



The Effect of Exogenous Calcium Treatments on Reducing Drought Stress Damages in Pepper Grown in In Vitro Conditions

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Received: 16 July 2025 / Accepted: 9 February 2026 / Published online: 18 February 2026
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Abstract

Drought stress severely limits plant growth and development. Calcium, beyond being an essential nutrient, functions as an important signaling-molecule in plant stress responses. Given its economic importance, increasing drought tolerance in *Capsicum annuum* L. varieties has become a priority. In this study, two pepper varieties with different drought tolerances, Hilvan and Inan, were exposed to drought stress induced by polyethylene glycol (PEG 6000; 3 mg L⁻¹) and/or calcium treatments (CT) (330 and 660 mg L⁻¹ CaCl₂). The seedlings were grown in a controlled growth chamber and evaluated for morphological, physiological, and biochemical responses after five weeks. PEG application promoted root growth in both varieties but significantly suppressed shoot growth, chlorophyll content, protein levels, and antioxidant capacity. These negative effects were more pronounced in Hilvan, indicating a higher sensitivity to drought. Inan exhibited relatively stable growth and metabolic response under PEG stress, indicating higher drought tolerance. CT significantly mitigated the damage caused by PEG in both varieties, with the level of mitigation differing between them. Inan exhibited marked increases in chlorophyll content, protein accumulation, root fresh weight, antioxidant enzyme activities, and phenolic compounds, accompanied by a decrease in MDA levels, indicating effective alleviation of oxidative stress. CT in Hilvan partially restored physiological balance, but the responses remained weaker compared to Inan. Overall, CT was more effective in mitigating drought-induced oxidative damage in the drought-sensitive Hilvan variety. These findings demonstrate that CT is beneficial in increasing tolerance in drought-sensitive pepper varieties, highlighting the importance of calcium-mediated signal transduction.

Keywords Abiotic stress · Calcium · Drought · Exogenous applications

Introduction

Plants, which are sessile organisms, interact with living and non-living factors in their environment, but in some cases, these interactions can negatively affect the life and development of plants. Stress refers to any adverse condition or harmful substance that negatively affects a plant's natural processes, such as metabolism, growth, and development (Lichtenthaler 1998; Ozturk et al. 2021; Tripathi et al. 2022; Zhan and Zhu 2025). Plants are generally exposed to various environmental stresses under natural and agricultural conditions (Turker et al. 2025; Turkeyilmaz Unal and Turker 2024). Drought stress is exemplified when the water available to plants in the soil decreases due to low soil moisture at a certain time (Dai 2011; Keyvan 2010). This lack of water can adversely affect plant growth, production, and life. Drought is one of the most common stress factors

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affecting plant life in many parts of the world, especially in arid and semiarid regions (Sharkey and Schrader 2006).

Drought stress is a complex type of stress and generally causes changes in the physiological, morphological, ecological, biochemical, and molecular characteristics of plants, negatively affecting the quantity and quality of plant growth and yield (Bhargava and Sawant 2013; Nezhadahmadi et al. 2013; Zlatev and Lidon 2012). The responses of plants to water scarcity depend on the plant species, age, stage of development, and especially the duration of drought (Sharkey and Schrader 2006). Plants aim to maintain cellular water balance under drought conditions by preventing water loss and increasing water uptake into the cell, thereby preserving the normal functions of stressed plant cells (Salehi-Lisar and Bakhshayeshan-Agdam 2020). In general, drought causes loss of leaf turgor pressure, etiolation, chlorosis, and premature leaf fall, reduction in leaf area, decrease in chlorophyll and stomatal numbers, thickening of the cell wall, cutinization of the leaf surface, deepening of stomata in succulent and xerophytic plants, and formation of tube leaves in cereals (Akhtar and Nazir 2013; Bernacchia and Furini 2004; Bhargava and Sawant 2013; Keyvan 2010; Mafakheri et al. 2010; Salehi-Lisar and Bakhshayeshan-Agdam 2016, 2020; Zare et al. 2023). During drought, organic compounds produced in the plant are used to expand the roots and create a branched and extensive root system to increase water uptake from the soil (Akhtar and Nazir 2013). Under drought stress, plants initiate osmoregulation mechanisms to balance intracellular water potential. The accumulation of osmoprotectants such as proline, soluble sugars, sugar alcohols, and quaternary ammonium compounds plays a crucial role. These osmotic regulators maintain cell turgor and protect reactive oxygen species by supporting antioxidant components and enzymes. Osmoprotectants, antioxidant enzymes, and antioxidant components also directly enhance the plant's ability to adapt to drought stress (Ozturk et al. 2021).

Pepper (*Capsicum annum* L.) (belonging to the Solanaceae family) is an annual herbaceous plant with white flowers that is cultivated as a vegetable and spice and used as a raw material in the pharmaceutical industry (Ceylan 2024; Rady et al. 2024). Pepper has various uses in Türkiye and around the world, and is an indispensable product in the food industry (as a spice and in sauces) and the healthcare industry (in the production of ointments and creams containing its active ingredient). Pepper (*C. annum*) is a highly valued product, cultivated for its high nutritional value, phytochemical compounds, and antioxidant properties (Angole-Tierrablanca et al. 2025). The Inan pepper variety is a plant produced for selection purposes and is widely used in agricultural production in Türkiye. The Inan pepper variety was created to be resistant to heat. The Hilvan

pepper variety, on the other hand, is a traditional seed and is preferred in local agricultural production. The fruits of both pepper varieties are characterized by their extremely spicy nature. The pepper varieties used in this study belong to the Urfa pepper group, which is traditionally cultivated in semi-arid regions and frequently exposed to water-limited environments (Kabay and Yaban 2019). These varieties are known to exhibit different physiological and biochemical responses under stress conditions, making them suitable for evaluating the effects of exogenous calcium applications on drought stress tolerance under in vitro conditions.

In addition to its role as an essential nutrient, Ca^{2+} functions as a central secondary messenger that mediates early signal transduction under abiotic stress (Gundogdu et al. 2026). Under non-stress conditions, cytosolic Ca^{2+} levels remain tightly controlled within nanomolar to low micromolar ranges, while intracellular stores, such as the vacuole, maintain millimolar concentrations. Upon exposure to an external stimulus, Ca^{2+} channels in the plasma membrane and organelles initiate rapid Ca^{2+} influx or release, generating specific Ca^{2+} signatures. These signatures constitute the earliest step of stress perception and subsequently activate decoding mechanisms that reprogram cellular metabolism and gene expression to initiate an adaptive response. Specific Ca^{2+} signatures activate calcium-dependent protein kinases (CDPKs) and other downstream pathways, enabling plants to coordinate stress tolerance responses (Ranf et al. 2008; Tuteja and Mahajan 2007).

In this study, the effects of drought stress and low-concentration exogenous calcium (CaCl_2) applications were investigated for the first time on two pepper varieties in Türkiye, namely the ancestral seed Hilvan and the pepper seed produced by Inan in the selection project, using polyethylene glycol (PEG). The mitigation of this stress effect was examined using various analytical methods.

Materials and Methods

Plant Materials

The seeds of the Hilvan variety required for the study were provided by the GAP Agricultural Research Institute Directorate of the Ministry of Agriculture and Forestry of the Republic of Türkiye, and the seeds of the Inan 3363 Urfa pepper variety were provided by BETA Ziraat ve Ticaret A.Ş.

Plant Cultivation and Treatments

The seeds of pepper (*Capsicum annum* L.) that were healthy, plump in appearance, and of similar size were

selected, sterilized, and then grown in MS basic nutrient medium to which 3% sucrose and 0.7% agar were added without hormones (Murashige and Skoog 1962). Polyethylene glycol 6000 (3 mg L⁻¹ PEG) to create drought stress and/or different concentrations of calcium (330 mg L⁻¹ CaCl₂, 660 mg L⁻¹ CaCl₂) were applied. The concentrations of polyethylene glycol (PEG) used to induce drought stress and calcium chloride (CaCl₂) applied to mitigate its effects were selected based on previously published *in vitro* studies (Koninskaya and Zagorskina 2020; Al-Mayahi 2019; Ibrahim et al. 2008; Okafor et al. 2022; Sidky and Gadalla 2013) and preliminary optimization trials. PEG concentrations were chosen to impose moderate osmotic stress without causing irreversible tissue damage, as commonly reported in *in vitro* drought simulation studies. Similarly, calcium concentrations were determinant within ranges previously shown to enhance stress tolerance and cellular stability in plant tissue cultures, while avoiding toxicity. This approach allowed the evaluation of calcium-mediated stress alleviation under controlled and physiologically relevant drought conditions. The control group was grown without any application. Both cultivars' seeds germinated and grew in Magenta GA-7 plant culture bowls under controlled growth chamber conditions (25 ± 2 °C, 16/8 h light/dark photoperiod). At the end of the 5th week, seedlings were harvested according to the randomized block design, morphological measurements were made on the seedlings, and they were put into the deep freezer (− 80 °C) for physiological-biochemical analyses. Ten biological replicates were used for morphological measurements and three biological replicates were used for biochemical analyses.

