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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Physiological and Biochemical Mechanisms in Two Alfalfa (*Medicago sativa* L.) Cultivars with Contrasting Salinity Tolerance

Yoncada (*Medicago sativa* L.) Tuzluluk Toleransı Farklı İki Çeşitte Fizyolojik ve Biyokimyasal Mekanizmalar

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Abstract

Salinity stress has been accepted as an abiotic stress factor that decreases agricultural productivity. Earlier studies have showed that salinity stress has a negative impact on plant growth, development, and yield, while also leading to quality losses. Among forage crops, alfalfa is one of the species that exhibits sensitivity to salinity stress. Thus, gaining insights into the physiological and biochemical mechanisms of alfalfa under salinity stress is essential for formulating effective strategies to improve its tolerance. This study aimed to reveal the physiological and biochemical mechanisms of two alfalfa cultivars showing different sensitivity to salinity stress when exposed to salt stress. Preliminary petri dish experiments conducted before the study revealed that the two alfalfa cultivars exhibited different sensitivities to salinity stress, with the 'Diane' being more tolerant compared to 'Bilensoy-80'. In the study, the effects of varying salinity levels (0, 50, 100, 150, 200 mM NaCl) were tested in alfalfa cultivars by performing growth parameters, physiological and biochemical status. The results indicated that the cultivar 'Diane' showed maintained root development, physiological and biochemical status, compared to control under salinity stress, except for 150 and 200 mM NaCl. In contrast, salinity stress in the 'Bilensoy-80' caused a significant reduction in growth parameters and severe impairments in physiological and biochemical traits, compared to control. The results collectively revealed that higher salinity tolerance in 'Diane' could be attributed to its increased free proline content and antioxidative defense mechanism, including total phenolic content, APX, CAT and GR enzyme biosynthesis. The findings of this study can be used in breeding strategies that aimed to develop salt-tolerant alfalfa plants. Additionally, it is recommended that the 'Diane' cultivar, which has been identified as more tolerant to salinity stress, be tested through field trials to evaluate its yield and adaptation capacity under saline conditions.

Keywords: Forage, NaCl stress, Osmotic stress, Antioxidant activity, Fodder crop

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Öz

Tuz stresi, tarımsal verimliliği azaltan abiyotik bir stres faktörü olarak kabul edilmektedir. Yapılan araştırmalar, tuz stresinin bitkilerde büyüme, gelişme ve verim üzerinde olumsuz etkiler yarattığını, aynı zamanda kalite kayıplarına yol açtığını ortaya koymaktadır. Özellikle yem bitkileri arasında yüksek besin değerine sahip olan yaygın yonca, tuz stresine karşı hassasiyet gösteren türlerden biridir. Bu nedenle, yaygın yoncanın tuz stresine karşı fizyolojik ve biyokimyasal tepkilerini anlamak, stres toleransını artırmak için gerekli olan programların ortaya konması adına büyük önem taşımaktadır. Bu çalışmada, tuz stresine farklı duyarlılık gösteren yaygın yonca çeşitlerinin tuz stresine maruz kaldığında gösterdiği fizyolojik ve biyokimyasal mekanizmaların ortaya konulması amaçlanmıştır. Araştırma öncesinde yapılan petri deneyleri, her iki çeşidin tuz stresine karşı farklı hassasiyet gösterdiğini ve 'Diane' çeşidinin 'Bilensoy-80' çeşidine kıyasla daha dayanıklı olduğunu ortaya koymuştur. Çalışmada, farklı tuzluluk seviyelerinin (0, 50, 100, 150, 200 mM NaCl) yaygın yonca çeşitlerinde büyüme parametreleri, fizyolojik ve biyokimyasal mekanizmaya etkileri analiz edilmiştir. Çalışma sonuçları 'Diane' çeşidinin tuz stresi altında 150 ve 200 mM NaCl uygulamaları dışında kontrol grubuna kıyasla kök gelişimini, fizyolojik ve biyokimyasal durumunu koruduğunu göstermiştir. Buna karşılık, 'Bilensoy-80' çeşidinde tuz stresi kontrol grubuna kıyasla, büyüme parametrelerinde belirgin bir azalmaya, fizyolojik ve biyokimyasal özelliklerde ise ciddi bozulmalara yol açmıştır. Elde edilen sonuçlar, 'Diane' çeşidinin yüksek tuz toleransının, serbest prolin ve toplam fenolik içeriğindeki artışın yanı sıra APX, CAT ve GR enzimlerinin artan biyosentezini içeren antioksidatif savunma mekanizmasına bağlı olabileceğini göstermektedir. Araştırma bulguları, tuz stresine dayanıklı yaygın yonca bitkisi geliştirmeyi hedefleyen ıslah programlarında kullanılabilir. Ayrıca, tuz stresine daha toleranslı olduğu belirlenen 'Diane' çeşidinin, tuzlu koşullarda verim ve adaptasyon kapasitesinin değerlendirilmesi amacıyla tarla denemeleriyle test edilmesi önerilmektedir.

