaction mixture also had a volume of 3 mL and contained 50 mM KH_2PO_4 (pH 7.8), 13 mM methionine, 75 µM nitroblue tetrazolium (NBT), 2 µM riboflavin, 0.1 mM EDTA, and 100 µL of enzyme extract. The reaction was initiated by adding riboflavin and exposing the mixture to a white light source for 15 min. The reaction was stopped by turning off the light. The intensity of NBT reduction was measured at 560 nm against a blank. One unit of SOD activity was defined as the amount of enzyme that caused a 50% inhibition of NBT reduction observed at 560 nm (Esim et al. 2013).

Statistical analysis

In the experiment, two different alfalfa varieties were used and 20 seeds were planted for each analysis. A total of 72 petri dishes, 8 for each concentration and alfalfa variety, were planted. Each petri dish was accepted as an experimental unit. The leaves and roots of the alfalfa seedlings were collected separately. Seedlings from the same petri dish were bulked together for analysis. Three biological replicates were performed for each analysis. In the study, "Treatment" was accepted as the source of variation and variance analysis was performed using one-way ANOVA test in SPSS 26.0 package program. Means were compared with Duncan Multiple Range test at $P < 0.05$ confidence interval.

Results

Morphological evaluations

In the diploid Bilensoy variety, leaf growth increased under salt stress (50 mM and 100 mM NaCl) compared to the control (Fig. 1B, C), but slight shrinkage occurred with CaO NP application (Fig. 1D, E). Combined treatments of NaCl and CaO NP showed continued leaf growth under salt stress, with higher CaO NP doses mitigating this growth (Fig. 1F–I). Maximum growth was observed with 50 mM NaCl+1.5 ppm CaO NP (Fig. 1G). For the tetraploid Alsancak variety, leaf growth persisted under salt stress (Fig. 1b, c), but CaO NP applications significantly reduced leaf size (Fig. 1d, e). In combined treatments, leaf growth was maintained, but the lowest growth occurred with 100 mM NaCl+0.5 ppm CaO NP (Fig. 1h). No significant differences were found in stem length for either variety (Bilensoy: $p=0.211$; Alsancak: $p=0.204$). However, root length differences were significant ($p<0.05$) (Fig. 2a, b). In Bilensoy, stem length decreased with increasing salt and NP concentrations, while the greatest increase occurred with 100 mM NaCl+1.5 ppm CaO NP. Root length decreased with higher salt concentrations

but increased with 1.5 ppm CaO NP, with the maximum observed at 50 mM NaCl+1.5 ppm CaO NP (Fig. 2a). In Alsancak, stem length increased most at 50 mM NaCl and 0.5 ppm CaO NP. Combined treatments showed greater stem length increases, peaking at 100 mM NaCl+1.5 ppm CaO NP. Root length showed the highest increase at 100 mM NaCl+0.5 ppm CaO NP, with the maximum increase under combined treatments at 50 mM NaCl+1.5 ppm CaO NP (Fig. 2b).

Real time PCR findings

In the Bilensoy variety, miR393a, miR166a, and miR156a expressions were statistically significant in leaves ($p<0.05$), and miR166a and miR156a were significant in roots ($p<0.05$). In Alsancak, miR393a expression was significant in both leaves and roots, while miR166a and miR156a were significant in roots ($p<0.05$) (Supplementary Table 4). In Bilensoy leaves, miR393a, miR166a, and miR159a were down-regulated under salt stress and NP treatments, while miR156a was up-regulated. The highest fold change in miR393a occurred at 100 mM NaCl+0.5 ppm CaO NP, while miR166a showed the lowest at 100 mM NaCl+1.5

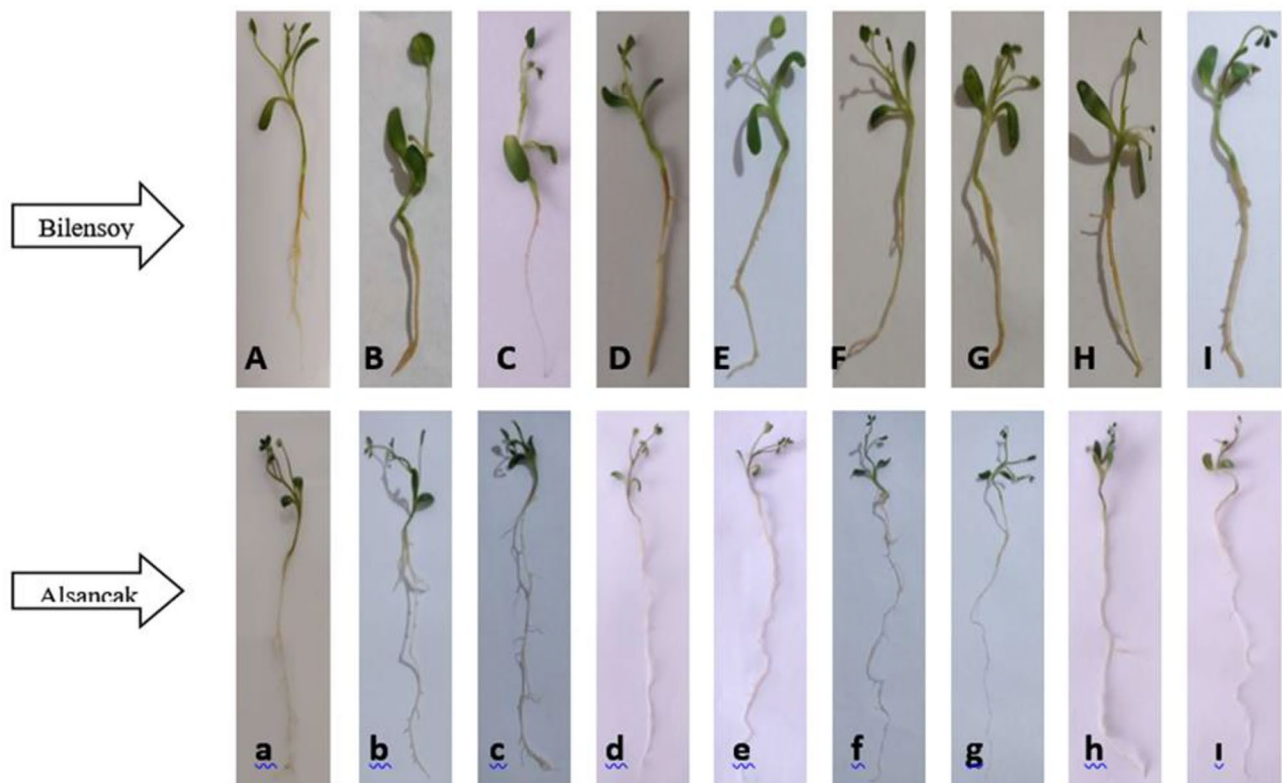


Fig. 1 Seedling growth of Bilensoy and Alsancak varieties. **A, a:** control; **B, b:** 50 mM NaCl; **C, c:** 100 mM NaCl; **D, d:** 0.5 ppm CaO; **E, e:** 1.5 ppm CaO; **F, f:** 50 mM NaCl+0.5 ppm CaO; **G, g:** 50 mM NaCl+1.5 ppm CaO; **H, h:** 100 mM NaCl+0.5 ppm CaO; **I, i:** 100

mM NaCl+1.5 ppm CaO. The small letters indicate the application dosages of the diploid Bilensoy variety, and the large letters indicate the application dosages of the tetraploid Alsancak variety