Determination of Root and Shoot Fresh-Dry Weights from Developing Seedlings

The lengths of the roots and shoots were measured in ten randomly selected seedlings based on a block design. The seedlings were separated at the root-shoot junction, and the fresh and dry weights of the roots and shoots were assessed separately. After fresh weight measurement, plant samples were dried in a forced-air oven at 78 ± 2 °C for 48 h, with minor modifications to the method described by Chehel-anan et al. (2025). This procedure was applied uniformly to all treatments. The drying duration was sufficient for *in vitro*-grown tissues, which have high water content.

Determination Of Chlorophyll Contents

To determine the chlorophyll (chlorophyll a, chlorophyll b, total chlorophyll) contents, the procedure of Witham et al. (1971) was used. Randomly taken 1 g of leaves from the control and experimental groups were cut into pieces and

crushed in a mortar with 2 mL of 80% acetone. The extract obtained by filtering through Whatman no:2 filter paper was completed to 10 mL with 80% acetone. Chlorophyll pigment absorption values (645 nm and 663 nm) of the obtained extracts were measured in VIS Spectrophotometer. By putting the values obtained from the optical density (D) measurements made at two different wavelengths of chlorophyll extract into their places in the equations given below, the contents of chlorophyll a, chlorophyll b, and total chlorophyll in 1 g were calculated as mg.

$$\begin{aligned} &\text{mg chlorophyll a/g tissue} \\ &= [12.7 (D_{663}) - 2.69 (D_{645})] (V/1000.W) \end{aligned}$$

$$\begin{aligned} &\text{mg chlorophyll b/g tissue} \\ &= [22.9 (D_{645}) - 4.68 (D_{663})] (V/1000.W) \end{aligned}$$

$$\begin{aligned} &\text{mg total chlorophyll/g tissue} \\ &= [20.2 (D_{645}) + 8.02 (D_{663})] (V/1000.W) \end{aligned}$$

In the equations, D indicates the optical density (absorbance value) of the chlorophyll extract at the specified wavelengths; V indicates the final volume of 80% acetone; W indicates the fresh weight of the extracted tissue in grams.

Determination of Total Protein Content

To determine the total protein content, 1 g of fresh leaf samples harvested from the control and treatment groups were extracted in 5 mL 0.05 M Na phosphate buffer, pH 7.8. The obtained extract was centrifuged in a microcentrifuge at 13,000 rpm for 30 min.

Total protein content determinations were made using BSA (Bovine Serum Albumin) standards according to Bradford (1976). This method is based on the principle that proteins form complexes with Coomassie Brilliant Blue reagent in phosphoric acid medium and that the formed complex shows maximum absorbance at 595 nm. 1 mL of reaction mixture containing Coomassie Brilliant Blue protein-dye was added to the supernatants taken in an appropriate volume after centrifugation. The absorbance values of the samples kept at room temperature for 10 min were taken at 595 nm with a VIS Spectrophotometer. These absorbance values obtained were treated to the calibration curve created with BSA standards (0.02–0.2 mg/mL), and the total soluble protein content in the plant samples of the treatment groups was determined as mg.g fresh weight⁻¹.

Determination of Antioxidant Capacities

Determination of Carotenoid Content

The absorbance value of the extracts prepared from pepper leaves according to the Witham et al. (1971) method at 450 nm wavelength was substituted into the formula below to calculate the mg carotenoid amount in 1 g of leaf fresh weight.

$$\text{mg total carotenoid/tissue} = 4.07 \times D_{450} - (0.0435 \times \text{Chla amount} + 0.367 \times \text{Chlb amount})$$

Determination of DPPH Radical Scavenging Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined according to the method of Brand-Williams et al. (1995). A series of dilutions was prepared using 0.1 mM DPPH solution, plant extracts, and methanol to determine the antioxidant activities of the extracts. 100 µl of each dilution was taken, respectively, and added to 2.9 mL of DPPH solution and incubated for 15 min at room temperature. After incubation, the absorbance values of the samples were measured at 517 nm. 0.1 mM DPPH solution was used as a blank tube, and the % DPPH scavenging effect of the samples was calculated by putting them into the formula.

Determination of Catalase (CAT) Enzyme Activity

Bergmeyer's (1970) method was used to determine CAT enzyme activities. 2.77 mL of 0.1 mM EDTA containing 0.05 M sodium-phosphate buffer (pH 7.0) and 30 µl of 3% H₂O₂ were added to 200 µl supernatants, and the change in absorption due to H₂O₂ consumption per minute was determined at 240 nm wavelength in a UV spectrophotometer. Specific enzyme activity was expressed as EU mg protein⁻¹. g fresh weight⁻¹.

Determination of Phenylalanine Ammonium Lyase (PAL) Activity

0.1 g of sample was homogenized with 50 mM sodium phosphate buffer (extraction) containing 1% polyvinylpyrrolidone (PVP) and 1 mM Phenylmethylsulfonyl (PMSF) at pH 6.5. Then, the homogenate was centrifuged at 10,000 rpm for 25 min at 4 °C. The collected supernatants from homogenates were used to determine phenylalanine ammonium lyase activity. The spectrophotometric method was used to calculate the phenylalanine ammonium lyase activity (Pascholati et al. 1986). The conversion of L-phenylalanine to

trans-cinnamic acid was recorded at 290 nm absorbance by reacting with 100 µl of enzyme extract and 1000 µl of 0.2% Phenylalanine solution at 37 °C for one hour. Phenylalanine ammonium lyase activity was determined by preparing the standard of cinnamic acid and expressed as µmol cinnamic acid hour⁻¹.

Determination of Total Phenolic Substance Content

0.1 g Leaf sample was subjected to homogenization with 5 mL of 80% methyl alcohol, incubated at 80 °C for 15 min, and centrifuged at 10,000 rpm for 10 min. The pellet was homogenized again with 2.5 mL of 80% methyl alcohol, incubated at 80 °C for 15 min, and centrifuged at 10,000 rpm for 10 min. The supernatant was dried at 80 °C and resuspended in 1 mL of 80% methyl alcohol. This suspension was used for the determination of total phenolic substances (Gayoso et al. 2004). The Folin-Ciocalteu method was used to determine the total phenolic substance (Singleton and Rossi 1965). 100 µl of the extract was incubated with 10 times diluted Folin-Ciocalteu for 5 min at room temperature, 750 µl of sodium bicarbonate solution was added to this solution, and incubated again at room temperature for 90 min. The absorbance change was recorded at 765 nm on the spectrophotometer, and the amount of phenolic substance was calculated using the gallic acid standard.

Determination of Total Proline Content

Proline contents were determined with 1 g samples according to the method of Bates et al. (1973). After the samples were homogenized in 10 mL of 3% sulfosalicylic acid, they were filtered through Whatman No. 2 filter paper. The filtrate was kept in a cool and dark environment for 24 h, then 2 mL of filtrate: acid ninhydrin: glacial acetic acid was added and incubated at 100 °C for 1 h. The reaction was stopped using an ice bath, 4 mL of cold toluene was added to the tubes and mixed, and the absorbance of the fraction containing toluene was taken at 520 nm on a Boeco brand Spectrophotometer. Standards were prepared to contain 0.1, 0.2, 0.3, 0.4, and 0.5 µmol.proline⁻¹, and the amount of proline was expressed as µmol.proline-g fresh weight⁻¹ using the calibration curve.

Determination of Malondialdehyde (MDA) Level

MDA level was measured in samples to determine lipid peroxidation. For this purpose, 0.2 g of fresh plant sample was homogenized with 4 mL of 0.1% trichloroacetic acid (TCA) solution and centrifuged at 12,500 revolutions/minute (rpm) for 5 min. A 1 mL aliquot was taken from the clear part and transferred to clean tubes, and 0.5% thiobarbituric acid

Table 1 Effects of PEG and calcium treatments on root and shoot development of Hilvan pepper variety (n:10)

Treatment groups	Root length (cm)	Root fresh weight (g)	Root dry weight (g)
Control	11.636±1.293a	0.358±0.019a	0.036±0.005a
PEG	15.800±2.502ab	0.497±0.050b	0.043±0.024ab
330 mg L ⁻¹ CaCl ₂	9.600±2.055ac	0.033±0.004c	0.011±0.002ac
660 mg L ⁻¹ CaCl ₂	10.000±1.464ac	0.094±0.015c	0.011±0.004ac
PEG+330 mg L ⁻¹ CaCl ₂	13.600±1.921a	0.474±0.133b	0.037±0.009a
PEG+660 mg L ⁻¹ CaCl ₂	14.500±3.460ab	0.241±0.023a	0.032±0.009a
Treatment groups	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Control	7.365±0.988a	0.223±0.015a	0.050±0.027a
PEG	5.900±1.183a	0.114±0.050ab	0.025±0.004b
330 mg L ⁻¹ CaCl ₂	8.150±0.881a	0.269±0.097ac	0.052±0.004a
660 mg L ⁻¹ CaCl ₂	7.550±0.208a	0.245±0.053a	0.051±0.003a
PEG+330 mg L ⁻¹ CaCl ₂	7.033±1.184a	0.144±0.072a	0.048±0.019a
PEG+660 mg L ⁻¹ CaCl ₂	6.943±1.236a	0.143±0.014a	0.047±0.012a