Anahtar Kelimeler: Yem, NaCl stresi, Osmotik stres, Antioksidan aktivite, Yem bitkisi

1. Introduction

Salinity stress, has been known as a global agricultural problem limiting productivity and sustainability (Mukhopadhyay et al., 2021; Beyaz and Kazankaya, 2024). It causes osmotic and ionic problems, lead to a significantly lower yielding and quality (Maryum et al., 2022). The severity of these effects varies not only among plant species but also between genotypes within a species, highlighting the need for a deeper understanding of tolerance mechanisms (Munns and Tester, 2008).

Plants have developed several adaptations to maintain their growth and development under salinity conditions (Demirkol, 2021). One of the key strategy to deal with salinity stress is antioxidative defense mechanism, which mitigates oxidative damage caused by increased rective oxygen species (ROS) (Begum et al., 2022). Studies showed that the biosynthesis of total phenolic contents and antioxidative enzymes are up-regulated by molecular regulation under salinity stress in stress-tolerant plants (Demirkol and Yılmaz, 2023). Moreover, enhanced antioxidative activity is linked to a several physiological and biochemical strategies, including cell membrane maintenance and protein structure protection (Yılmaz, 2019). Furthermore, increased osmolytes like free proline, glycine betaine, and soluble sugar biosynthesis have been identified as a key factor in stabilizing cellular structures and maintaining water potential under salinity stress conditions (Ashraf and Harris, 2004; Ghaffari et al., 2019). Earlier studies have also showed the involvement of plant hormones in modulating stress responses by triggering defense-related signal mechanisms (Waadt et al., 2022). These interconnected mechanisms collectively contribute to the ability of some genotypes to cope with salinity stress more effectively than others (Demirkol and Yılmaz, 2023).

Previous studies have reported that many cultivated crop species are susceptible to salinity stress (Ondrasek et al., 2022), including forage legumes which is crucial in ruminant nutrition and soil sustainability (Sharavdorj et al., 2021). Alfalfa is recognized as a main forage legume due to its high nutritional quality and nitrogen-fixing ability (Lei et al., 2018). Nonetheless, its sensitivity to abiotic stresses presents a significant limitation (Bicakci et al., 2020). While numerous studies have examined the impact of salinity on alfalfa, a clear understanding of the physiological and biochemical mechanisms differentiating salt-tolerant and salt-sensitive genotypes remains lacking. Most research has focused on general stress responses rather than dissecting the distinct adaptive strategies employed by tolerant and sensitive varieties. Unraveling these differences is essential not only for advancing our knowledge of salinity tolerance in alfalfa but also for developing targeted strategies to enhance its productivity in saline environments. Thus, elucidating the physiological and biochemical mechanisms that confer salinity tolerance in alfalfa remains a critical research priority. This study aimed to highlight the salinity tolerance mechanism in two alfalfa cultivars differing in salinity sensitivity in a view of physiological and biochemical status.

2. Materials and Methods

2.1. Plant materials

The alfalfa cultivars 'Bilensoy-80' and 'Diane' were selected for the experiment because of their contrasting salinity tolerance. This contrast was further supported by a preliminary laboratory study conducted under petri dish conditions (data not shown), which confirmed that 'Bilensoy-80' is more sensitive, whereas 'Diane' shows greater tolerance, under NaCl stress. 'Bilensoy-80' is a Turkish cultivar widely cultivated in Central Anatolia and is characterized by high forage yield, whereas 'Diane' is an introduced cultivar known for its high forage quality and tolerance to drought stress. The seeds were obtained from the General Directorate of Agricultural Research and Policies and Ulusoy Tohumculuk. Prior to sowing, the seeds were surface-sterilized with 70% (v/v) ethanol for 1 min, following the protocol of Demirkol and Yılmaz (2023), and subsequently rinsed three times with distilled water.