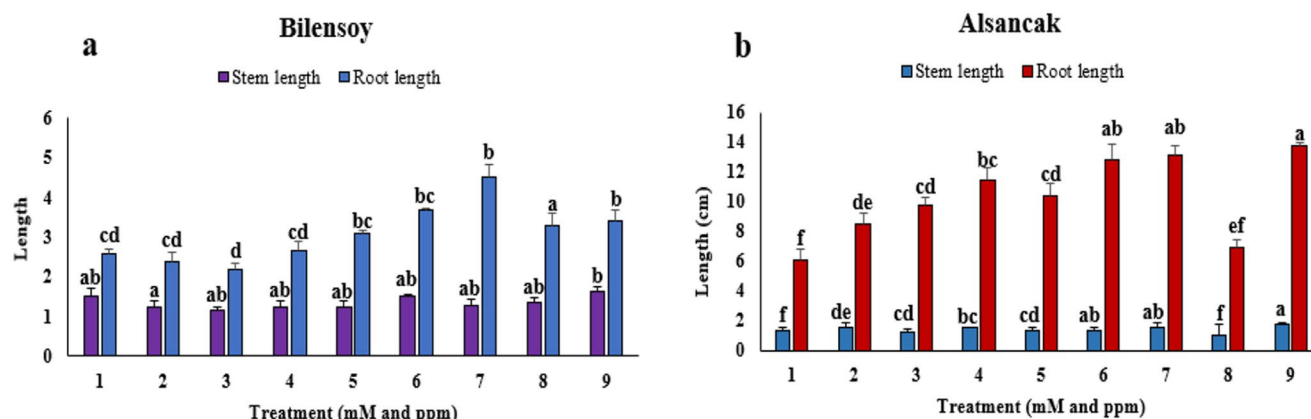


Fig. 2 Stem length and root length in different salt stress and NP-salt treatments of Bilensoy (a) and Alsancak (b). 1: Control; 2: 50 mM NaCl; 3: 100 mM NaCl; 4: 0.5 ppm CaO; 5: 1.5 ppm CaO; 6: 50 mM NaCl+0.5 ppm CaO; 7: 50 mM NaCl+1.5 ppm CaO; 8: 100 mM NaCl+0.5 ppm CaO; 9: 100 mM NaCl+1.5 ppm CaO NP. In the fig-

ure, the columns represent the stem and root lengths at each treatment (mM and ppm). Standard error bars are displayed on the columns. Significant differences among the groups were determined using analysis of variance (ANOVA) ($p < 0.05$), and homogeneous subsets identified by Duncan's multiple comparison test are indicated with letters

ppm CaO NP. MiR159a was up-regulated at certain doses, with the highest increase at 50 mM NaCl+1.5 ppm CaO NP (Fig. 3; Table 3). In Alsancak leaves, miR393a and miR166a were down-regulated, while miR156a and miR159a were up-regulated. During NP treatments, miR393a, miR166a, and miR159a were down-regulated, and miR156a was up-regulated. The most significant down-regulation for miR393a, miR166a, and miR159a occurred at 100 mM NaCl+0.5 ppm CaO NP, 100 mM NaCl+1.5 ppm CaO NP, and 50 mM NaCl+1.5 ppm CaO NP, respectively. MiR156a displayed both up- and down-regulation depending on the treatment (Fig. 3; Table 3). In Bilensoy roots, miR393a was down-regulated, while miR166a, miR156a, and miR159a were up-regulated, with the highest fold change at specific combinations of salt and NP treatments. Combined NP and salt treatments resulted in miR393a down-regulation at all doses except 50 mM NaCl+1.5 ppm CaO NP (Fig. 3; Table 3). In Alsancak roots, miR393a and miR166a were down-regulated, while miR156a was up-regulated in response to salt and NP treatments. MiR159a showed varied regulation depending on the treatment. Combined treatments generally led to down-regulation of miR393a, miR166a, and miR159a, while miR156a was up-regulated except at 100 mM NaCl+1.5 ppm CaO NP (Fig. 3; Table 3).

Hydrogen peroxide content

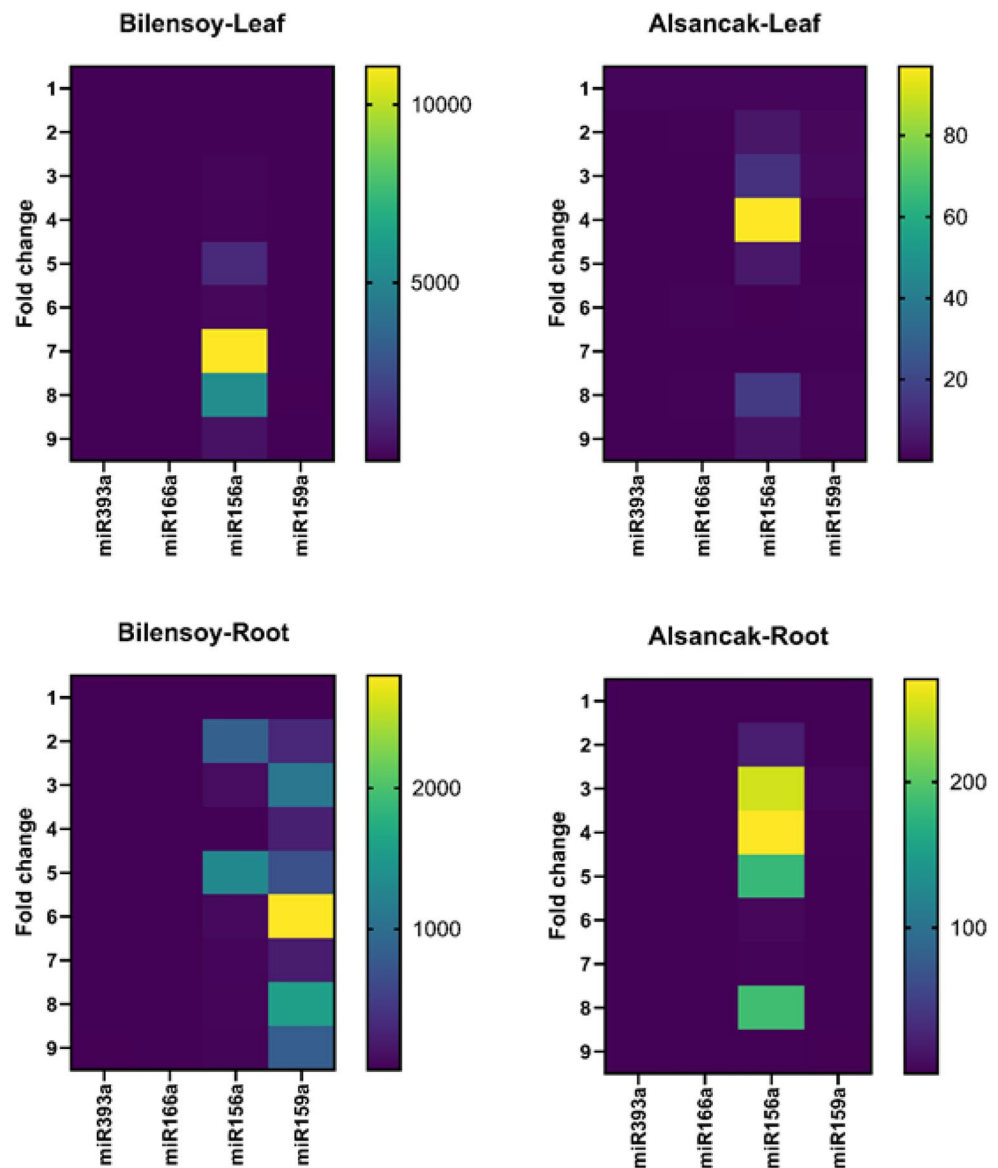
According to one-way ANOVA ($p < 0.05$) (Supplementary Table 5), H_2O_2 damage in Bilensoy leaves was highest at 100 mM NaCl+1.5 ppm CaO NP. The greatest reduction occurred at 50 mM NaCl+0.5 ppm CaO NP, while an increase was noted at 100 mM NaCl+0.5 ppm CaO NP (Fig. 4a, Supplementary Table 12). In Alsancak leaves, H_2O_2 damage was highest at 100 mM NaCl+0.5 ppm CaO

NP, with the greatest reduction at 100 mM NaCl+1.5 ppm CaO NP (Fig. 4b, Supplementary Table 12). In Bilensoy roots, H_2O_2 damage peaked at 100 mM NaCl+0.5 ppm CaO NP, while the greatest reduction occurred at 100 mM NaCl+1.5 ppm CaO NP (Fig. 4a). In Alsancak roots, the highest H_2O_2 damage was observed at 100 mM NaCl, with a reduction at 1.5 ppm CaO NP, and the greatest decrease occurred under 100 mM NaCl+1.5 ppm CaO NP (Fig. 4b, Supplementary Table 12).