Different letters indicate that the difference is statistically significant, $p < 0.05$

Table 2 Effects of PEG and calcium treatments on root and shoot development of Inan pepper variety (n:10)

Treatment groups	Root length (cm)	Root fresh weight (g)	Root dry weight (g)
Control	15.250±0.850a	0.516±0.230a	0.071±0.012a
PEG	16.600±0.600ab	0.340±0.025ab	0.050±0.010a
330 mg L ⁻¹ CaCl ₂	14.500±0.058ac	0.575±0.364ac	0.078±0.017a
660 mg L ⁻¹ CaCl ₂	14.800±0.451ac	0.556±0.032ac	0.072±0.018a
PEG+330 mg L ⁻¹ CaCl ₂	15.300±0.950a	0.473±0.113a	0.070±0.012a
PEG+660 mg L ⁻¹ CaCl ₂	15.900±0.321a	0.410±0.122a	0.068±0.005a
Treatment groups	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Control	8.167±1.138a	0.484±0.095a	0.059±0.020a
PEG	5.400±0.115b	0.204±0.071b	0.028±0.009b
330 mg L ⁻¹ CaCl ₂	9.100±1.266a	0.590±0.084a	0.065±0.011a
660 mg L ⁻¹ CaCl ₂	8.200±1.571a	0.460±0.136a	0.054±0.029a
PEG+330 mg L ⁻¹ CaCl ₂	8.500±1.479a	0.432±0.046a	0.051±0.002a
PEG+660 mg L ⁻¹ CaCl ₂	8.300±1.559a	0.367±0.055a	0.053±0.009a

Different letters indicate that the difference is statistically significant, $p < 0.05$

(TBA) dissolved in 4 mL of 20% TCA was added. The samples were kept in a water bath at 95 °C for 30 min and then quickly cooled and centrifuged at 10,000 rpm for 10 min. After centrifugation, absorbance readings of the clear parts of the samples were taken on a spectrophotometer set at 532 and 600 nm wavelengths (Zhou and Leul 1999). MDA $\text{nmol g}^{-1} \text{FW} = (A_{532} - A_{600}) \times V (4 \text{ mL}) \times 1000 / 155 \times W$ (g plant) (3.5).

A532: absorbance reading at 532 nm wavelength

A600: absorbance reading at 600 nm wavelength

Statistical Evaluation

All measurements and analysis data were evaluated in SPSS version 16.0 program with Variance analysis (Tukey 1954) according to a $p < 0.05$ significance level. Standard error and

standard deviation values of the averages were also calculated in the same program.

Results

Measurements of Plant Growth

The growth parameters of Hilvan variety seedlings are shown in Table 1, and Inan variety seedlings are in Table 2.

The root length of the Hilvan variety increased compared to the control in all treatments except 330 and 660 mg L⁻¹ CaCl₂ treatments. The root fresh and dry weights decreased in 330 and 660 mg L⁻¹ CaCl₂ and PEG+660 mg L⁻¹ CaCl₂ treatments, and an increase occurred in other groups. While the changes in root length and dry weight were not significant, all increases and decreases in root fresh weight, except

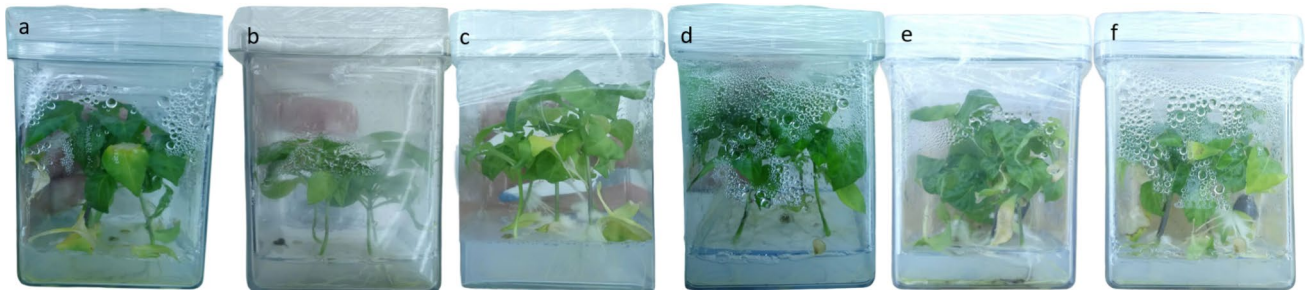


Fig. 1 Phenotypic characteristics of Hilvan pepper varieties. **a** Control **b** 3 mg L⁻¹ PEG **c** 330 mg L⁻¹ **d** 660 mg L⁻¹ **e** 3 mg L⁻¹ PEG + 330 mg L⁻¹ **f** 3 mg L⁻¹ PEG + 660 mg L⁻¹

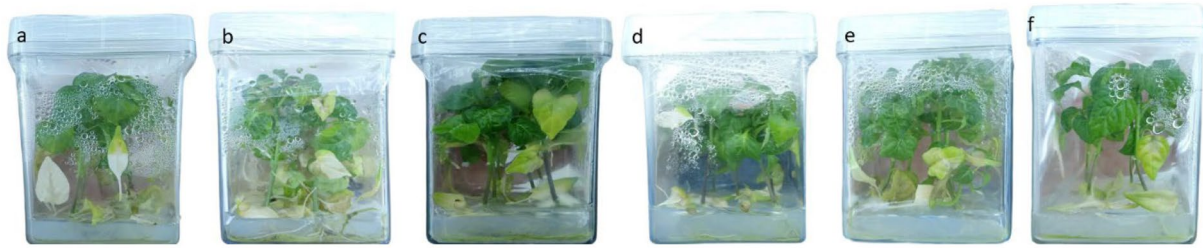


Fig. 2 Phenotypic characteristics of Inan pepper varieties. **a** Control **b** 3 mg L⁻¹ PEG **c** 330 mg L⁻¹ **d** 660 mg L⁻¹ **e** 3 mg L⁻¹ PEG + 330 mg L⁻¹ **f** 3 mg L⁻¹ PEG + 660 mg L⁻¹

in the PEG + 660 mg L⁻¹ CaCl₂ group, were significant. The highest increase was 38.83% in PEG treatment, and the highest decrease was 90.78% in 330 mg L⁻¹ CaCl₂ treatment ($p < 0.05$) (Table 1).

Hilvan variety shoot length, fresh weight, and dry weight decreased compared to the control in all treatment groups except 330 and 660 mg L⁻¹ CaCl₂. While the changes in shoot length and fresh weight were not significant, the decrease in dry weight in PEG treatment (90%) was found significant ($p < 0.05$) (Table 1).

Inan pepper variety, 330 and 660 mg L⁻¹ CaCl₂ treatments caused a decrease in root length and increases in root fresh and dry weights compared to the control, while the opposite situation was observed in other treatments (Table 2).

The increases and decreases that occurred were not significant (Table 2). The shoot length decreased only with PEG treatment. Shoot fresh and dry weights increased only by 330 mg L⁻¹ CaCl₂ treatment. From the obtained data, only the decreases in shoot length (33.88%), fresh (57.85%), and dry (52.54%) weights were significant ($p < 0.05$) (Table 2).

The phenotypic traits of Hilvan and Inan pepper varieties grown in MS medium are shown in Figs. 1 and 2.

The Inan pepper cultivar used in this study is known for its drought tolerance, which provides important context for the observed phenotypic responses. In our study, Inan plants exhibited relatively weaker growth and leaf chlorosis under control conditions compared to PEG-treated plants. This may be associated with suboptimal nutrient balance or limited calcium availability under non-stress conditions. As a

drought-tolerant cultivar, Inan may activate adaptive physiological mechanisms under mild PEG-induced stress, resulting in improved growth performance. Furthermore, calcium supplementation (330 mg L⁻¹ CaCl₂) likely enhanced membrane stability and chlorophyll preservation, thereby alleviating visible stress symptoms. The Hilvan pepper cultivar, similar to Inan, originates from the Urfa region and is inherently adapted to semi-arid conditions, which explains its overall capacity to survive PEG-induced drought stress. However, in contrast to the Inan cultivar, Hilvan exhibited more pronounced reductions in shoot vigor and leaf turgor under PEG treatment, indicating a comparatively lower phenotypic resilience. Under control conditions, Hilvan plants maintained moderate growth with fewer chlorotic symptoms, suggesting that basal growth performance in this cultivar is less dependent on stress-induced adaptive responses. Calcium supplementation, particularly at 330 mg L⁻¹ CaCl₂, noticeably improved shoot compactness and leaf integrity under both control and PEG conditions. This response likely reflects the role of calcium in stabilizing cellular membranes, supporting water relations, and mitigating drought-induced morphological damage. Collectively, these observations suggest that while both Urfa-origin cultivars possess inherent drought tolerance. However, Hilvan relies more strongly on exogenous calcium supply to maintain phenotypic stability under osmotic stress.