2.2. Growth and stress conditions

The experiment was conducted in the greenhouse of the Agricultural Research Center at Tokat Gaziosmanpaşa University in 2024. Seeds were sown in plastic pots (15 cm diameter × 20 cm height), each filled with 3 kg of field soil. The soil had a clay-loam texture, with the following properties: organic matter 1.96% (low), pH 7.80 (alkaline), electrical conductivity 0.01% (non-saline), and $CaCO_3$ content of 4.97% (moderately calcareous). The available macronutrient contents were 4.05 kg da^{-1} P_2O_5 (deficient) and 39 kg da^{-1} K_2O (sufficient). Regarding micronutrients, Zn (0.36 mg kg^{-1}) and Fe (2.61 mg kg^{-1}) were deficient, whereas Mn (17.14 mg kg^{-1}) and Cu (0.92 mg kg^{-1}) were sufficient (*Table 1*).

Table 1. Analysis results of the research soil

Traits	Value
Texture	Clay loam
Organic matter	1.96
pH	7.80
P_2O_5 (kg da^{-1})	4.05
K_2O (kg da^{-1})	39
Tuz (%)	0.01
$CaCO_3$ (%)	4.97
Zn (mg kg^{-1})	0.36
Fe (mg kg^{-1})	2.61
Mn (mg kg^{-1})	17.14
Cu (mg kg^{-1})	0.92

The experiment was conducted in greenhouse of the Agricultural Research Center in Tokat Gaziosmanpaşa University in 2024. The greenhouse conditions were at an average day/night temperature of 21±2°C/15±2°C and a relative humidity of 60±5%. The seeds were planted in pots filled with field soil. The pots were irrigated with a half-strength Hoagland nutrient solution (pH 6) (*Table 2*) at 80% of field capacity every two days, since a considerable decrease in soil water content was observed by the second day. A similar approach has also been reported in previous studies (Cornacchione and Suarez, 2017). Three-week-old seedlings at the two-leaf stage were used to impose salinity stress at an early developmental phase. Plants were divided into five groups and irrigated with salinity treatments of 0, 50, 100, 150, and 200 mM NaCl, which are commonly applied concentrations in alfalfa salt stress studies (Babakhani et al., 2011). The electrical conductivity of the irrigation solutions was measured as approximately 1.2, 5.4, 10.2, 15.6, and 19.8 dS m^{-1} for 0, 50, 100, 150, and 200 mM NaCl, respectively. All solutions were prepared using distilled water. Salt solutions were applied in increments of 50 mM NaCl every other day until the target concentration was reached. During the study, drainage was not allowed in the pots in order to maintain the targeted salinity levels within the root zone throughout the experiment. Plants were harvested four weeks after the completion of salinity stress treatments, as visible morphological damages were observed within this period, consistent with earlier reports (Mueller and Teuber, 2008). For physiological and biochemical analyses, fully expanded upper leaves were collected from each plant at the end of the salinity stress period, following the approach used in similar salinity studies (Ali et al., 2014; Akhtar et al., 2015).

Table 2. Composition of half-strength Hoagland nutrient solution used in the experiment

Compount	Concentration (mmol L^{-1})
KNO_3	2.5
$Ca(NO_3)_2 \cdot 4H_2O$	2.5
$NH_4H_2PO_4$	0.5
$MgSO_4 \cdot 7H_2O$	1.0
Fe-EDTA	0.05
H_3BO_3	0.023
$MnCl_2 \cdot 4H_2O$	0.005
$ZnSO_4 \cdot 7H_2O$	0.0005
$CuSO_4 \cdot 5H_2O$	0.0002
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0001

2.3. Growth parameters

The roots and shoots of the harvested seedlings were measured to determine their lengths then dried at 60°C for 72 hours to evaluate dry weights of the roots and shoots.

2.4. Relative water content

The relative water content of the seedlings was assessed using the following formula: 100 x (fresh weight - dry weight) / (turgid weight - dry weight) (Farrant 2000).