Soluble sugar content

The differences between the groups were statistically significant ($p < 0.05$) (Supplementary Table 6). In Bilensoy leaves, soluble sugar content increased most at 100 mM NaCl but decreased at 1.5 ppm CaO NP. In combined treatments, the greatest decrease was observed at 100 mM NaCl+1.5 ppm CaO NP, compared to the control and salt treatments (Fig. 4c, Supplementary Table 12). In Alsancak leaves, soluble sugar content increased the most at 100 mM NaCl+1.5 ppm CaO NP. The highest increase in combined treatments occurred at 50 mM NaCl+0.5 ppm CaO NP, compared to the control. Compared to salt stress alone, increases were observed at 50 mM NaCl+0.5 ppm and 50 mM NaCl+1.5 ppm CaO NP, while decreases were noted at 100 mM NaCl+0.5 ppm and 100 mM NaCl+1.5 ppm (Fig. 4d, Supplementary Table 12). In Bilensoy roots, soluble sugar content increased most at 100 mM NaCl+0.5 ppm CaO NP but decreased at 1.5 ppm CaO NP. In combined treatments, the greatest decrease was at 50 mM NaCl+0.5 ppm CaO NP (Fig. 4c, Supplementary Table 12). In Alsancak roots, soluble sugar content increased the most at 100 mM NaCl+1.5 ppm CaO NP, but decreased at 0.5 ppm CaO NP. In combined treatments, sugar content increased at 50 mM NaCl+0.5 ppm and 50 mM NaCl+1.5

Fig. 3 Heatmap of diploid Bilensoy and tetraploid Alsancak varieties under applied dosages. 1: Control; 2: 50 mM NaCl; 3: 100 mM NaCl; 4: 0.5 ppm CaO; 5: 1.5 ppm CaO; 6: 50 mM NaCl+0.5 ppm CaO; 7: 50 mM NaCl+1.5 ppm CaO; 8: 100 mM NaCl+0.5 ppm CaO; 9: 100 mM NaCl+1.5 ppm CaO



ppm CaO NP, but decreased at 100 mM NaCl+0.5 ppm and 100 mM NaCl+1.5 ppm. The highest increase, compared to salt stress, was at 50 mM NaCl+1.5 ppm CaO NP, while the largest decrease occurred at 100 mM NaCl+0.5 ppm CaO NP (Fig. 4d, Supplementary Table 12).

Lipid peroxidation level

The differences between the groups were statistically significant ($p < 0.05$) (Supplementary Table 7). In Bilensoy leaves, MDA levels were highest at 100 mM NaCl+1.5 ppm CaO NP. In combined nanoparticle and salt stress treatments, MDA levels increased at 50 mM NaCl+0.5 ppm and 100 mM NaCl+0.5 ppm CaO NP but decreased at 50 mM NaCl+1.5 ppm and 100 mM NaCl+1.5 ppm CaO NP. The greatest reduction compared to salt stress alone was seen at

50 mM NaCl+1.5 ppm CaO NP (Fig. 4e, Supplementary Table 12). In Alsancak leaves, MDA levels peaked at 100 mM NaCl but decreased at 1.5 ppm CaO NP. In combined treatments, the greatest reduction in MDA levels occurred at 100 mM NaCl+0.5 ppm CaO NP (Fig. 4f, Supplementary Table 12). In Bilensoy roots, MDA levels were highest at 100 mM NaCl+0.5 ppm CaO NP. Combined treatments led to the greatest increase at 100 mM NaCl+1.5 ppm CaO NP, while the largest reduction was seen at 50 mM NaCl+1.5 ppm CaO NP (Fig. 4e, Supplementary Table 12). In Alsancak roots, MDA levels were highest at 100 mM NaCl, but decreased at 0.5 ppm CaO NP. The greatest reduction under combined stress was observed at 100 mM NaCl+1.5 ppm CaO NP (Fig. 4f, Supplementary Table 12).

Table 3 Fold change obtained from real-time PCR result with $2^{-\Delta\Delta ct}$ method

Treatment	Bilensoy		Alsancak	
	Leaf (Mean $\pm$ SE)	Root (Mean $\pm$ SE)	Leaf (Mean $\pm$ SE)	Root (Mean $\pm$ SE)
miR393a				
1	1,0000 $\pm$ 0,00000 ^{bc}	1,0000 $\pm$ 0,00000 ^a	1,0000 $\pm$ 0,00000 ^a	1,0000 $\pm$ 0,00000 ^a
2	0,4750 $\pm$ 0,01810 ^c	0,4732 $\pm$ 0,19579 ^a	0,2803 $\pm$ 0,11200 ^b	0,1031 $\pm$ 0,11200 ^b
3	0,5958 $\pm$ 0,11620 ^c	0,5724 $\pm$ 0,12493 ^a	0,1642 $\pm$ 0,07399 ^b	0,0395 $\pm$ 0,07399 ^c
4	0,5779 $\pm$ 0,06383 ^c	0,5565 $\pm$ 0,13605 ^a	0,2215 $\pm$ 0,02681 ^b	0,0350 $\pm$ 0,02681 ^c
5	0,5833 $\pm$ 0,03231 ^c	0,3893 $\pm$ 0,11765 ^a	0,1653 $\pm$ 0,04790 ^b	0,0271 $\pm$ 0,04790 ^c
6	0,8185 $\pm$ 0,16778 ^{bc}	0,3146 $\pm$ 0,16174 ^a	0,2803 $\pm$ 0,18948 ^b	0,0309 $\pm$ 0,18948 ^c
7	2,6481 $\pm$ 0,53405 ^a	1,0051 $\pm$ 0,76028 ^a	0,1489 $\pm$ 0,04317 ^b	0,0122 $\pm$ 0,04317 ^d
8	2,8396 $\pm$ 0,08854 ^a	0,4406 $\pm$ 0,02593 ^a	0,1476 $\pm$ 0,04185 ^b	0,0120 $\pm$ 0,04185 ^d
9	1,4492 $\pm$ 0,20947 ^b	0,2249 $\pm$ 0,09817 ^a	0,1516 $\pm$ 0,04869 ^b	0,0113 $\pm$ 0,04869 ^d
miR166a				
1	1,0000 $\pm$ 0,00000 ^a	1,0000 $\pm$ 0,00000 ^{cd}	1,0000 $\pm$ 0,00000 ^a	1,0000 $\pm$ 0,00000 ^a
2	0,6928 $\pm$ 0,05987 ^{ab}	3,0940 $\pm$ 0,79661 ^b	0,5187 $\pm$ 0,10115 ^b	0,1335 $\pm$ 0,03263 ^{bc}
3	0,5766 $\pm$ 0,01798 ^{ab}	4,7741 $\pm$ 0,69006 ^a	0,3541 $\pm$ 0,03547 ^b	0,2240 $\pm$ 0,11285 ^b
4	0,5968 $\pm$ 0,08626 ^{ab}	1,7532 $\pm$ 0,00000 ^{cd}	0,3729 $\pm$ 0,03607 ^b	0,1659 $\pm$ 0,05632 ^{bc}
5	0,5390 $\pm$ 0,21364 ^{ab}	2,2581 $\pm$ 0,41774 ^{bc}	0,3340 $\pm$ 0,02196 ^b	0,1080 $\pm$ 0,02500 ^{bc}
6	0,4197 $\pm$ 0,19591 ^{ab}	1,0529 $\pm$ 0,11990 ^{cd}	0,7145 $\pm$ 0,37299 ^{ab}	0,0960 $\pm$ 0,03345 ^{bc}
7	0,8545 $\pm$ 0,24740 ^{ab}	3,1317 $\pm$ 0,16266 ^b	0,3729 $\pm$ 0,07148 ^b	0,0755 $\pm$ 0,00756 ^{bc}
8	0,6593 $\pm$ 0,32013 ^{ab}	1,3334 $\pm$ 0,01386 ^{cd}	0,4516 $\pm$ 0,09557 ^b	0,0582 $\pm$ 0,01018 ^c
9	0,2769 $\pm$ 0,10732 ^a	0,6457 $\pm$ 0,12808 ^d	0,3299 $\pm$ 0,00229 ^b	0,0478 $\pm$ 0,00298 ^c
miR156a				
1	1,0000 $\pm$ 0,00000 ^c	1,0000 $\pm$ 0,00000 ^b	1,0000 $\pm$ 0,00000 ^b	1,0000 $\pm$ 0,00000 ^c
2	2,4006 $\pm$ 1,88652 ^c	848,2849 $\pm$ 560,26992 ^{ab}	5,3337 $\pm$ 3,60461 ^b	20,8692 $\pm$ 3,72081 ^{bc}
3	103,3398 $\pm$ 81,48315 ^c	78,3169 $\pm$ 61,75268 ^b	13,0055 $\pm$ 2,44946 ^b	251,3493 $\pm$ 57,33754 ^a
4	67,9088 $\pm$ 58,32898 ^c	9,8083 $\pm$ 8,69872 ^b	96,9194 $\pm$ 60,66710 ^a	270,4046 $\pm$ 131,30250 ^a
5	1255,4667 $\pm$ 1001,23500 ^c	1304,0520 $\pm$ 466,52027 ^a	6,0814 $\pm$ 0,69251 ^b	180,8211 $\pm$ 58,03532 ^{abc}
6	187,0301 $\pm$ 98,99542 ^c	65,1379 $\pm$ 1,57994 ^b	0,0094 $\pm$ 0,00324 ^b	4,7099 $\pm$ 0,79224 ^c
7	11058,0197 $\pm$ 688,94169 ^a	29,1625 $\pm$ 21,05085 ^b	0,0133 $\pm$ 0,00356 ^b	1,9285 $\pm$ 0,11349 ^c
8	5363,4762 $\pm$ 1063,83670 ^b	24,1059 $\pm$ 20,52585 ^b	15,7161 $\pm$ 13,72991 ^b	186,7527 $\pm$ 15,49792 ^{ab}
9	495,4138 $\pm$ 286,03089 ^c	20,0242 $\pm$ 18,29512 ^b	4,2813 $\pm$ 3,44620 ^b	0,1998 $\pm$ 0,01933 ^c
miR159a				
1	1,0000 $\pm$ 0,00000 ^a	1,0000 $\pm$ 0,00000 ^a	1,0000 $\pm$ 0,00000 ^a	1,0000 $\pm$ 0,00000 ^a
2	0,4579 $\pm$ 0,19729 ^a	304,3019 $\pm$ 263,79768 ^a	1,6115 $\pm$ 0,51722 ^a	0,0831 $\pm$ 0,00403 ^b
3	0,4656 $\pm$ 0,21737 ^a	1099,1412 $\pm$ 851,86146 ^a	2,2252 $\pm$ 1,13843 ^a	3,5354 $\pm$ 1,85357 ^b
4	0,0084 $\pm$ 0,00145 ^a	238,3485 $\pm$ 229,53323 ^a	0,5645 $\pm$ 0,04684 ^a	1,2968 $\pm$ 0,10762 ^b
5	0,5550 $\pm$ 0,40427 ^a	686,6321 $\pm$ 282,13094 ^a	0,1883 $\pm$ 0,10290 ^a	0,7130 $\pm$ 0,41984 ^b
6	1,0604 $\pm$ 0,76723 ^a	2796,7054 $\pm$ 2607,99897 ^a	0,4963 $\pm$ 0,23571 ^a	0,0955 $\pm$ 0,01412 ^b
7	1,0951 $\pm$ 0,62205 ^a	204,1155 $\pm$ 186,60683 ^a	0,2271 $\pm$ 0,02818 ^a	0,0226 $\pm$ 0,00273 ^b
8	0,0066 $\pm$ 0,00494 ^a	1564,5999 $\pm$ 1454,70349 ^a	0,9576 $\pm$ 0,84492 ^a	0,1366 $\pm$ 0,04385 ^b
9	0,9536 $\pm$ 0,78751 ^a	811,7804 $\pm$ 772,92616 ^a	0,9497 $\pm$ 0,87793 ^a	0,0097 $\pm$ 0,00212 ^b