Chlorophyll Contents

In the Hilvan variety, there was an increase in chl_a, chl_b, and total chl contents in the group where only PEG and low-concentration CaCl₂ were used together. The highest decreases were chl_a (80.54%), chl_b (79.03%), and total chl (80.05%) in PEG treatment. The decreases were significant ($p < 0.05$) (Fig. 3).

In the Inan variety, increases were detected in the chl_a and total chl values in the groups treated with 330 mg L⁻¹ CaCl₂, 660 mg L⁻¹ CaCl₂, and calcium-treated groups. The chl_b values increased in the treatments of 330 mg L⁻¹ CaCl₂, 660 mg L⁻¹ CaCl₂, and PEG+330 mg L⁻¹ CaCl₂ compared to the control. Among the increases in the groups treated with calcium at different concentrations, only those in the groups treated with calcium at different concentrations were found to be significant. All of those decreases in chl_a and in total chl at PEG were found to be significant ($p < 0.05$) (Fig. 4).

Total Protein Content

Data on total protein content are given in Fig. 5. Total protein content increased in all treatment groups except the PEG treatment in the Hilvan variety compared to the control group. The increases that occurred were not at the level of significance, while the decrease was at the level of

significance (79.37%) ($p < 0.05$). In the Inan variety, there were increases but not significant in all treatment groups (Fig. 5).

Antioxidant Capacities

Carotenoid Content

Carotenoid contents decreased in all groups except PEG+330 mg L⁻¹ CaCl₂ treatment in Hilvan variety, and 660 mg L⁻¹ CaCl₂, PEG+330 mg L⁻¹ CaCl₂, and PEG+660 mg L⁻¹ CaCl₂ treatments in Inan variety. While the increases and decreases in the Inan variety are not significant, the decreases in the Hilvan variety are significant ($p < 0.05$) (Fig. 6).

DPPH Radical Scavenging Activity

The findings regarding DPPH radical scavenging activity are in Fig. 7. In the Hilvan pepper variety, radical scavenging activity increased in groups where only calcium treatment was made, while it decreased in other treatment groups. In the Inan variety, increases were determined in the PEG+330 mg L⁻¹ CaCl₂ treatment group with calcium treatments, and decreases were determined in other groups. The highest decrease was in PEG+660 mg L⁻¹ CaCl₂

Fig. 3 Effects of PEG and calcium treatments on chlorophyll content in Hilvan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG+330 mg L⁻¹ CaCl₂, 6: PEG+660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

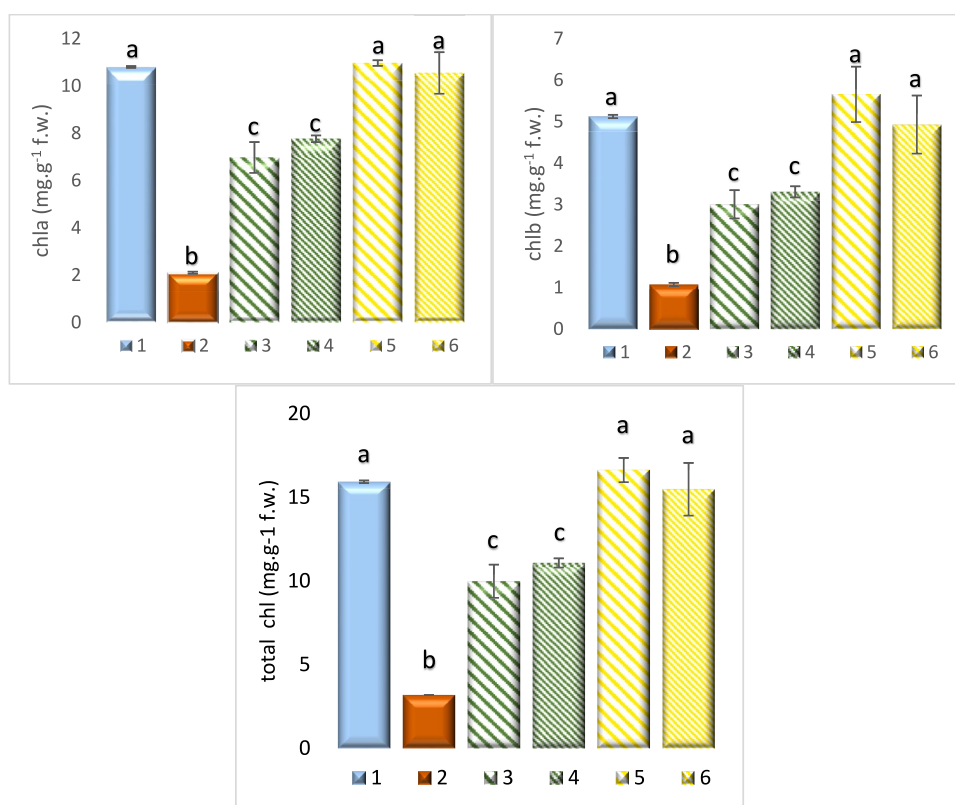


Fig. 4 Effects of PEG and calcium treatments on chlorophyll content in Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG+330 mg L⁻¹ CaCl₂, 6: PEG+660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

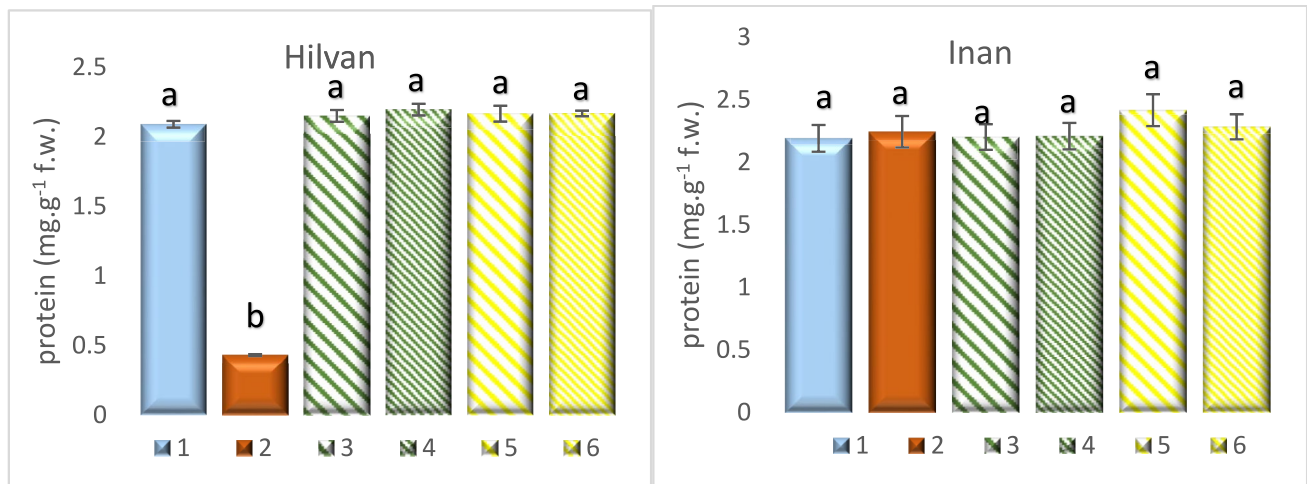
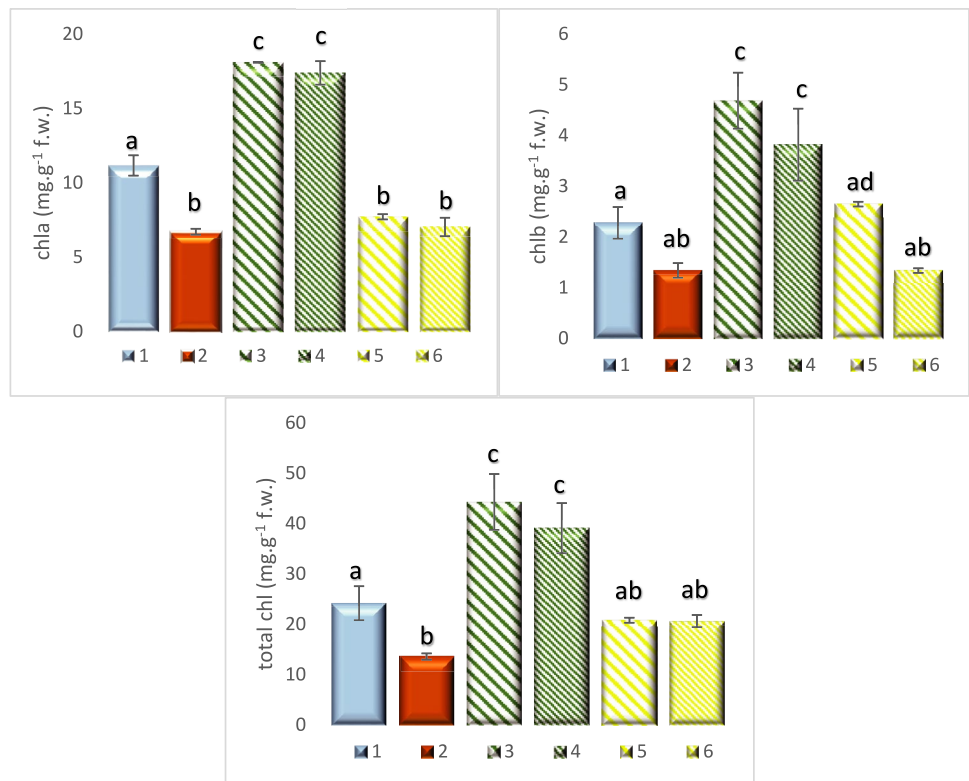