2.5. Free proline content

The amount of free proline was determined through the protocol of Bates et al. (1973). The homogenization with 3% sulfosalicylic acid with the samples were mixed in ninhydrin, glacial acetic acid and sulfosalicylic acid. Then 1 ml of toluene was added to this reaction and centrifuged at 5 000 × g for 30 seconds. Finally, the absorbance of the upper phase at 520 nm was read in UV-Vis spectrophotometer.

2.6. Total chlorophyll content

Total chlorophyll content was determined spectrophotometrically after extraction with 80% (v/v) acetone. Absorbance values were recorded using a UV–Vis spectrophotometer. Chlorophyll concentrations were calculated using the corrected extinction coefficients and simultaneous equations for buffered 80% aqueous acetone as given by Porra et al. (1989) with the following equation;

$$\text{Total chlorophyll } (\mu\text{g mL} - 1) = 17.16\,A663 + 7.34\,A645 \qquad \text{(Eq.1)}$$

2.7. Malondialdehyde contents

The protocol of Ohkawa et al. (1979) was used to determine malondialdehyde (MDA) content in alfalfa plants. A sample of the fresh leaves was homogenized with trichloroacetic acid then centrifuged for 10 min at 15 000 × g. The supernatant was was mixed with 0.5% thiobarbituric acid dissolved in 20% trichloroacetic acid. Then the heated mixture was centrifuged for 15 min at 10 000 × g. The resulting supernatant was used to absorbance at 532 nm and 600 nm using a UV-Vis Spectrophotometer.

2.8. Total phenolic content

The protocol of Slinkard and Singleton (1977) was used to determine total phenolic content in alfalfa plants. The methanolic extract of the sample was mixed with Folin-Ciocalteu reagent then Na_2CO_3. Finally the absorbance of the reaction was determined at 760 nm using a UV-Vis Spectrophotometer, with gallic acid serving as the calibration standard.

2.9. Antioxidative enzymes

In this study, it was planned to determine the antioxidative enzyme activities which were preferred in previous abiotic stress physiology studies and successfully used in the differentiation of resistant and sensitive varieties. In the study, protein ratios were determined in accordance with the research of Bradford (1976) before the protocol of determination of enzyme activities. Afterwards, the enzymatic activities of APX, CAT, SOD, and GR were assessed following the methods outlined by Wang et al. (1991), Chance and Maehly (1955), Beauchamp and Fridovich (1971), and Sgherri et al. (1994), respectively.

2.10. Statistical analyses

The variables were statistically analyzed by one-way ANOVA followed by the Tukey test ($p < 0.05$ and $p < 0.01$), using Minitab software (Minitab Inc., State College, PA). Before performing ANOVA in the research, the data were tested for normality and homogeneity.

3. Results and Discussion

3.1. Growth parameters

The root development of the 'Bilensoy-80' exhibited a significant decline under salinity stress at 100 mM NaCl and higher, whereas shoot growth was adversely affected at a lower threshold, with a significantly reduction observed at 50 mM NaCl, compared to controls (0 mM NaCl) (*Figure 1A, 1B, 1C, 1D*). For the 'Diane', the first statistically significant reduction in root development under salinity stress was observed at 200 mM NaCl and higher, while reductions in shoot dry weight and shoot length were first recorded at 100 mM and 150 mM NaCl, respectively, compared to controls (*Figure 1A, 1B, 1C, 1D*).

The ability to maintain or enhance root development under saline conditions represents a crucial adaptive strategy in crops to minimize the negative effects of salt stress (Zou et al., 2022). The growth parameters assessed in this study indicated that the lower reduction under salinity conditions in root and shoot development in 'Diane' confirmed its higher salinity tolerance than 'Bilensoy-80'. Moreover, the data highlighted that shoot growth is more adversely affected by salinity than root development. This observation has been reported in previous studies, which indicate that salinity stress causes photosynthetic degradation in shoots, making them more susceptible to damage than roots (Munns and Tester, 2008). Furthermore, the better root performance of 'Diane' under high salinity levels suggests an improved ability for osmotic adjustment and ion compartmentalization, allowing sustained water uptake and reduced Na^+ toxicity. Similar root-based tolerance mechanisms have been observed in forage pea genotypes (Demirkol and Yılmaz, 2023). These findings imply that genotypic variation in root system response is a major determinant of overall salinity tolerance in alfalfa.