Values with different superscripts in the same column differ significantly at $P < 0.05$ according to Duncan's test; expressed as lowercase = difference in line. SE: Standard Error. 1: Control; 2: 50 mM NaCl; 3: 100 mM NaCl; 4: 0.5 ppm CaO; 5: 1.5 ppm CaO; 6: 50 mM NaCl+0.5 ppm CaO; 7: 50 mM NaCl+1.5 ppm CaO; 8: 100 mM NaCl+0.5 ppm CaO; 9: 100 mM NaCl+1.5 ppm CaO NP

Proline level

The one-way ANOVA analysis showed statistically significant differences between the groups ($p < 0.05$) (Supplementary Table 8). In Bilensoy leaves, proline levels increased the most under 100 mM NaCl, while a decrease was observed at 0.5 ppm CaO NP. In combined treatments of nanoparticles and salt stress, the largest reduction in proline levels compared to the control and salt stress alone occurred at

50 mM NaCl+1.5 ppm CaO NP (Fig. 4g, Supplementary Table 12). For Alsancak leaves, proline levels were highest at 100 mM NaCl and 1.5 ppm CaO NP. In combined treatments, proline levels increased at 50 mM NaCl+0.5 ppm CaO NP and 100 mM NaCl+0.5 ppm CaO NP, but decreased at 50 mM NaCl+1.5 ppm CaO NP and 100 mM NaCl+1.5 ppm CaO NP. The greatest reduction compared to salt stress alone was seen at 100 mM NaCl+1.5 ppm CaO NP (Fig. 4h). In Bilensoy roots, proline levels were highest

