


Physiological and Biochemical Insights into Drought Tolerance of Alfalfa Cultivars with Different Sensitivities

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ABSTRACT

Drought is one of the most critical abiotic stresses limiting alfalfa (*Medicago sativa* L.) productivity worldwide. This study investigated the physiological and biochemical responses of two contrasting alfalfa cultivars, 'Bilensoy-80' (sensitive) and 'Diane' (tolerant), exposed to drought stress induced by polyethylene glycol (PEG 6000) at different water potentials (0, -0.5, -1.0, -1.5, -2.0 MPa). Growth traits, relative water content, total chlorophyll content, osmolyte accumulation, lipid peroxidation, total phenolics, and activities of major antioxidant enzymes were evaluated. The results showed that the reductions in growth parameters, relative water content, and total chlorophyll content started to be observed at earlier drought stress levels in Bilensoy-80, whereas Diane maintained stability until more severe drought levels, compared with the control. This enhanced performance was largely attributed to increased free proline biosynthesis and the activity of key antioxidative enzymes such as catalase (CAT), and glutathione reductase (GR). Overall, the findings provide valuable insights into the mechanisms of drought tolerance in alfalfa and may inform breeding programs aimed at improving forage crop performance under water-limited conditions.

Key words: Antioxidant activity, forage, osmotic stress, water deficiency

Farklı Duyarlılıklara Sahip Yaygın Yonca Çeşitlerinde Kuraklık Toleransına İlişkin Fizyolojik ve Biyokimyasal Etkiler

ÖZ

Kuraklık, dünya genelinde yaygın yonca (*Medicago sativa* L.) verimliliğini sınırlayan en kritik abiyotik streslerden biridir. Bu çalışma, farklı kuraklık toleransına sahip iki yonca çeşidinin, 'Bilensoy-80' (hassas) ve 'Diane' (tolerant), farklı su potansiyellerinde (0, -0.5, -1.0, -1.5, -2.0 MPa) polietilen glikol (PEG 6000) ile indüklenen kuraklık stresine karşı fizyolojik ve biyokimyasal tepkilerini ortaya koymuştur. Büyüme özellikleri, bağıl su içeriği, toplam klorofil miktarı, ozmolit birikimi, lipid peroksidasyonu, toplam fenolik miktarı ve antioksidatif enzimlerin aktiviteleri değerlendirilmiştir. Sonuçlar, büyüme parametreleri, bağıl su içeriği ve toplam klorofil miktarındaki azalışların Bilensoy-80 çeşidinde daha erken kuraklık seviyelerinde başladığını, buna karşın Diane çeşidinin kontrole kıyasla daha şiddetli kuraklık seviyelerine kadar stabilitesini koruduğunu göstermiştir. Bu gelişmiş performans, büyük ölçüde serbest prolin biyosentezinin artışı ve katalaz (CAT) ile glutatyon redüktaz (GR) gibi temel antioksidatif enzimlerin aktivitesine bağlanmıştır. Genel olarak, elde edilen bulgular, yaygın yoncada kuraklık toleransı mekanizmaları hakkında bilgiyi ortaya koymakta ve su kısıtlı koşullarda yem bitkisi performansının iyileştirilmesine yönelik ıslah programlarına katkı sunmaktadır.

Anahtar kelimeler: Antioksidan aktivite, yem, osmotik stres, su eksikliği.

INTRODUCTION

Drought has emerged as a major ecological challenge globally. Drought stress could result in yield reduction or crop failure (Zhang et al., 2018). It adversely influences crop performance by lowering leaf water potential and disrupting key physiological processes, including respiration and photosynthesis (Khandal et al., 2017).

To withstand drought, plants have evolved multiple adaptive mechanisms that support their growth and development under limited water availability (Zhang et al., 2020). In stress-tolerant species, drought has been reported to stimulate the accumulation of phenolic compounds and to enhance the activity of key antioxidant enzymes, including ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD), through molecular regulation (Demirkol and Yilmaz, 2023). Consequently, investigating these enzymatic defenses alongside other physiological and biochemical traits represents a promising strategy to sustain and improve plant productivity under drought conditions.