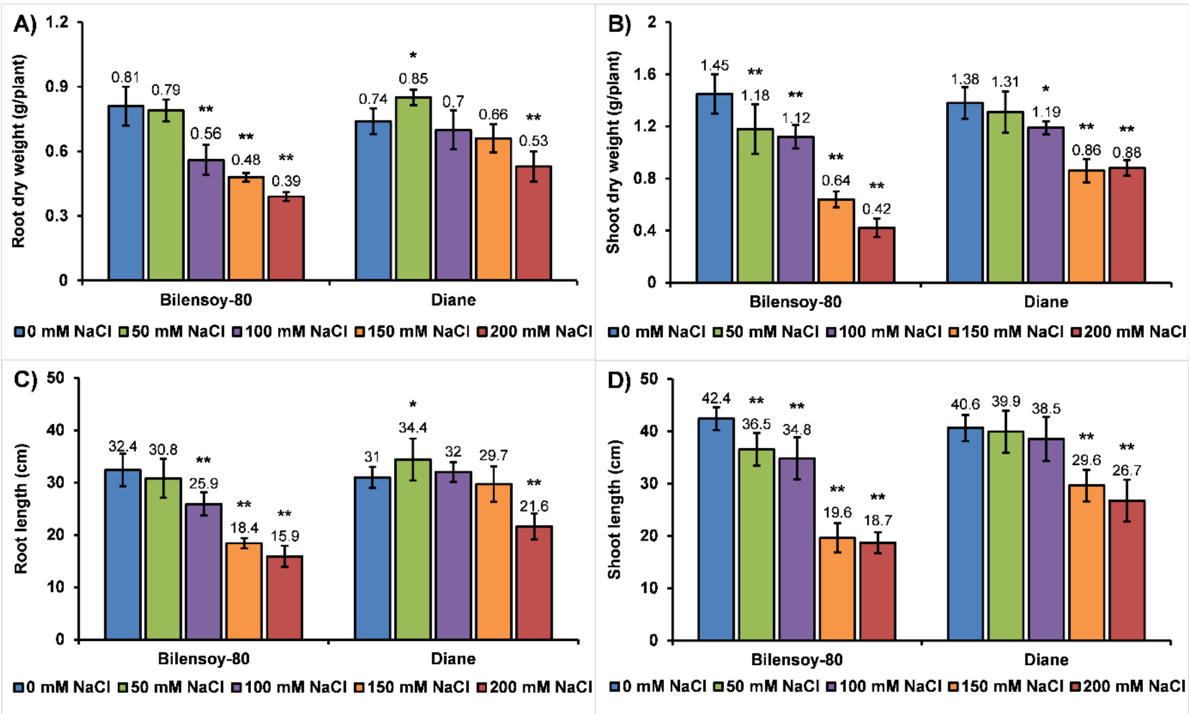

Figure 1. Growth parameters of alfalfa cultivars under varying conditions; A. Root dry weight, B. Shoot dry weight, C. Root length, D. Shoot length. Bars represent the mean values ± standard errors from four replicates. Asterisks showed differences between control (0 mM) and NaCl-treated plants within each cultivar (*p < 0.05 or **p < 0.01).

3.2. Biochemical status

Both cultivars exhibited a significant reduction in relative water content under salinity stress; however, a dramatic decline was observed in 'Bilensoy-80' at 200 mM NaCl, compared to controls (*Figure 2A*). This reduction in water content under salinity conditions reflects a disruption of cellular osmotic balance, which can limit turgor-driven cell expansion (Jeddi et al., 2025). In addition, the reduced water content in the plant leaves induces chlorophyll degradation mechanism has been reported to leading to a decrease in chlorophyll quantity (Pour-Aboughadareh et al., 2017).

The decreases were observed in free proline content in 'Bilensoy-80', whereas an extreme increase was detected in 'Diane' under 100 mM NaCl, compared to control (*Figure 2B*), suggesting that the enhanced synthesis of free proline plays a crucial role in salt stress tolerance in salinity tolerant alfalfa plants. Free proline enables plants to cope with various stressors, including salinity, by maintaining turgor pressure and redox homeostasis (Rajasheker et al., 2019). However, the increase in the 'Diane' could not be sustained beyond the 100 mM NaCl stress level (*Figure 2B*), suggesting that while proline accumulation contributes to salt stress tolerance, its regulation varies according to severity of stress in alfalfa. In addition, the transient proline accumulation observed in 'Diane' may reflect a dynamic balance between synthesis and utilization.