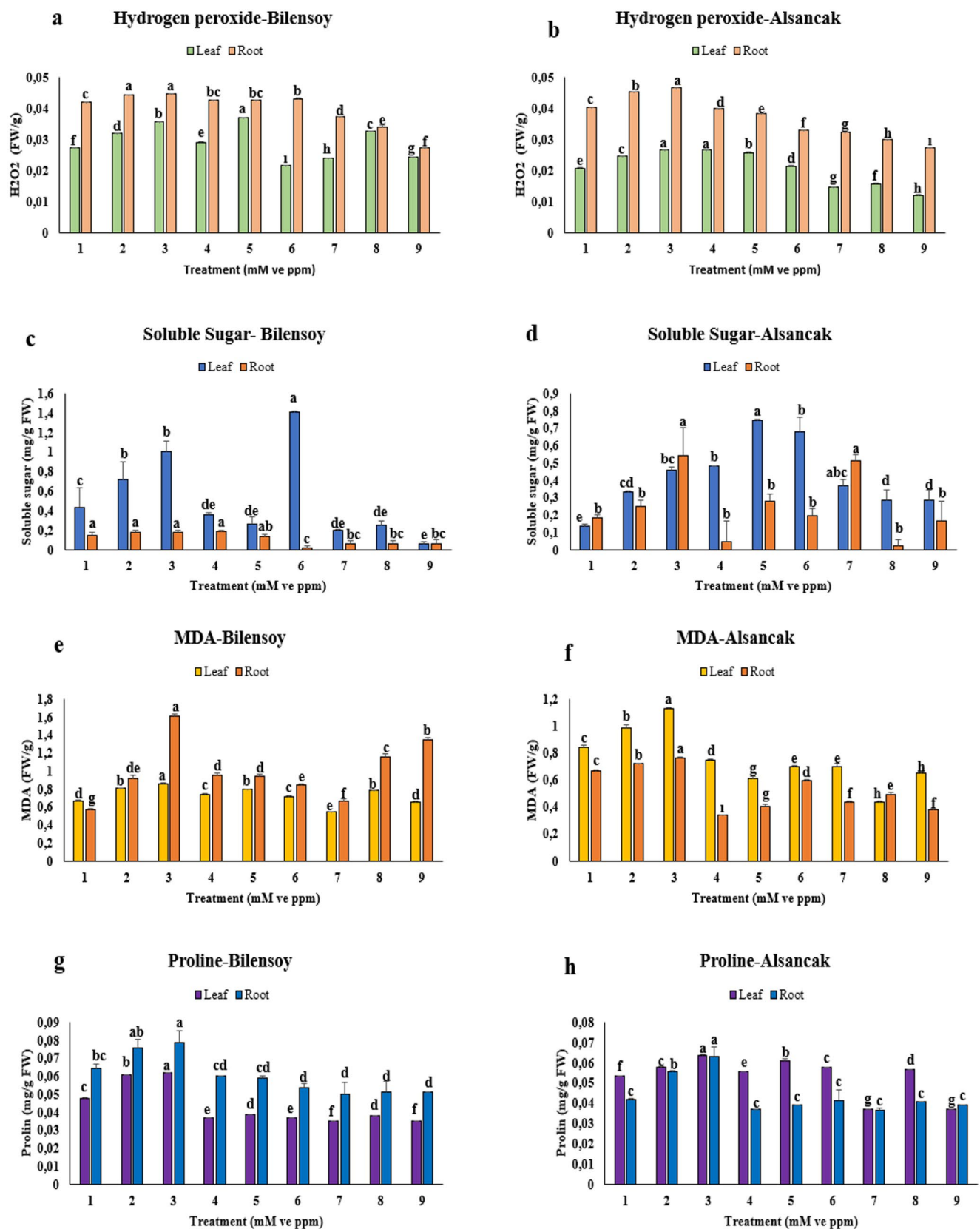


Fig. 4 H₂O₂, Soluble sugar, MDA amount and proline level in different salt stress and NP-salt applications. a: Bilensoy leaf and root hydrogen peroxide amount; b: Alsancak leaf and root hydrogen peroxide amount; c: Bilensoy leaf and root soluble sugar amount; d: Alsancak leaf and root soluble sugar amount; e: Bilensoy leaf and root MDA amount; f: Alsancak leaf and root MDA amount; g: Bilensoy leaf and root proline level; h: Alsancak leaf and root proline level; 1: Control; 2: 50 mM NaCl; 3: 100 mM NaCl; 4: 0.5 ppm CaO; 5: 1.5 ppm CaO; 6: 50 mM NaCl+0.5 ppm CaO; 7: 50mM NaCl+1.5 ppm CaO; 8: 100mM NaCl+0.5 ppm CaO; 9: 100 mm NaCl+1.5 ppm CaO. In the figure, the columns represent the stem and root lengths at each treatment (mM and ppm). Standard error bars are displayed on the columns. Significant differences among the groups were determined using analysis of variance (ANOVA) ($p < 0.05$), and homogeneous subsets identified by Duncan's multiple comparison test are indicated with letters

at 100 mM NaCl, with a decrease at 1.5 ppm CaO NP. The most significant reduction in proline levels under combined treatments was observed at 50 mM NaCl+1.5 ppm CaO NP (Fig. 4g, Supplementary Table 12). In Alsancak roots, proline levels peaked at 100 mM NaCl but decreased at 0.5 ppm CaO NP. The largest reduction in proline levels under combined treatments compared to the control and salt stress alone was at 50 mM NaCl+1.5 ppm CaO NP (Fig. 4h).

Photosynthetic pigment content

The one-way ANOVA analysis revealed statistically significant differences between the groups ($p < 0.05$) (Supplementary Table 9). In Bilensoy leaves, chlorophyll a content showed the greatest decrease at 100 mM NaCl, but increased at 1.5 ppm CaO NP. The largest decrease in combined treatments was at 100 mM NaCl+0.5 ppm CaO NP, while the highest increase was at 100 mM NaCl+1.5 ppm CaO NP (Fig. 5a, Supplementary Table 13). In Alsancak, chlorophyll a content decreased the most at 100 mM NaCl and 0.5 ppm CaO NP, while it increased at lower NaCl concentrations combined with CaO NP. The highest increase under salt stress was at 50 mM NaCl+1.5 ppm CaO NP (Fig. 5b). For chlorophyll b, Bilensoy showed a similar pattern, with the highest decrease at 100 mM NaCl and the highest increase at 100 mM NaCl+1.5 ppm CaO NP (Fig. 5a). In Alsancak, chlorophyll b content decreased at higher NaCl and CaO NP concentrations but increased under combined treatments at 50 mM NaCl (Fig. 5b). Total chlorophyll in Bilensoy followed the same trend, with the greatest increase at 100 mM NaCl+1.5 ppm CaO NP (Fig. 5c). In Alsancak, total chlorophyll decreased at higher NaCl concentrations but increased under lower NaCl+CaO NP treatments, with the highest increase at 50 mM NaCl+1.5 ppm CaO NP (Fig. 5d). In terms of total carotenoids, Bilensoy saw the largest decrease at 100 mM NaCl and the highest increase at 50 mM NaCl+0.5 ppm CaO NP (Fig. 5e). In Alsancak, total carotenoids increased at 50 mM NaCl but decreased at higher NaCl and CaO NP concentrations, with the largest increase

observed at 50 mM NaCl+1.5 ppm CaO NP (Fig. 5f, Supplementary Table 13).

Antioxidant enzyme activity findings

The one-way ANOVA analysis shows statistically significant differences between the groups ($p < 0.05$) (Supplementary Table 10). In Bilensoy leaves, POD levels increased the most at 100 mM NaCl but decreased at 1.5 ppm CaO NP. In combined salt and nanoparticle stress, the highest increase was at 100 mM NaCl+1.5 ppm CaO NP, while 50 mM NaCl+CaO NP combinations also showed increases (Fig. 6a, Supplementary Table 14). In Alsancak leaves, POD levels increased at 100 mM NaCl and 1.5 ppm CaO NP, with the greatest increase at 100 mM NaCl+1.5 ppm CaO NP (Fig. 6b). In Bilensoy roots, POD levels increased at 100 mM NaCl and 0.5 ppm CaO NP, but decreased at 1.5 ppm CaO NP. The greatest reduction under combined stress was at 50 mM NaCl+0.5 ppm CaO NP (Fig. 6a). In Alsancak roots, POD levels increased at 100 mM NaCl and 1.5 ppm CaO NP, with the highest increase at 100 mM NaCl+1.5 ppm CaO NP (Fig. 6b, Supplementary Table 14). For SOD levels, the analysis (Supplementary Table 11) shows that in Bilensoy leaves, SOD levels increased most at 100 mM NaCl and 1.5 ppm CaO NP, with the highest increase at 100 mM NaCl+1.5 ppm CaO NP (Fig. 6c). In Alsancak leaves, SOD levels increased at 100 mM NaCl and 1.5 ppm CaO NP but decreased at 0.5 ppm CaO NP. The greatest increase was observed at 100 mM NaCl+1.5 ppm CaO NP (Fig. 6d). In Bilensoy roots, SOD levels increased most at 100 mM NaCl but decreased at 1.5 ppm CaO NP. Under combined stress, the greatest reduction was at 50 mM NaCl+0.5 ppm CaO NP (Fig. 6c). In Alsancak roots, SOD levels increased at 100 mM NaCl but decreased at 1.5 ppm CaO NP, with the highest increase in combined stress at 100 mM NaCl+1.5 ppm CaO NP (Fig. 6d).

Discussion

Salt stress negatively impacts plant height, leaf number, shoot and root length, while increasing necrotic areas (Shahid et al. 2020). Similar effects have been observed in maize (QN701 cultivar), where plant height, leaf number, and biomass decreased under salt stress, while leaf area increased (Dikobe et al. 2021). Low salt concentrations have also been shown to enhance shoot and root lengths in forage cowpea, but higher concentrations cause significant reductions (Bakirov et al. 2022). In this study, leaves of both the diploid Bilensoy and tetraploid Alsancak varieties showed growth under salinity stress (Fig. 1B, C, b and c). However, Bilensoy exhibited reduced stem and root