Fig. 5 Effects of PEG and calcium treatments on total protein content in Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG+330 mg L⁻¹ CaCl₂, 6: PEG+660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

treatment for both varieties and was found to be significant (Fig. 7).

CAT Enzyme Activity

CAT activity increased compared to the control in all treatments except 660 mg L⁻¹ CaCl₂ in the pepper Hilvan variety and PEG+660 mg L⁻¹ CaCl₂ in the Inan variety. The highest enzyme activities were in the Hilvan with 49.171 EU.g⁻¹

PEG+660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

f.w. and PEG+660 mg L⁻¹ CaCl₂ treatment, and in the Inan with 76.670 EU.g⁻¹ f.w. and 330 mg L⁻¹ CaCl₂ treatment ($p < 0.05$) (Fig. 8).

PAL Enzyme Activity

PAL activity decreased in the Hilvan variety with 660 mg L⁻¹ CaCl₂, Inan variety with 330 mg L⁻¹ CaCl₂, and in PEG+660 mg L⁻¹ CaCl₂ treatments, while increases were

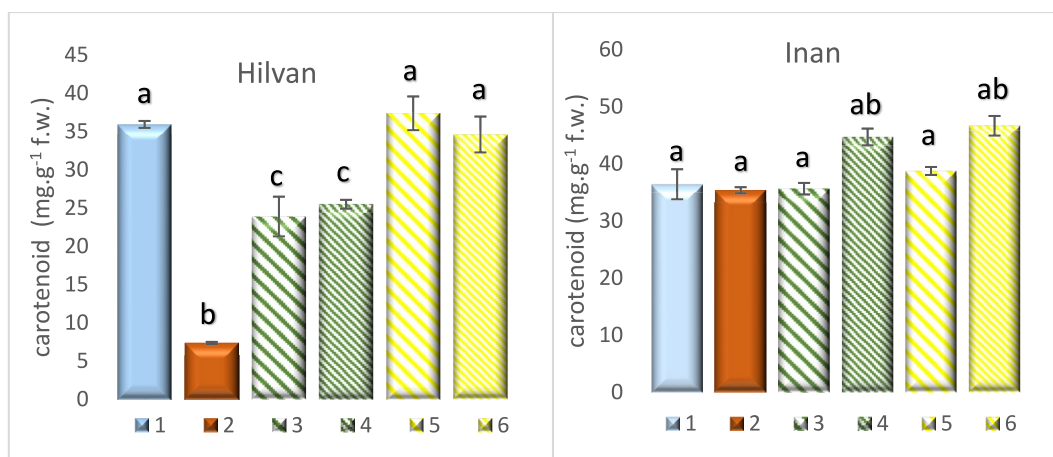


Fig. 6 Effects of PEG and calcium treatments on carotenoid content in Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG + 330 mg L⁻¹ CaCl₂, 6:

PEG + 660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

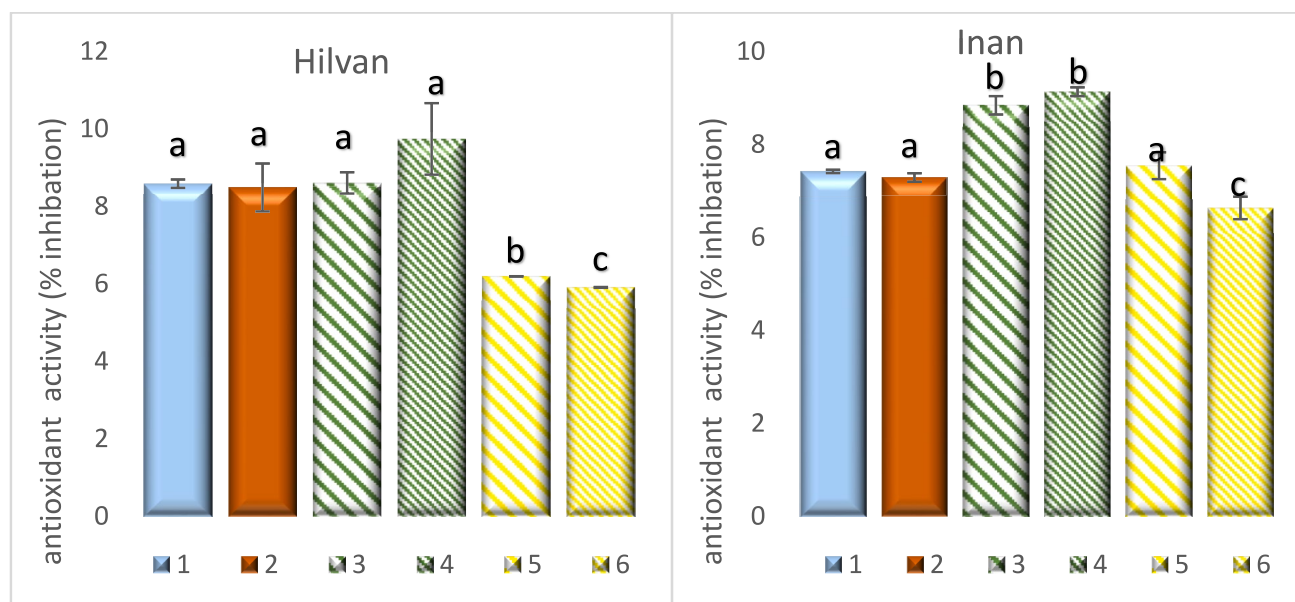


Fig. 7 Effects of PEG and calcium treatments on DPPH radical scavenging activity Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG + 330 mg L⁻¹

CaCl₂, 6: PEG + 660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

detected in other treatment groups compared to the control. The highest increase was in the Hilvan variety (13.14%) with low concentration of calcium, and the Inan variety (19.34%) with high concentration of calcium, and was found significant ($p < 0.05$) (Fig. 9).

Total Phenolic Substance Content

Total phenolic substance content increased in both pepper varieties in all treatment groups compared to the control. All increases were at the level of significance in the Hilvan variety. The highest increase was in Hilvan with 42.15% and

PEG + 330 mg L⁻¹ CaCl₂, and in Inan with 18.75% and 660 mg L⁻¹ CaCl₂ treatments (Fig. 10) ($p < 0.05$).

Proline Content

In the Hilvan variety, the proline content increased in PEG and 660 mg L⁻¹ CaCl₂ treatments, and in the Inan variety, only in PEG treatment, while it decreased in all other treatments compared to the control. While the highest increases were in PEG treatment in both varieties, the highest decreases were in PEG + 660 mg L⁻¹ CaCl₂ treatment ($p < 0.05$) (Fig. 11).