Identifying contrasting genotypes with known sensitivity or tolerance to drought is crucial for unraveling the physiological and biochemical mechanisms underlying plant responses to water deficit. Comparative evaluation of tolerant and susceptible genotypes provides valuable insights into stress-adaptive traits, helping to clarify the complex regulatory networks that contribute to resilience under drought conditions (Rehman et al., 2023).

Alfalfa (*Medicago sativa* L.) is a widely cultivated forage legume valued for its high nutritional quality, substantial biomass production, and nitrogen-fixing ability, making it a cornerstone of global agriculture (Lei et al., 2018). Despite these advantages, alfalfa is considered highly vulnerable to drought stress (Zhang et al., 2019). Earlier investigations have shown that water deficit conditions markedly reduce both yield and forage quality in alfalfa (Liu et al., 2018; Putnam, 2021). Several studies have indicated that drought tolerance in alfalfa may involve physiological and biochemical adjustments such as osmolyte accumulation and activation of antioxidant defense systems (Zhang et al., 2018; Kang et al., 2023). However, the extent to which these responses differ among cultivars with contrasting drought sensitivity remains insufficiently characterized. In particular, integrated and comparative evaluations of growth performance, water status, osmolyte dynamics, oxidative damage, phenolic metabolism, and antioxidative enzyme activities under well-defined and progressive osmotic stress conditions are limited. Therefore, a comprehensive assessment of these traits in alfalfa cultivars differing in drought tolerance is necessary to improve our understanding of the physiological mechanisms underlying drought adaptation. This underscores the necessity of developing strategies that enhance drought tolerance while minimizing yield and quality losses in alfalfa. The present study was designed to investigate the physiological and biochemical mechanisms associated with drought adaptation in alfalfa cultivars differing in their sensitivity to drought.

MATERIALS AND METHODS

Plant materials

The alfalfa cultivars, 'Bilensoy-80' and 'Diane', were selected for the experiment. In a preliminary study, the drought stress tolerance of the cultivars was assessed in petri dishes, confirming that 'Bilensoy-80' was more susceptible to salinity than 'Diane' (data not shown). Prior to the experiment, the seeds were surface-sterilized with 70% (v/v) ethanol for 1 min, following the protocol of Demirkol and Yilmaz (2023). Afterwards, the seeds were washed three times with sterile distilled water.

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 CaCO₃ content of 4.97% (moderately calcareous). The available macronutrient contents were 4.05 kg da⁻¹ P₂O₅ (deficient) and 39 kg da⁻¹ K₂O (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 ₂ O ₅ (kg da ⁻¹)	4.05
K ₂ O (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 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. In the study, polyethylene glycol (PEG) was used to impose controlled osmotic stress, allowing precise regulation of water potential and reproducibility across treatments. Although PEG-induced stress does not fully replicate field drought conditions, it is widely applied to simulate water deficit at early growth stages and to compare genotype-specific responses under standardized conditions (Demirkol et al., 2023). 45 days after sowing, PEG 6000 was incorporated into Hoagland's nutrient solution to generate osmotic potentials of 0, -0.5, -1.0, -1.5, and -2.0 MPa, calculated according to the method of Michel and Kaufmann (1973). The imposed drought stress was maintained for a period of seven days, following the experimental framework described in earlier studies (Zhang et al., 2018). Then, alfalfa plants were harvested for the analyses.

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

Compound	Concentration (mmol L ⁻¹)
KNO ₃	2.5
Ca(NO ₃) ₂ ·4H ₂ O	2.5
NH ₄ H ₂ PO ₄	0.5
MgSO ₄ ·7H ₂ O	1.0
Fe-EDTA	0.05
H ₃ BO ₃	0.023
MnCl ₂ ·4H ₂ O	0.005
ZnSO ₄ ·7H ₂ O	0.0005
CuSO ₄ ·5H ₂ O	0.0002
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.0001

Growth parameters

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

Relative water content

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

Free proline content

Free proline content in alfalfa was assessed according to the method described by Bates et al. (1973). Fresh leaf tissues were extracted in 3% sulfosalicylic acid and centrifuged at 10 000 × g for 10 min. The resulting supernatant was reacted with a mixture containing ninhydrin, glacial acetic acid, and sulfosalicylic acid.