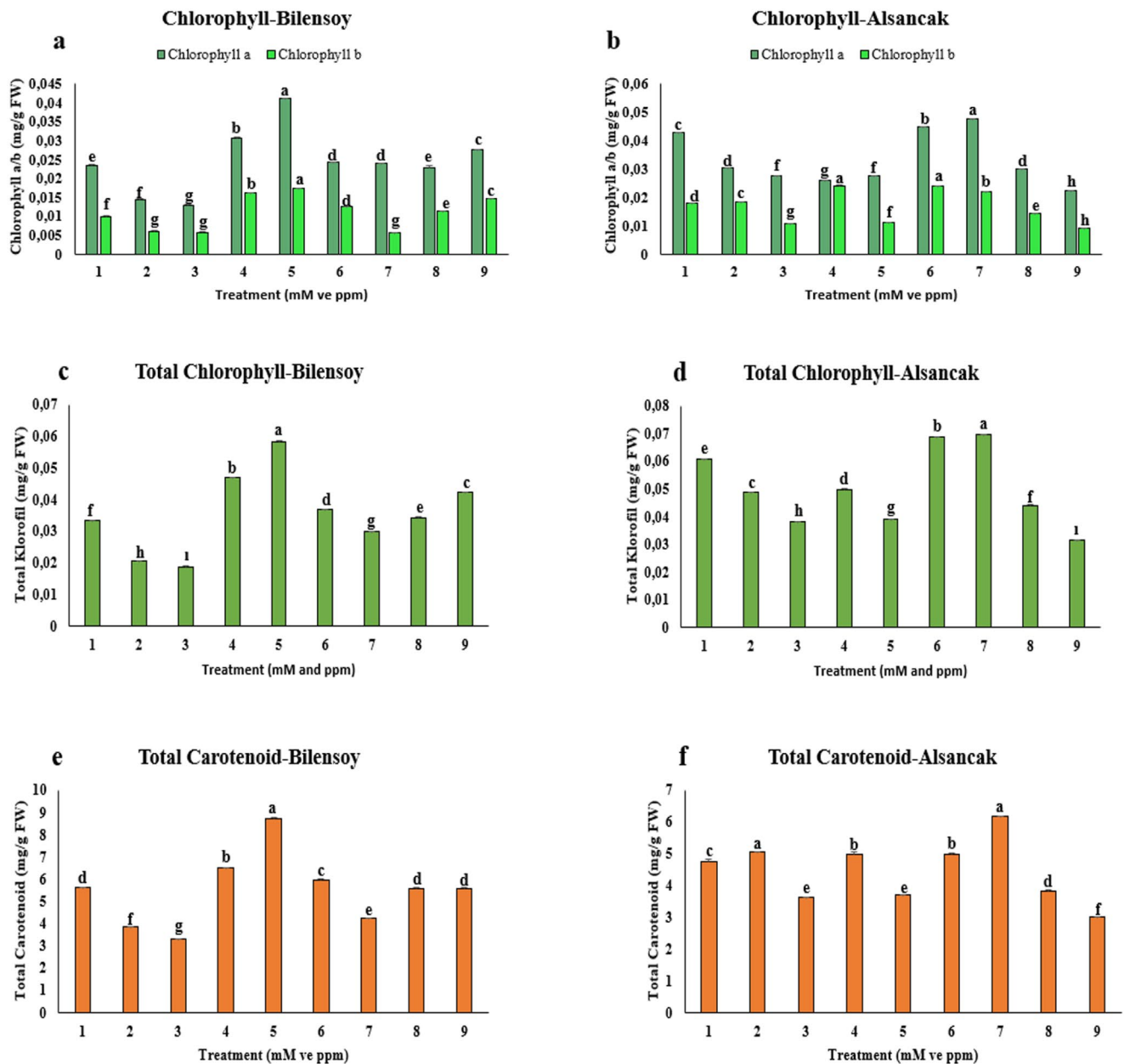


Fig. 5 Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid content in different salt stress and NP-salt applications. a: Bilensoy chlorophyll a and chlorophyll b; b: Alsancak chlorophyll a and chlorophyll b; c: Bilensoy total chlorophyll; d: Alsancak total chlorophyll; e: Bilensoy total carotenoid; Alsancak total carotenoid. 1: Control; 2: 50 mM NaCl; 3: 100 mM NaCl; 4: 0.5 ppm CaO; 5: 1.5 ppm CaO; 6: 50 mM NaCl + 0.5 ppm CaO; 7: 50 mM NaCl + 1.5 ppm CaO;

8: 100 mM NaCl + 0.5 ppm CaO; 9: 100 mM NaCl + 1.5 ppm CaO. In the figure, the columns represent the stem and root lengths at each treatment (mM and ppm). Standard error bars are displayed on the columns. Significant differences among the groups were determined using analysis of variance (ANOVA) ($p < 0.05$), and homogeneous subsets identified by Duncan's multiple comparison test are indicated with letters

lengths, while Alsancak showed variable responses depending on salt concentration (Figs. 1B, C, b and c and 2a and b). These findings suggest that the contrasting responses may result from differences in ploidy or stress tolerance mechanisms. Nanoparticles are known to positively influence plant growth and stress tolerance (Gelaw 2022; Khodakovskaya et al. 2013). For instance, Fe-NPs have improved

root length in blackberry plants, while other nanoparticles show variable effects (Rastogi et al. 2017). In this study, CaO NP applications inhibited leaf growth in both varieties (Fig. 1D, E, d and e). Root length responses, however, differed: Bilensoy showed a decrease (Figs. 1D and E and Fig. 2a), whereas Alsancak displayed an increase (Figs. 1d and e and 2b). The enhanced root growth in Alsancak may

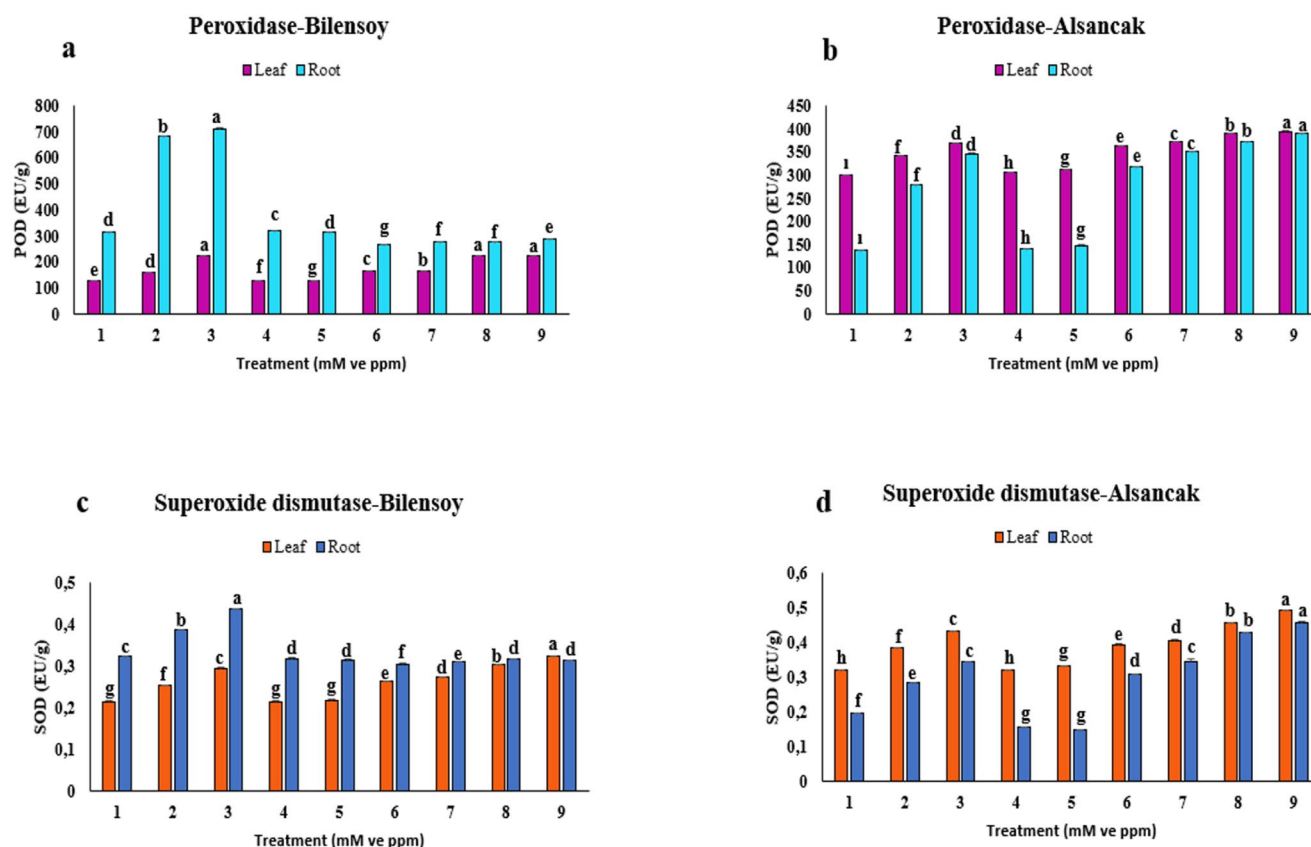


Fig. 6 Different salt stress and the level of POD and SOD in NP-salt applications. a: Bilensoy-leaf and root POD level; b: Alsancak-leaf and root POD level; c: Bilensoy-leaf and root SOD level; d: Bilensoy-Alsancak-leaf and root SOD level. 1: Control; 2: 50 mM NaCl; 3: 100 mM NaCl; 4: 0.5 ppm CaO; 5: 1.5 ppm CaO; 6: 50 mM NaCl+0.5 ppm CaO; 7: 50 mM NaCl+1.5 ppm CaO; 8: 100 mM NaCl+0.5 ppm

CaO; 9: 100 mM NaCl+1.5 ppm CaO NP. In the figure, the columns represent the stem and root lengths at each treatment (mM and ppm). Standard error bars are displayed on the columns. Significant differences among the groups were determined using analysis of variance (ANOVA) ($p < 0.05$), and homogeneous subsets identified by Duncan's multiple comparison test are indicated with letters

be attributed to its greater nuclear size, which aids in abiotic stress tolerance (Del Pozo and Ramirez-Parra 2015). Under combined NP and salt stress, both varieties maintained relatively balanced growth (Figs. 1 and 2). Stem and root length increases, observed only in Alsancak under NP treatment, were also evident in both varieties under combined CaO NP and salt treatments (Figs. 1F–I and f–i and 2a and b). This suggests that Bilensoy is more sensitive to the synergistic effects of salt stress and NP applications than Alsancak. While no statistically significant differences in stem length were observed between treatment groups for either variety, root length differences were statistically significant (Supplementary Table 5). These findings align with existing morphological data and reinforce the role of nanoparticles in mitigating stress effects.