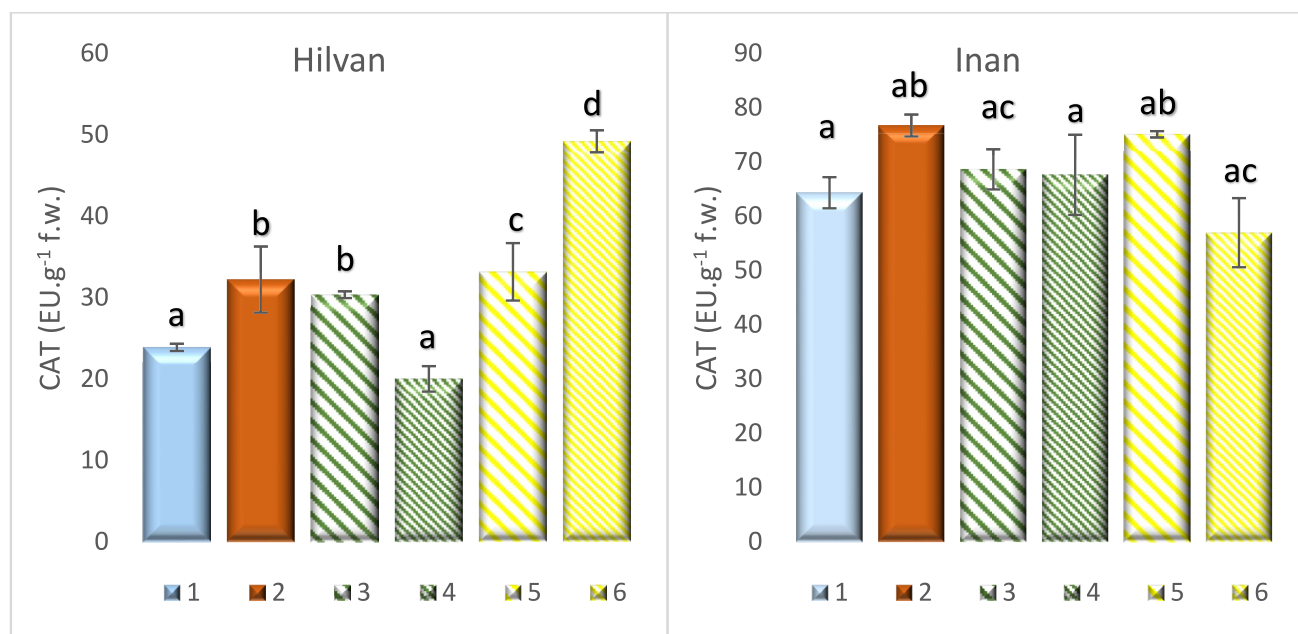


Fig. 8 Effects of PEG and calcium treatments on CAT enzyme activity of Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG+330 mg L⁻¹ CaCl₂, 6:

PEG+660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

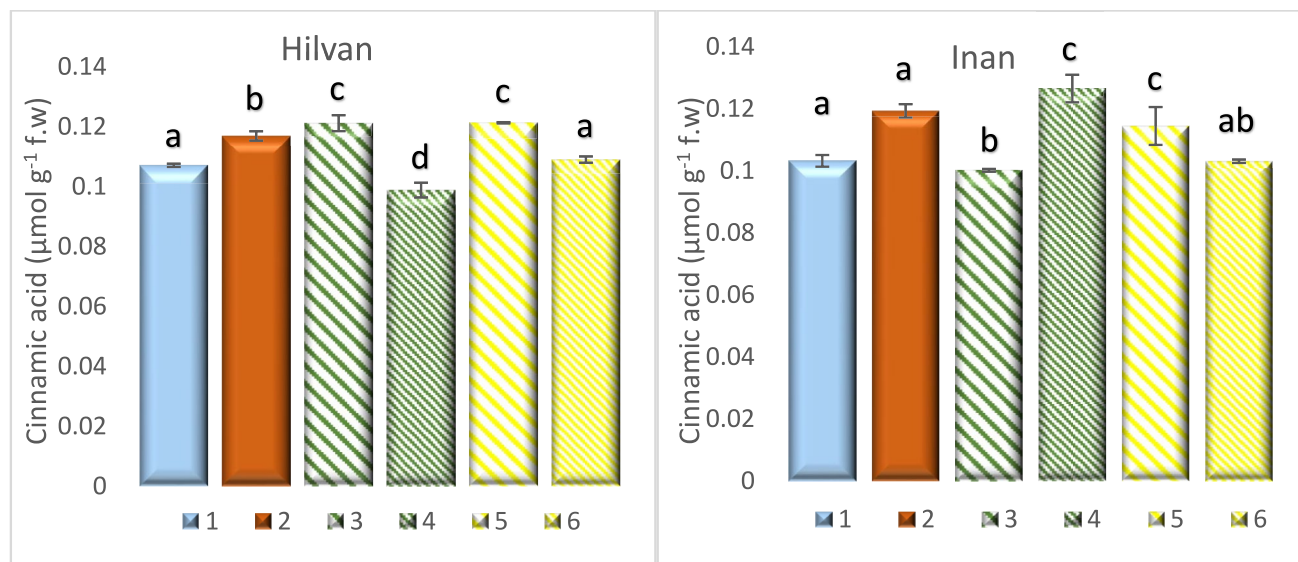


Fig. 9 Effects of PEG and calcium treatments on PAL enzyme activity of Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG+330 mg L⁻¹ CaCl₂, 6:

PEG+660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

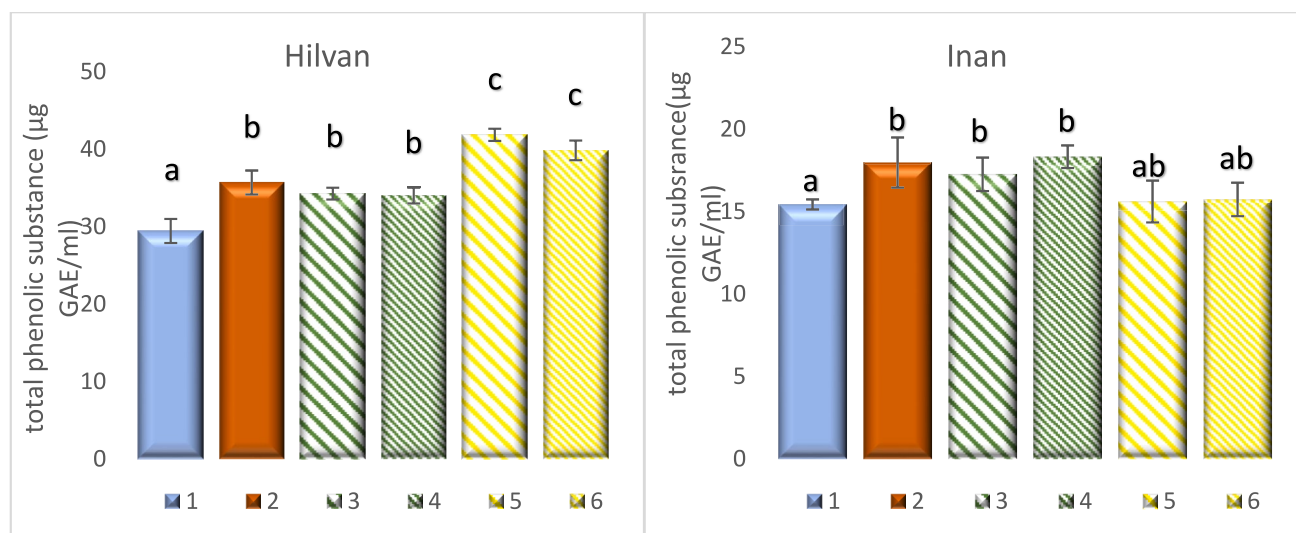


Fig. 10 Effects of PEG and calcium treatments on total phenolic substance content of Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG + 330 mg L⁻¹ CaCl₂, 6: PEG + 660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

CaCl₂, 6: PEG + 660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

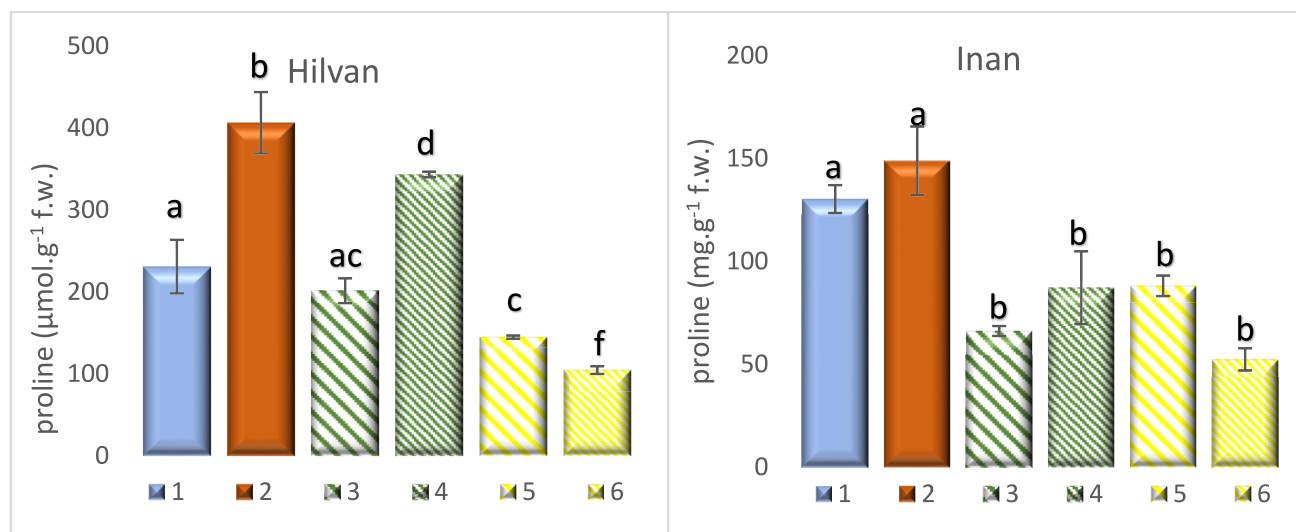


Fig. 11 Effects of PEG and calcium treatments on total proline content of Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG + 330 mg L⁻¹ CaCl₂, 6: PEG + 660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

PEG + 660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

Malondialdehyde (MDA) Level

While the amount of MDA increased with drought treatment in the Hilvan pepper variety, all other treatments caused a decrease. In the Inan variety, increases were detected in the groups where only PEG and PEG and calcium were treated together, and decreases were detected in the others. The amount of MDA in the group where only PEG was treated increased significantly in Hilvan (45.53%) and Inan (82.65%) varieties ($p < 0.05$) (Fig. 12).