Subsequently, 1 mL of toluene was added, and the mixture was briefly centrifuged at $5\,000 \times g$ for 30 s. The absorbance of the colored phase was then recorded at 520 nm using a UV–Vis spectrophotometer.

Total chlorophyll content

Total chlorophyll content in alfalfa was quantified following the method outlined by Arnon (1949). Fresh leaf samples were extracted with acetone and centrifuged at $10\,000 \times g$ for 15 min. The absorbance of the clarified extract was subsequently measured at 645 and 663 nm using a UV–Vis spectrophotometer.

Malondialdehyde contents

Malondialdehyde (MDA) levels in alfalfa were determined according to the method of (Ohkawa et al., 1979). Fresh leaves were homogenized in trichloroacetic acid and centrifuged at $15\,000 \times g$ for 10 min. The collected supernatant was reacted with 0.5% thiobarbituric acid prepared in 20% trichloroacetic acid, followed by heating and centrifugation at $10\,000 \times g$ for 15 min. The absorbance of the resulting solution was then measured at 532 and 600 nm using a UV–Vis spectrophotometer.

Total phenolic content

Total phenolic content in alfalfa was quantified following the procedure described by Slinkard and Singleton (1977). For the analysis, the methanolic extract of the plant material was reacted sequentially with Folin–Ciocalteu reagent and sodium carbonate. The resulting color intensity was measured spectrophotometrically at 760 nm, and gallic acid was employed to construct the standard calibration curve.

Antioxidative enzymes

In the study, the enzymatic activity of ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) was detected using procedures developed by Wang et al. (1991), Chance and Maehly (1955), Beauchamp and Fridovich (1971), and Sgherri et al., (1994), respectively.

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.

RESULTS AND DISCUSSION

This study aimed to elucidate the physiological and biochemical mechanisms underlying drought tolerance in alfalfa cultivars with contrasting sensitivity to water deficit. It should be noted that PEG-induced osmotic stress represents a simplified model of drought conditions. Nevertheless, this approach enables the identification of intrinsic physiological differences between cultivars under controlled water deficit, which may contribute to drought tolerance under natural conditions.

Under drought stress conditions, the first significant decline in root dry weight was observed in Bilensoy-80 at -1.0 MPa, whereas in Diane this reduction started to appear at -1.5 MPa, compared with their controls (Figure 1A). For shoot dry weight, Bilensoy-80 exhibited an earlier decrease at -0.5 MPa, while Diane maintained stability until -1.5 MPa (Figure 1B). Root length in Bilensoy-80 started to decline significantly at -1.5 MPa, in contrast to Diane, where the reduction was delayed until -2.0 MPa (Figure 1C). Similarly, shoot length decreased in Bilensoy-80 at -0.5 MPa, but in Diane the reduction was observed after -1.5 MPa, compared with their controls (Figure 1D). These findings clearly indicate that Diane has better resilience to progressive drought stress compared with Bilensoy-80. In addition to sustaining shoot growth, under drought stress compared with the control, the enhanced root development was more pronounced in Diane, which is recognized as a fundamental adaptive strategy enabling plants to explore deeper soil layers and mitigate the impact of water deficit (Bothe et al., 2018).