Plants develop complex defense mechanisms to cope with salt stress, with microRNAs (miRNAs) playing a key role in post-transcriptional regulation (Xu et al. 2021). MiRNAs regulate gene expression under stress conditions, influencing plant responses to salt stress (Islam et al. 2022).

For instance, miR156 has been shown to be upregulated in alfalfa under salt stress (Arshad et al. 2017), and miR156, miR166, and miR393 are upregulated in both wild-type and mutant alfalfa lines, whereas miR159 shows differential regulation (Ma et al. 2020). In rice, miR393a is down-regulated under salt stress (Gao et al. 2011). In this study, salt stress caused down-regulation of miR159a, miR166a, and miR393a in the leaves of the diploid Bilensoy variety, while miR156a was upregulated. In the roots of Bilensoy, miR156a, miR159a, and miR166a were upregulated, while miR393a was downregulated (Fig. 3; Table 3). In the tetraploid Alsancak variety, miR156a and miR159a were upregulated in the leaves, while miR166a and miR393a were downregulated. In the roots of Alsancak, miR156a was upregulated, while miR393a and miR166a were down-regulated. MiR159a exhibited differential responses under different salt concentrations (Fig. 3; Table 3). These results confirm species-specific differences in miRNA expression under salt stress. Nanoparticles can modulate gene expression related to stress tolerance (Gelaw 2022). Studies show

that nanoparticles influence miRNA expression profiles involved in plant development and stress responses (Ruotolo et al. 2018). For example, low concentrations of aluminum oxide nanoparticles upregulated miR156, miR157, and miR172 in tobacco, while higher concentrations affected several miRNAs, including miR156, miR159, and miR399 (Burklew et al. 2012). In this study, CaO NP application in Bilensoy resulted in downregulation of miR159a, miR166a, and miR393a, while miR156a was upregulated. In combined NP and salt stress treatments, miR156a and miR393a were upregulated, while miR166a and miR159a were downregulated. In the roots of Bilensoy, miR159a, miR166a, and miR156a were upregulated under NP and combined NP and salt stress treatments, while miR393a was downregulated. In Alsancak, miR159a, miR166a, and miR393a were downregulated in both leaves and roots under combined NP and salt stress, while miR156a was upregulated (Fig. 3; Table 3). These differences in miRNA expression likely result from variations in NP dosage and stress defense mechanisms between the diploid and tetraploid species. Statistically significant differences in miRNA expression were observed for miR393a in Bilensoy leaves and Alsancak leaves and roots ($p < 0.05$), miR166a in the roots of both varieties ($p < 0.05$), and miR156a in the leaves and roots of Bilensoy and in the roots of Alsancak ($p < 0.05$) (Supplementary Table 6).

Hydrogen peroxide (H_2O_2) is a common cellular metabolite produced through enzymatic and non-enzymatic reactions during processes like photosynthesis and respiration (Hajihashemi et al. 2020). It plays a role in various cellular functions under both normal and stress conditions. Under stress, H_2O_2 accumulates, contributing to oxidative stress in plants (Hernandez et al. 2010). In salt stress, H_2O_2 levels in alfalfa increase compared to controls (Chen and Wang, 2021), and similar increases were observed in *Trigonella foenum-graecum*, particularly in the Ardestanian variety, with a decrease in the Mashhadian variety under salinity (Noohpishheh et al. 2021). In our study, both Bilensoy and Alsancak varieties showed increased H_2O_2 levels in leaves and roots under salt stress compared to controls (Fig. 4a, b). The release of engineered nanoparticles (ENPs) has raised environmental concerns due to their potential toxicity. Nanoparticles can alter physiological and biochemical processes in plants, affecting growth and seed production. They have been shown to reduce oxidative damage and enhance plant resistance to stress (Khan et al. 2021). In salt stress conditions, H_2O_2 levels in pearl millet increased significantly, especially at 150 mM NaCl (Khan et al. 2021), and in *Linum usitatissimum*, H_2O_2 levels increased but decreased after AgNP treatment (Khalofah et al. 2021). In our study, NP application increased H_2O_2 levels in the leaves of both varieties compared to controls (Fig. 4a, b). In roots, Bilensoy exhibited increased H_2O_2 , while Alsancak

showed a reduction (Fig. 4a, b). When CaO NPs were combined with salt stress, H_2O_2 levels decreased, especially in the leaves and more significantly in the roots of Alsancak (Fig. 4b). One-way ANOVA results confirmed statistically significant differences between groups ($p < 0.05$) (Supplementary Table 7).

In alfalfa plants under salt stress, soluble sugar content increased significantly (El Moukhtari et al. 2021). A similar trend was observed in *Oenanthе javanica*, where the salt-tolerant V11E0022 genotype had higher soluble sugar levels than the sensitive V11E0135 genotype, with both showing increased sugar levels in leaves and roots compared to controls (Kumar et al. 2021a, b). In our study, soluble sugar levels increased in both Bilensoy and Alsancak varieties under salt stress in both leaves and roots (Fig. 4c, d). Salt stress also significantly elevated total soluble sugar content in *Linum usitatissimum*, with further increases observed following AgNP application (Khalofah et al. 2021). Similarly, sugar content increased in rice seedlings exposed to ZnO NPs (Sheteiwy et al. 2016). In Bilensoy leaves, CaO NP application reduced soluble sugar levels, whereas Alsancak showed an increase (Fig. 4c, d). In roots, sugar content increased at 0.5 ppm CaO NP in Bilensoy, but decreased at 1.5 ppm, while Alsancak showed a decrease at 0.5 ppm and an increase at 1.5 ppm (Fig. 4c, d). Under combined NP and salt stress (50 mM NaCl + 0.5 ppm CaO), soluble sugar levels increased sharply in both varieties' leaves, but while Bilensoy showed a decrease in other NP + salt combinations, Alsancak exhibited increases. In the roots, Bilensoy showed a general decrease in soluble sugar under NP + salt stress, while Alsancak exhibited variable responses depending on dosage (Fig. 4c, d). These findings suggest that stress responses depend on nanoparticle dosage and variety sensitivity, with Bilensoy showing quicker responses due to higher sensitivity, while Alsancak displayed more variability. One-way ANOVA results confirmed statistically significant differences between groups ($p < 0.05$) (Supplementary Table 8). These results, while consistent with some studies, also highlight discrepancies that may depend on species, nanoparticle type, and concentration, warranting further investigation.

During oxidative stress, malondialdehyde (MDA) is a key marker of lipid peroxidation, indicating the effects of reactive oxygen species (ROS) (Teixeira da Silva Nazarovets, 2023). MDA levels typically increase under salt stress in various plant species (Hernandez et al. 2010; Kumar et al. 2021a, b). In *Brassica oleracea* roots, MDA levels increased with rising NaCl concentrations (Hernandez et al. 2010). Similarly, salt stress induced lipid peroxidation, reflected in increased MDA content in the leaves and roots of *Oenanthе javanica* varieties (Kumar et al. 2021a, b). In this study, MDA levels increased in both leaves and roots of Bilensoy and Alsancak varieties under salt stress (Fig. 4e, f). In *Linum usitatissimum*, AgNPs were shown to reduce MDA levels in both salt-stressed and non-stressed

plants (Khalofah et al. 2021). In Bilensoy, CaO NP application led to increased MDA levels in both leaves and roots, while in Alsancak, CaO NP application decreased MDA levels (Fig. 4e, f). In pearl millet, salt stress elevated MDA levels, but low concentrations of AgNPs reduced MDA levels, while a slight increase occurred at 30 mM AgNP treatment. A combined treatment of 20 mM AgNPs and 150 mM NaCl reduced MDA levels (Khan et al. 2021). Similarly, in our study, the reduction in MDA observed under combined NP+salt stress suggests that NP treatments may aid in damage repair, enhancing plant resilience under salt stress (Fig. 4e, f). One-way ANOVA confirmed statistically significant differences between groups ($p < 0.05$) (Supplementary Table 9).