Discussion

Drought stress is a multifaceted abiotic stress factor that damages plant physiological mechanisms at different stages, from seed germination to plant growth and development. Plants can respond to drought stress and adapt by modifying various morphological, physiological, biochemical, and antioxidant defense mechanisms. Morphological measurements were made, and photosynthetic pigment content, MDA content, total protein content, proline content and antioxidant capacity (enzymatic and non-enzymatic) were determined on the plants grown in in vitro in the study.

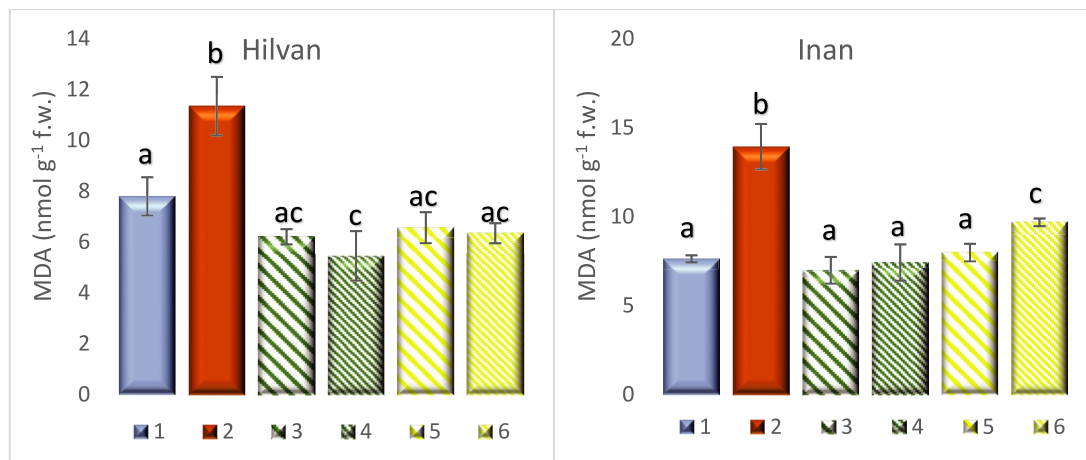


Fig. 12 Effects of PEG and calcium treatments on Malondialdehyde level of Hilvan and Inan pepper variety (n:3) (1: Control, 2: PEG, 3: 330 mg L⁻¹ CaCl₂, 4: 660 mg L⁻¹ CaCl₂, 5: PEG+330 mg L⁻¹ CaCl₂,

6: PEG+660 mg L⁻¹ CaCl₂) (Different letters indicate that the difference is statistically significant, $p < 0.05$)

The responses of two different pepper cultivars to drought stress and the effects of calcium on stress reduction were investigated.

A study conducted by Hosseini et al. (2019) on sugar beets showed that while drought stress alone caused only a limited decrease in shoot growth, Ca²⁺ application significantly increased shoot dry weight, plant height, and overall biomass under water-deficient conditions. Specifically, calcium-treated plants continued shoot development despite ongoing drought stress, indicating that Ca²⁺ supports the allocation of assimilates to above-ground organs while maintaining root functionality. Shoaib et al. (2022) reported that early and rapid elongation in roots is one of the basic characteristics of drought tolerance. The relationship between the decrease in growth rate and the osmotic stress and the tolerance level of the plant has been reported in many studies. (Aazami et al. 2010; Hamayun et al. 2010; Jahan et al. 2023; Manoj Kulkarni and Uday Deshpande 2007). PEG 6000 treatments to South African native grass species (*Antheophora pubescens* and *Heteropogon contortus*) were conducted. The results showed that at -0.3 MPa water potential, root length increased while shoot length decreased (Van den Berg and Zeng 2006). Toosi et al. (2014) stated that when they treated PEG at different concentrations on *Brassica juncea* (L.), hypocotyl length and weight decreased, and root length increased, especially in low-concentration treatments. These findings firmly align with the results of our study. It is known that calcium protects cells against drought by increasing cell wall cohesion and improving cell hydration levels, and positively affects growth (Abbas et al. 2021; El-Beltagi et al. 2025; Mohammed and Al-Maliki 2022; Mustafa et al. 2021). In addition, Akhtar et al. (2022) observed that calcium alleviated drought stress in their study examining the effects of exogenous

calcium, potassium, and boron treatments on the growth and yield characteristics of durum wheat under drought. The results of the conducted study on pepper varieties clearly affirm this understanding of plant cells. Overall, the morphological responses observed in our study follow the same general pattern reported in previous studies, where drought stress alters root–shoot allocation and calcium application contributes to the stabilization of growth responses under water deficit conditions. Our findings suggest that, under drought conditions, plants tend to allocate more resources toward root growth to improve water uptake, while shoot growth is restrained. Calcium appears to stabilize morphological responses under such stress by maintaining osmotic balance and enhancing cell wall integrity. Mechanistically, the root–shoot adjustments under PEG and CaCl₂ treatments likely involve osmotic signaling and Ca²⁺-mediated regulation of cell expansion. Calcium may influence ion transport, turgor maintenance, and cell wall rigidity, enabling coordinated growth under water deficit while preventing excessive root elongation at high doses. These observations suggest that Ca²⁺ acts as both a structural stabilizer and a signalling molecule, modulating osmotic balance, cell wall cohesion, and root–shoot resource allocation under drought, which aligns with the reported drought-tolerance mechanisms in other species.

In recent studies, it has been shown that PEG-induced drought stress causes a pronounced decline in chlorophyll a, chlorophyll b, and total chlorophyll contents, whereas calcium-containing treatments significantly restore pigment levels compared to drought alone (Begum et al. 2024; Zhao et al. 2024; Ntanasi et al. 2025; Dewi et al. 2025). In particular, treatments lacking calcium under PEG stress exhibited the lowest chlorophyll contents, while the presence of calcium remarkably alleviated pigment degradation (Begum et

al. 2024; Zhao et al. 2024). Numerous studies have examined another stress parameter of drought on plants, specifically changes in chlorophyll content (Ntanasi et al. 2025; Dewi et al. 2025). This decrease in chlorophyll content under drought is due to the destruction of pigments and the instability of the pigment-protein complex (Xu et al. 2013). In a study, it was stated that drought stress has a negative effect on the leaf pigment content of plants, but calcium treatment eliminated this negative effect (Naeem et al. 2018). In the same study, it was found that calcium treatment caused a 13% increase in the amount of chlorophyll a, 20% in the amount of chlorophyll b, and 31% in the amount of total carotenoids in plants under drought conditions compared to the control group (Naeem et al. 2018). In another study, the effects of drought stress on the yield and economic value of *Rosa* spp. were investigated, along with how exogenous calcium (Ca^{2+}) application alleviates these adverse impacts. Specifically, this study examined the influence of exogenous Ca^{2+} on the antioxidant system and photosynthetic properties of rose plants subjected to drought stress induced by polyethylene glycol 6000 (PEG6000). Drought stress was found to significantly reduce parameters, such as chlorophyll content, leaf and root biomass, while increasing the accumulation of reactive oxygen species. Exogenous Ca^{2+} application enhanced the net photosynthetic rate and stomatal conductance of leaves, and improved water use efficiency. Moreover, organ-specific physiological responses were observed depending on Ca^{2+} concentration: 5 mmol L^{-1} Ca^{2+} promoted photosynthesis and antioxidant activity in leaves, whereas 10 mmol L^{-1} Ca^{2+} enhanced antioxidant activity in roots. Overall, exogenous Ca^{2+} application contributed to increased drought resistance in rose plants (Zhao et al. 2024). Similar results were observed in the study conducted by Xu et al. (2013). The study showed that CaCl_2 (5 and 10 mM) treatments applied to plants under drought conditions resulted in a significant increase in chlorophyll content compared to other groups (Xu et al. 2013). Ben Amor et al. (2010) stated that calcium treatment was quite effective in increasing the amount of chlorophyll in abiotic stress experiments conducted for European searocket. Our study produced results that are consistent with the findings presented in the existing literature, thereby enhancing the credibility of our research outcomes. The mitigation of chlorophyll degradation by calcium may result from Ca^{2+} -mediated stabilization of the pigment-protein complex and thylakoid membrane structure, thereby protecting the photosynthetic apparatus from osmotic and oxidative damage induced by drought stress. Calcium may contribute to the preservation of protein content under drought stress by reducing oxidative damage to cellular proteins and supporting the synthesis or stabilization of stress-related proteins, thereby maintaining metabolic activity under adverse