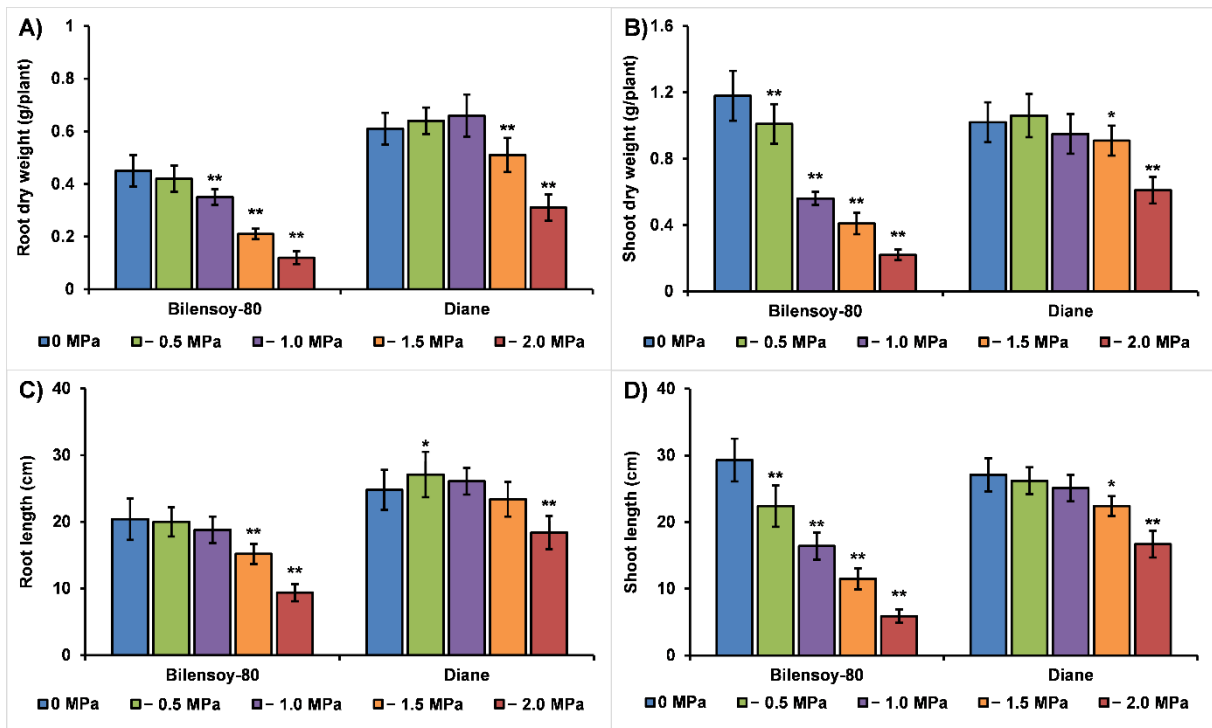


Figure 1. Growth parameters; A. Root dry weight (g/plant), B. Shoot dry weight (g/plant), C. Root length (cm), D. Shoot length (cm) of the alfalfa cultivars in different sets.

The columns represent means and the bars represent standard errors of the four replicates. Asterisks indicate a significant difference between control (0 MPa) and drought stressed plants in a cultivar, * $p < 0.05$ or ** $p < 0.01$.

In Bilensoy-80, relative water content started to decrease at -1.0 MPa drought stress level, compared with the control, while in Diane the first significant decrease appeared at -1.5 MPa (Figure 2A). Previous reports indicate that reductions in leaf water status often accelerate chlorophyll breakdown, which in turn diminishes chlorophyll concentration under drought conditions (Pour-Aboughadareh et al., 2017; Khan et al., 2024). Regarding osmolyte accumulation, Bilensoy-80 exhibited a significant increase in free proline content at -0.5 MPa; however, proline levels decreased under more severe stress conditions when compared with the control (Figure 2B). The reduction in proline content under stress may be attributed to metabolic impairment caused by excessive stress intensity. Under abiotic stresses, the capacity for proline biosynthesis may be limited due to reduced enzymatic activity, impaired nitrogen metabolism, or increased oxidative damage (Sadighfard and Demirkol, 2025). By contrast, Diane exhibited increases in free proline accumulation at -1.0 , -1.5 , and -2.0 MPa (approximately 35%, 80% 10%, respectively), compared with the control (Figure 2B). Free proline is known to play a crucial role in drought stress adaptation by sustaining turgor and contributing to redox equilibrium, thereby enhancing plant survival under adverse conditions (Ullah et al., 2022).