Proline is an essential osmolite in plant stress responses, playing a key role in maintaining osmotic balance under salt stress (Mohamed et al. 2017). Elevated proline levels are associated with increased salt tolerance in various plant species (El Moukhtari et al. 2021; Yazicilar and Bezirganoglu 2023). Salt-tolerant plants typically have higher proline concentrations than sensitive ones (Kumar et al. 2021a, b). In our study, proline levels increased in both Bilensoy and Alsancak varieties under salt stress compared to the control (Fig. 4g, h). Similarly, in *Linum usitatissimum*, proline levels increased under salt stress, and this effect was further enhanced by AgNPs (Khalofah et al. 2021). In Bilensoy leaves, proline levels decreased with CaO NP treatments (Fig. 4g), while in Alsancak, proline levels increased under NP application (Fig. 4g). In the roots, proline content decreased in both varieties with NP treatments (Fig. 4g, h). In *Medicago sativa*, the tolerant genotype (Mesa-Sirsa) showed a higher increase in proline under salt stress compared to the sensitive genotype (Bulldog 505) (El-Shal et al. 2022). Nano-fertilizers such as K_2SO_4 , ZnO, and SiO_2 significantly elevated proline levels under salt stress. In this study, combined NP and salt stress treatments reduced proline levels in Bilensoy leaves but showed variability in the roots of Alsancak. These findings suggest that NP treatments help regulate proline production by alleviating stress. The differences in response may indicate that Alsancak exhibits greater stress tolerance due to its enhanced proline accumulation. One-way ANOVA analysis showed statistically significant differences between groups ($p < 0.05$) (Supplementary Table 10). The variation in proline levels between this study and others could be attributed to differences in the type and concentration of nanoparticles used, underscoring the complexity of nanoparticle interactions and their impact on plant stress responses.

Salt stress negatively affects chlorophyll and carotenoid levels, leading to reduced photosynthetic efficiency (Arif et al. 2020). However, nanoparticle (NP) treatments can mitigate this by enhancing chlorophyll and carotenoid content under stress conditions (Gohari et al. 2020; Faizan et al. 2021), particularly in more tolerant varieties (Kumar et al. 2021a, b). For instance, NP applications under salt stress in alfalfa have been

shown to improve photosynthetic pigments (El Moukhtari et al. 2021). In this study, Bilensoy showed a decrease in chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids under salt stress, while NP treatments increased these pigments (Fig. 5a, c, e). In Alsancak, chlorophyll a, chlorophyll b, and total chlorophyll levels decreased under high salt stress (100 mM NaCl), whereas total carotenoid levels increased at 50 mM NaCl but decreased at 100 mM NaCl. NP treatment with 0.5 ppm CaO NPs led to a decrease in chlorophyll a, while chlorophyll b and total carotenoids decreased with 1.5 ppm CaO NPs. Chlorophyll b only increased with 0.5 ppm CaO NP application (Fig. 5b, d, f). NP+salt stress combinations enhanced chlorophyll and carotenoid content in Bilensoy, with variable effects in Alsancak depending on NP dosage. These findings suggest that lower NP dosages may be more effective in promoting recovery. One-way ANOVA analysis confirmed statistically significant differences between groups ($p < 0.05$) (Supplementary Table 11).

Salinity stress induces reactive oxygen species (ROS) production, leading to oxidative stress in plants, which activate both enzymatic and non-enzymatic antioxidant defenses. Enzymes like superoxide dismutase (SOD) and peroxidase (POD) typically increase under salt stress. However, high nanoparticle (NP) concentrations can exacerbate ROS production, causing cellular damage and disrupting protein and DNA function (Alkharabsheh et al. 2021). In this study, POD and SOD activities increased in both leaves and roots of Bilensoy and Alsancak under salt stress, with sharper increases in the roots (Fig. 6a–d). POD activity in Bilensoy decreased in leaves but increased at low NP dosages in roots, while it decreased at higher dosages (Fig. 6a). In Alsancak, POD activity increased in both leaves and roots (Fig. 6b). SOD activity increased in Bilensoy leaves (Fig. 6c) but decreased in roots (Fig. 6c), while in Alsancak, SOD activity increased at higher NP dosages in leaves (Fig. 6d) but decreased in roots (Fig. 6d). Combined NP and salt stress treatments enhanced POD and SOD activities in Bilensoy leaves, while POD activity decreased in roots. In Alsancak, both POD and SOD activities increased in leaves and roots (Fig. 6b–d). These results suggest that antioxidant enzyme activities increase with higher salt stress and NP dosages, though variations in response may be due to differences in stress tolerance and NP dosage. One-way ANOVA confirmed statistically significant differences between groups ($p < 0.05$) (Supplementary Table 12).

Polyploidy plays a crucial role in plant defense mechanisms against abiotic stresses, influencing molecular, morphological, and physiological responses. Tetraploid plants are generally more tolerant to abiotic stresses than diploids, exhibiting improved morphological and physiological traits as well as miRNA responses. For example, tetraploid rice has better germination under salt stress, lower seed death rates, and higher chlorophyll a/b ratios. Tetraploids also

accumulate more free proline (Jiang et al. 2013). MiRNAs are thought to contribute to stress tolerance in polyploid plants (Dong et al. 2016). In *Robinia pseudoacacia*, the tetraploid form shows fewer negative effects from salt stress compared to diploids, with enhanced enzyme activity related to ROS detoxification, photosynthesis, and defense (Wang et al. 2013). In our study, the tetraploid Alsancak alfalfa variety displayed similar responses to the above findings in both morphological and physiological adaptations to abiotic salt stress and miRNA gene expression changes. This provides strong evidence that the tetraploid Alsancak alfalfa variety may be more tolerant to salt stress.

Conclusions

This study examined the responses of diploid Bilensoy and tetraploid Alsancak alfalfa varieties to salt stress, revealing that salt stress has different effects on plant morphology, gene expression, and physiological responses. It was observed that salt stress caused changes in plant height, leaf number, and root structures. Under salt stress, Bilensoy showed a reduction in root and stem lengths, while Alsancak exhibited variable responses depending on salt concentration. The effects of calcium oxide nanoparticles (CaO NP) were also investigated. In Bilensoy, root length decreased, while in Alsancak, it increased. This suggests that Alsancak's larger nuclear size might allow it to develop a more effective defense mechanism against salt stress. Furthermore, combined NP and salt stress applications enhanced Alsancak's root development, while Bilensoy displayed a more sensitive response. Gene expression analysis through miRNA profiling showed that salt stress induced different miRNA regulations in the two alfalfa varieties. In Bilensoy, miR159 and miR166 were suppressed in roots, while miR156 was upregulated. In contrast, Alsancak exhibited an upregulation of both miR156 and miR159. These differences reflect the effects of NP dosages and gene responses under salt stress. Physiological analyses revealed that H₂O₂ levels increased under salt stress; however, NP treatments reduced H₂O₂ levels, especially in Alsancak's roots. Similarly, MDA levels, which increased due to salt stress, were mitigated by NP applications, demonstrating a protective role. Soluble sugar levels rose under salt stress, with variations dependent on NP doses and plant varieties. Proline levels were higher in Alsancak under combined salt and NP treatments, while they decreased in Bilensoy with NP applications. In conclusion, the tetraploid Alsancak variety exhibited greater resistance to salt stress compared to diploid Bilensoy. This increased tolerance was supported by NP applications, miRNA responses, and physiological adjustments. The genetic makeup and nuclear size of tetraploid plants play a significant role in developing protective mechanisms against salt stress.

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Data availability All the data supporting the findings of this study are available within the paper.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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