In the 'Bilensoy-80', extremely higher up-regulations were recorded in MDA levels under 100, 150, and 200 mM NaCl stress conditions, compared to the control (1.5, 3.2, and 4.1 folds, respectively). Although similar increases were observed in the 'Diane' cultivar, the extent of the increase was less pronounced compared to that in 'Bilensoy-80' (*Figure 2C*). The significantly higher up-regulations of MDA content in 'Bilensoy-80' indicates its sensitibility against oxidative stress, as MDA is a well-known biomarker of lipid peroxidation (Demirci-Cekic et al., 2022). In contrast, the relatively lower increases observed under salinity stress at 150 and 200 mM NaCl in 'Diane' (*Figure 2C*). This finding is consistent with the concept that tolerant cultivars restrict membrane lipid peroxidation through more efficient antioxidant systems. The lower MDA accumulation in 'Diane' suggests a better capacity to detoxify reactive oxygen species (ROS), preventing irreversible damage to chloroplast membranes and photosynthetic machinery (Duan et al., 2025).

Total chlorophyll content of the 'Bilensoy-80' started to decrease at 50 mM NaCl, whereas the first decrease was observed at 100 mM NaCl in 'Diane' (*Figure 3A*). It has been revealed that the reducing water in the plant leaves causes degradation chlorophyll pathway, lead to a decrease in quantity of chlorophyll (Demirkol and Yılmaz, 2023; Pour-Aboughadareh et al., 2017). The delayed reduction in chlorophyll in 'Diane' indicates a more efficient maintenance of photosynthetic pigments under osmotic stress. This could be attributed to enhanced antioxidant enzyme activity protecting chlorophyll from ROS-mediated degradation. Such responses are typical of tolerant alfalfa genotypes, which sustain photosynthetic efficiency under salinity (Lundell and Biligetu, 2024).

Total phenolic content of the 'Bilensoy-80' started to decrease at 100 mM NaCl. However, 100 mM NaCl stress led to an increase in total phenolic content of 'Diane', while no change was observed under other stress treatments, compared to control (*Figure 3B*). This suggests that the 'Diane' activate the antioxidative defense system to mitigate oxidative damage induced by salinity stress. A previous study reported that moderate salinity stress increases total polyphenol content in *Thymus vulgaris* (Zrig et al., 2016). These findings indicating that salinity stress stimulates the biosynthesis of phenolics as part of a protective mechanism in salinity tolerant alfalfa plants. The contrasting response between the two cultivars further supports that phenolic metabolism is tightly linked to genetic differences in stress perception and signaling.