For oxidative stress markers, MDA concentrations in Bilensoy-80 increased significantly at -1.0 , -1.5 , and -2.0 MPa stress levels, compared with the control, whereas in Diane the increases were detected at -1.5 and -2.0 MPa (Figure 2C). The substantial increase of MDA in Bilensoy-80 shows its susceptibility to oxidative damage, as MDA is a substance produced by membrane lipids in response to reactive oxygen species, can be used as a drought indicator to evaluate the degree of plasma membrane damage and the plants' ability to tolerate drought stress (Zhang et al., 2021). In contrast, the comparatively moderate increases in Diane confirm its better oxidative stress tolerance than Bilensoy-80.

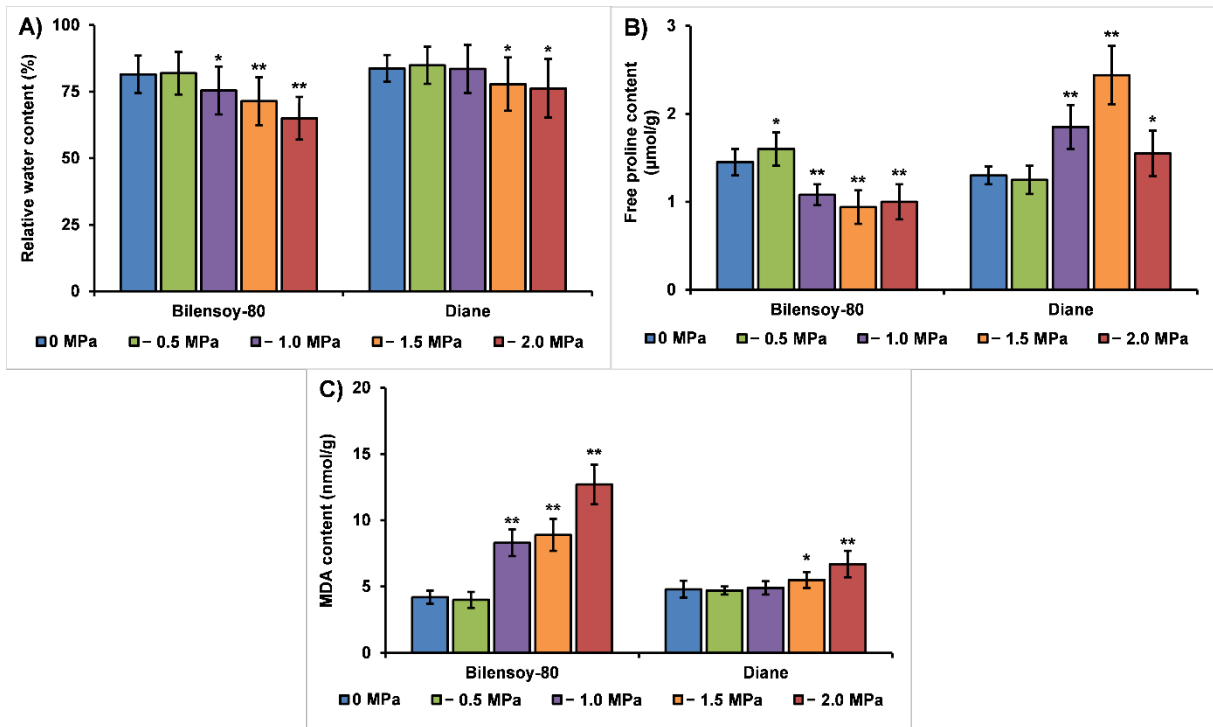


Figure 2. Biochemical status; A. Relative water content (%), B. Free proline content (µmol/g), C. MDA content (nmol/g) of the alfalfa cultivars in different sets.

The columns represent means and the bars represent standard errors of the four replicates. Asterisks indicate a significant difference between control (0 MPa) and drought stressed plants in a cultivar, *p < 0.05 or **p < 0.01.

In Bilensoy-80, the declines in total chlorophyll content started to be observed at -0.5 MPa drought stress level, compared with the control, whereas in Diane this reduction was only observed under more severe stress levels of -1.5 and -2.0 MPa (Figure 3A). This observation was closely associated with the relative water content results described earlier, suggesting a parallel response between leaf hydration and pigment stability. Total phenolic content remained statistically unchanged in both cultivars, compared with their controls despite increasing drought intensity, suggesting that phenolic metabolism in these genotypes was relatively insensitive to water deficit (Figure 3B).

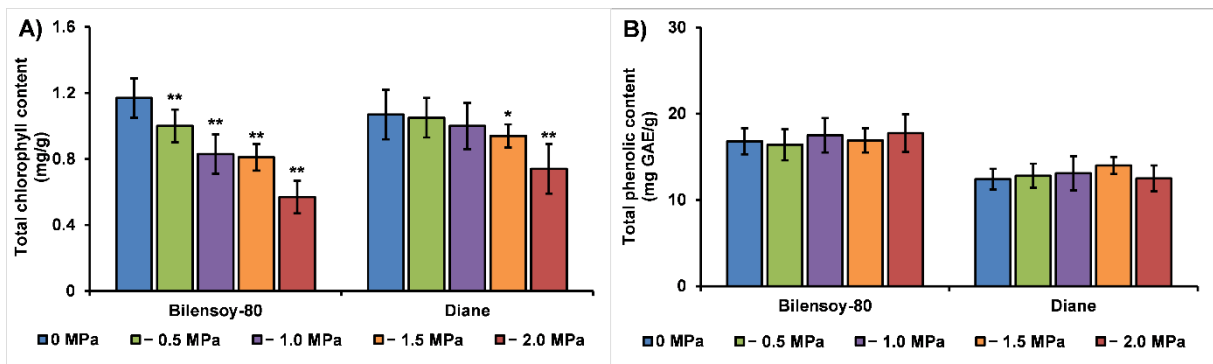


Figure 3. Biochemical status; A. Total chlorophyll content (mg/g), B. Total phenolic content (mg GAE/g) of the alfalfa cultivars in different sets.

The columns represent means and the bars represent standard errors of the four replicates. Asterisks indicate a significant difference between control (0 MPa) and drought stressed plants in a cultivar, *p < 0.05 or **p < 0.01.

Regarding antioxidative enzymes, Bilensoy-80 exhibited a marked decrease in CAT activity at -2.0 MPa drought stress level, compared with the control, while Diane showed significant increases at -1.0 and -1.5 MPa (Figure 4B). A similar pattern was observed for GR activity; Bilensoy-80 displayed consistent reductions across all stress levels, compared with the control, whereas Diane exhibited increase at moderate drought stress levels (-1.0 and -1.5 MPa). In contrast, APX and SOD activities remained statistically unaffected in both genotypes

under drought conditions. The antioxidative enzyme results showed a coordinated enhancement in CAT, and GR activities in Diane, suggesting that the activation of these enzymes may have contributed to a more robust antioxidant defense system, thereby conferring greater resilience to drought stress (Figure 4A–D). The role of such enzymatic up-regulation in improving stress tolerance has also been reported in different crop species, including alfalfa (Hasanuzzaman et al., 2020; Mushtaq et al., 2020; Demirkol, 2021).