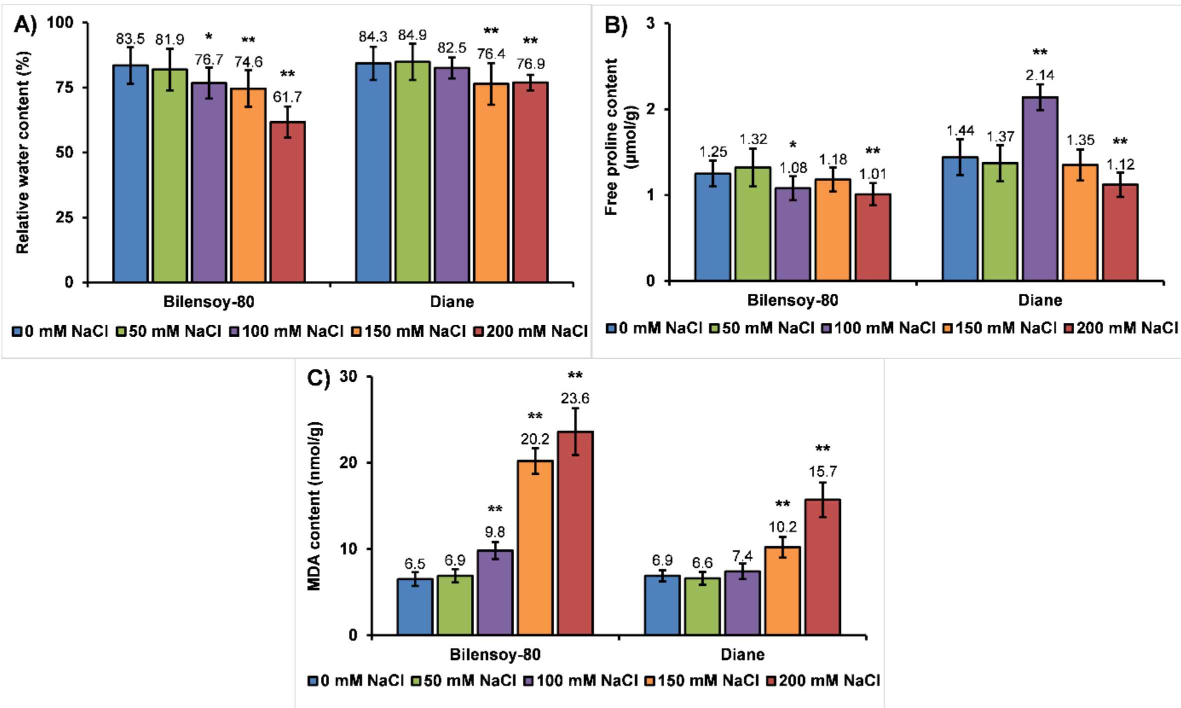

Figure 2. Biochemical status of alfalfa cultivars under varying conditions; A. Relative water content (%), B. Free proline content (µmol/g), C. MDA content (nmol/g). Bars represent the mean values ± standard errors from four replicates. Asterisks showed differences between control (0 mM) and NaCl-treated plants within each cultivar (*p < 0.05 or **p < 0.01).

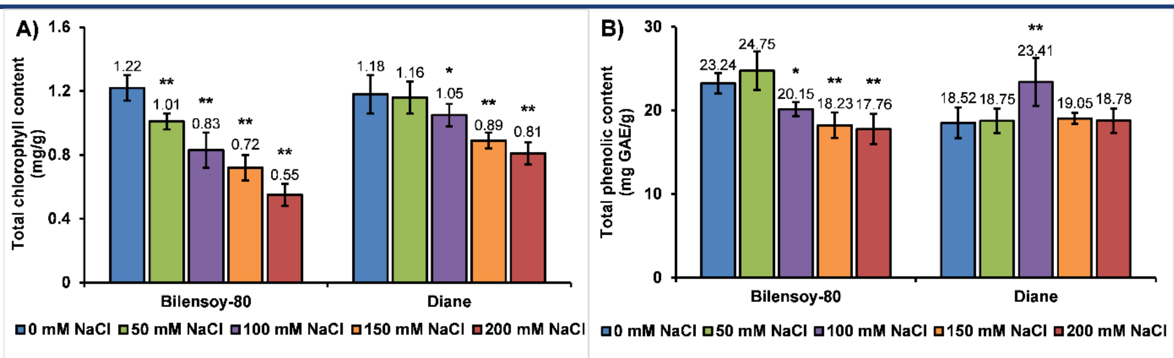

Figure 3. Biochemical status of alfalfa cultivars under varying conditions; A. Total chlorophyll content (mg/g), B. Total phenolic content (mg GAE/g). Bars represent the mean values ± standard errors from four replicates. Asterisks denote significant differences between control (0 mM) and NaCl-treated plants within each cultivar (*p < 0.05 or **p < 0.01).

3.3. Antioxidative enzymes

Under salinity stress conditions, decreases in APX, CAT, and SOD activities were observed in 'Bilensoy-80', while no significant change was detected in GR activity, compared to controls (*Figure 4A, 4B, 4C, 4D*). In contrast, 'Diane' exhibited increased activities of APX, CAT, and GR activities under salinity stress, compared to controls (*Figure 4A, 4B, 4C, 4D*), suggesting that these upregulated enzymes strengthened its antioxidant defense mechanism and enhanced salinity tolerance (*Figure 4A, 4B, 4C, 4D*). APX and CAT act synergistically to eliminate hydrogen peroxide, while GR maintains the redox balance through regeneration of reduced glutathione (Rao et al., 2025).

The up-regulation of these enzymes has been known to contribute to greater salinity tolerance in various crops (Hasanuzzaman et al., 2020; Mushtaq et al., 2020; Riseh et al., 2024).

These integrated responses collectively reduce oxidative stress and maintain metabolic stability under salinity, highlighting the physiological basis for its superior tolerance relative to 'Bilensoy-80'.

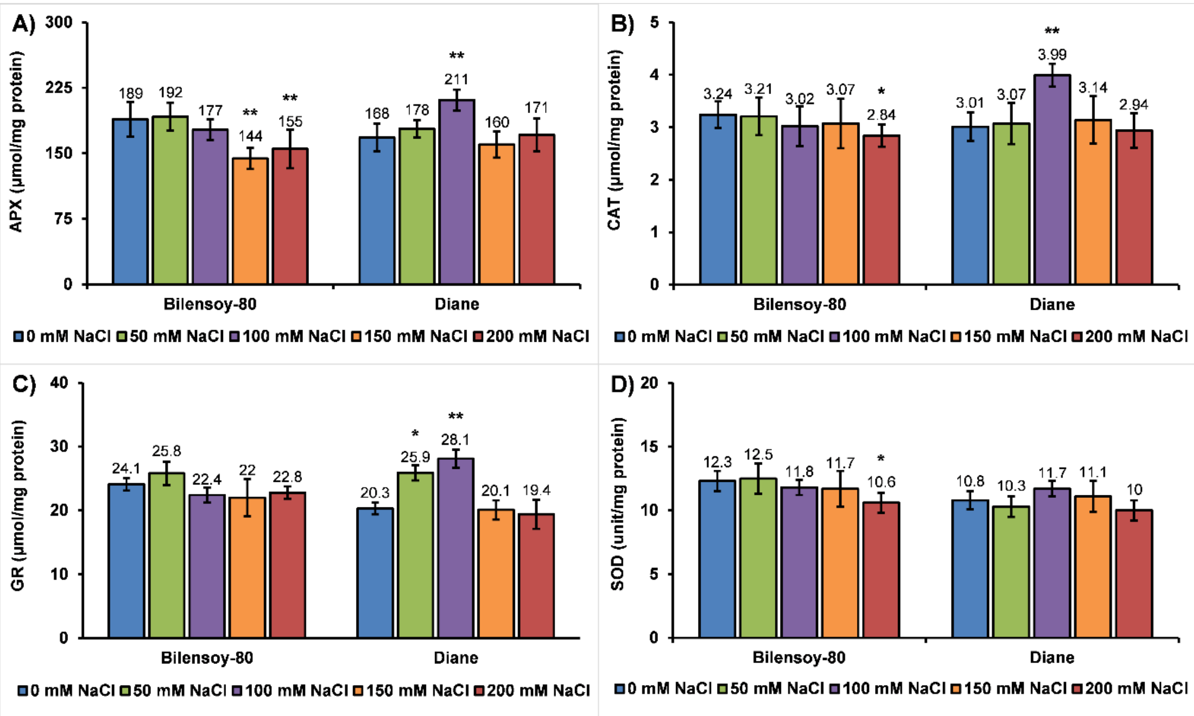

Figure 4. Antioxidative enzymes of alfalfa cultivars under varying conditions; A. APX (µmol/mg protein), B. CAT (µmol/mg protein), C. GR (µmol/mg protein), D. SOD (unit/mg protein). Bars represent the mean values ± standard errors from four replicates. Asterisks showed differences between control (0 mM) and NaCl-treated plants within each cultivar (*p < 0.05 or **p < 0.01).

4. Conclusions

The findings showed that the salinity-tolerant cultivar 'Diane' exhibited better growth performance, physiological stability, and biochemical status compared to 'Bilensoy-80' under salinity stress. This enhanced tolerance was primarily associated with the up-regulation of mechanisms for free proline accumulation, total phenolic content, and the activity of key antioxidative enzymes (APX, CAT, and GR). Furthermore, the results indicate that 'Diane' possesses significant potential for cultivation in salt-affected environments, providing valuable insights for breeding programs aimed at enhancing salinity tolerance in crops.

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Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

There is no conflict of interest between the article authors.

Authorship Contribution Statement

Concept: Demirkol, G.; Design: Demirkol, G.; Data Collection or Processing: Demirkol, G.; Statistical Analyses: Demirkol, G.; Literature Search: Demirkol, G.; Writing, Review and Editing: Demirkol, G.

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