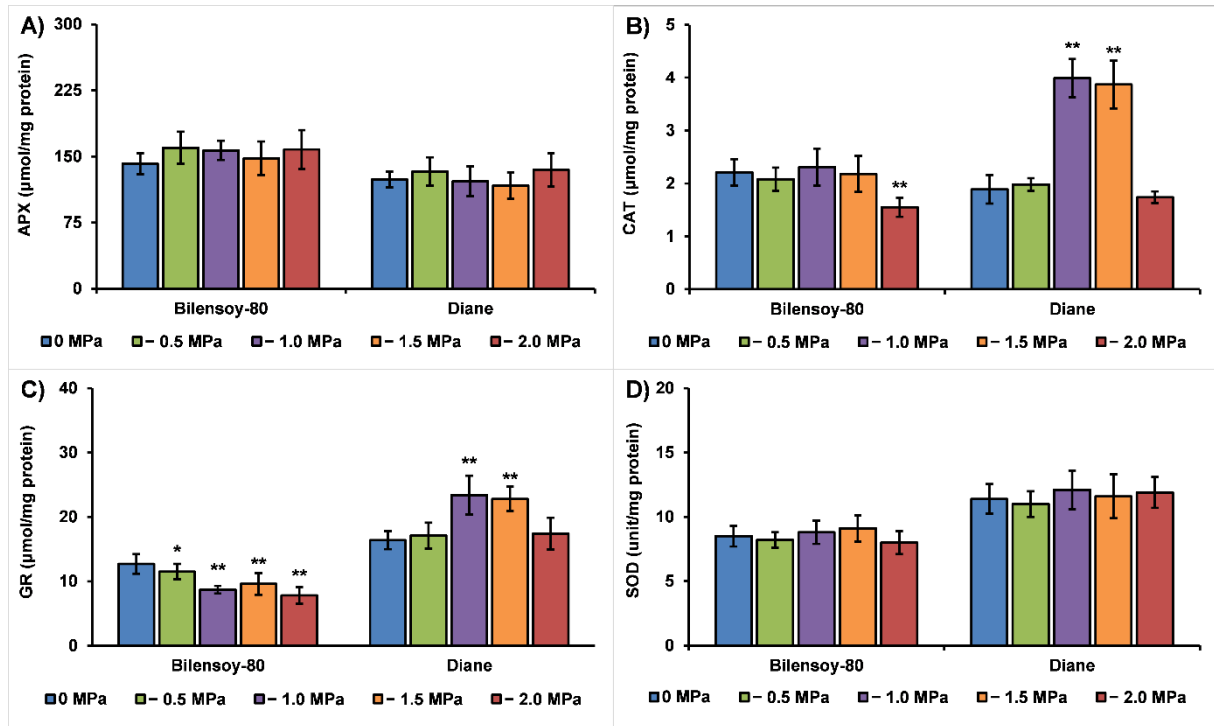


Figure 4. Antioxidative enzymes; A. APX ($\mu\text{mol}/\text{mg}$ protein), B. CAT ($\mu\text{mol}/\text{mg}$ protein), C. GR ($\mu\text{mol}/\text{mg}$ protein), D. SOD (unit/mg protein) of the alfalfa cultivars in different sets.

The columns represent means and the bars represent standard errors of the four replicates. Asterisks indicate a significant difference between control (0 MPa) and drought stressed plants in a cultivar, * $p < 0.05$ or ** $p < 0.01$.

In the study, the superior performance of the ‘Diane’ cultivar under severe drought stress may be attributed to the coordinated regulation of osmolyte accumulation and antioxidant defense mechanisms. Enhanced free proline accumulation may contribute to osmotic adjustment and cellular protection, while increased activities of antioxidant enzymes such as CAT and GR likely mitigate oxidative damage by scavenging reactive oxygen species. The interaction between these mechanisms may collectively enhance stress tolerance by maintaining cellular homeostasis under water deficit conditions.

Despite the valuable insights provided, this study has certain limitations. The experiments were conducted under controlled greenhouse conditions using PEG-induced osmotic stress, which may not fully reflect field drought scenarios. Future studies integrating field experiments and gene expression analyses would further clarify the mechanisms underlying drought tolerance in these alfalfa cultivars.

CONCLUSION

The results of this study demonstrated that the drought-tolerant cultivar ‘Diane’ maintained superior growth, physiological stability, and biochemical balance compared with ‘Bilensoy-80’ under water deficit levels. This improved performance was largely attributed to enhanced free proline biosynthesis and increased activity of key antioxidant enzymes (CAT and GR). These findings highlight the potential of ‘Diane’ for cultivation in drought-prone regions and provide valuable information for breeding programs targeting improved drought resilience in forage crops.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Gürkan DEMİRKOL :Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; software; writing— original draft; writing—review and editing.

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