conditions. This protective effect is likely associated with Ca^{2+} -dependent stabilization of thylakoid membranes and pigment-protein complexes, which limits drought-induced pigment degradation. Therefore, calcium-mediated preservation of photosynthetic structures represents a central mechanism underlying drought stress alleviation. In conclusion, Ca^{2+} reduces water loss in plants, enhances chlorophyll content in leaves, restores the activity of the PSII reaction center, ensures efficient photosynthetic electron transport, maintains the stability of the thylakoid membrane, increases cyclic electron flow, and minimizes damage to the photosynthetic system (Wei et al. 2025). Our findings are in agreement with previous reports demonstrating that calcium application mitigates drought-induced chlorophyll degradation and supports photosynthetic stability under PEG-induced osmotic stress. The observed responses reinforce the role of calcium as a key regulator of photosynthetic protection mechanisms during drought conditions. The improvement in chlorophyll content under calcium supplementation suggest that CaCl_2 acts as a physiological modulator, stabilizes the pigment-protein complex, and enhances the efficiency of the photosynthetic mechanism under osmotic stress.

Total protein content is widely used as a physiological indicator of plant responses to drought stress; however, its behavior varies depending on plant species, genotype, and stress intensity. For example, some authors reported that protein levels of plants decrease in drought (Mahmood et al. 2021; Masoumi et al. 2021), while others reported that drought does not have a negative effect on total protein amount or even causes increases (Trejo-Paniagua et al. 2024; Mohammed and Hussen 2023). This contradiction may be due to the expression of specific proteins (HSP, etc.) synthesized intensively during stress conditions being higher than the total protein content of plants under normal conditions (Akhzari and Pessarakli 2016; Naikwade 2023; Sharma and Dubey 2019). For example, in a study conducted on corn plants exposed to drought stress and not exposed to it, it was observed that there were differences in protein contents between the groups with and without calcium treatment. In the study, it was determined that calcium treatment to plants grown under normal and drought conditions significantly increased the protein content in both conditions (Abbas et al. 2021). The patterns observed in our study are consistent with previous reports indicating that drought stress can disrupt protein homeostasis, while calcium application contributes to the stabilization of protein metabolism through Ca^{2+} -dependent signaling and stress-responsive protective mechanisms. Previous studies have indicated that when plants are exposed to the correct amount of Ca or its derivatives, their total protein content increases significantly (Feng et al. 2023; Guo et al. 2021). Since Ca ions also play an important role in the activity of signaling molecules

that can stimulate cellular pathways associated with protein synthesis, exogenous CaCl_2 applications may be the reason for higher protein content. The observed reduction in total protein under PEG-induced drought stress in Hilvan may be attributed to inhibited protein synthesis due to osmotic stress and increased proteolytic activity, as well as oxidative damage to cellular proteins. Calcium supplementation likely stabilizes cellular homeostasis by modulating Ca^{2+} -dependent signaling pathways that activate stress-responsive proteins, including chaperones and enzymes involved in antioxidant defense. This regulation can protect proteins from denaturation and degradation, thereby maintaining metabolic activity under stress. In Inan, the relative stability of total protein suggests inherently more efficient stress signaling and protective mechanisms, which may include enhanced expression of heat-shock proteins (HSPs) and other stress-related proteins that are less dependent on exogenous calcium for their induction. Taken together, the results suggest that the Hilvan pepper variety is quite sensitive to drought and that Ca applications mitigate the harmful effects of drought stress. Considering that drought stress has recently significantly disrupted agricultural production, it is thought that biotechnological and more affordable applications, as in our study, may offer alternatives for tackling this major problem.

Recent studies further support the role of calcium in preserving carotenoid content under drought stress (Ayyaz et al. 2024; Naeem et al. 2018). It has been demonstrated that drought markedly reduces carotenoid and xanthophyll levels, whereas exogenous calcium application significantly enhances the biosynthesis and maintenance of these pigments under water deficit conditions. In particular, calcium supplementation has been shown to improve relative water content (RWC), stabilize photosynthetic efficiency, and positively correlate carotenoid accumulation with biomass production and gas exchange parameters under drought stress. Calcium treatments mitigated drought-induced carotenoid losses (Ayyaz et al. 2024). Our results indicate that PEG-induced drought stress negatively affects carotenoid accumulation in Hilvan peppers, but calcium application, particularly at low and moderate doses, partially or fully mitigates this effect. The combination of PEG with calcium-maintained carotenoid levels close to the control, reducing the adverse effects of drought. In Inan peppers, PEG alone did not significantly reduce carotenoid content, and calcium treatments, especially at higher doses, enhanced carotenoid accumulation. This suggests that calcium plays a protective role for the photosynthetic apparatus under water deficit conditions in both cultivars. Consistent with our findings, Naeem et al. (2018) found that drought stress decreased the amount of carotenoids in maize compared to control, while foliar calcium treatments increased the amount of

carotenoids. In another study, the physiological effects of exogenous CaCl_2 treatment on the *Dunaliella parva* plant were investigated. The study reported increases in carotenoid levels with exogenous CaCl_2 application (80/120 ppm) (Zou et al. 2023). The increase in the content of carotenoids shows that calcium protects the photosynthetic apparatus and alleviates the effects of drought. Our results are consistent with the literature findings. Drought-induced reductions in carotenoid levels in the Hilvan cultivar may result from oxidative degradation of pigments and inhibited biosynthesis due to osmotic stress and ROS accumulation. Calcium supplementation likely mitigates these effects by stabilizing chloroplast membranes and the pigment–protein complexes, reducing ROS-mediated carotenoid oxidation. Additionally, Ca^{2+} -dependent signaling may upregulate enzymes involved in carotenoid biosynthesis, supporting pigment maintenance under stress. In Inan, the relatively stable carotenoid content suggests more robust intrinsic protective mechanisms, possibly including efficient antioxidant enzyme activity and tighter regulation of pigment biosynthetic pathways, which are further enhanced by exogenous calcium applications. The patterns observed in our study are consistent with previous reports indicating that drought stress can disrupt protein homeostasis, while calcium treatments contribute to the stabilization of protein metabolism through Ca^{2+} -dependent signaling and stress-responsive protective mechanisms. The Hilvan pepper variety was found to be more sensitive to drought than the Inan pepper variety, and greater increases in carotenoid levels were observed with exogenous CaCl_2 treatment. It is considered that the CaCl_2 application is highly effective in mitigating the harmful effects of drought induced by PEG.

It is known that drought stress can induce significant changes in total antioxidant capacity, including DPPH radical scavenging activity, largely depending on genotype and stress severity. Rezayian et al. (2018) demonstrated that DPPH radical scavenging activity decreased in canola plants under drought conditions and that calcium and penconazole treatments increased radical scavenging activity. Calcium was found to be more effective in increasing DPPH radical scavenging activity. Similarly, it was determined that DPPH radical scavenging activities increased when calcium treatment was applied to tomato plants under drought stress (Patanè et al. 2018). In another study, calcium nitrate was applied to *C. annum* plants, and it was stated that antioxidant activity increased under salinity conditions with the application of calcium (Akladios and Mohamed 2018). In some plant systems, drought exposure leads to increased DPPH activity through the accumulation of phenolic and flavonoid compounds and enhanced PAL activity, whereas prolonged or severe stress may overwhelm the non-enzymatic antioxidant pool, resulting in reduced radical scavenging capacity

(Hossain et al. 2024; Gusain et al. 2025). The observed changes in DPPH radical scavenging activity under drought and calcium treatments likely reflect the balance between ROS generation and antioxidant defense capacity. In Hilvan, decreases in DPPH activity under PEG may result from excessive ROS accumulation overwhelming the non-enzymatic antioxidant pool, whereas calcium supplementation enhances radical scavenging by stabilizing cellular redox homeostasis and potentially upregulating phenolic and flavonoid biosynthesis. In Inan, relatively stable or increased DPPH activity suggests more effective intrinsic antioxidant defenses, which may further be supported by calcium through maintenance of phenolic compounds and modulation of ROS-scavenging pathways. These mechanisms collectively contribute to cultivar-dependent mitigation of oxidative stress under PEG-induced drought. When comparing the increases observed in the Hilvan and Inan varieties, the higher increase in the Hilvan variety suggests that it activates its antioxidant defense systems to a greater extent because it is more sensitive to drought stress. Overall, our results demonstrate that non-enzymatic antioxidant responses to drought are strongly genotype- and stress-intensity-dependent, as evidenced by the contrasting DPPH responses of the Hilvan and Inan cultivars. Calcium supplementation appears to play a central role in stabilizing this response by supporting redox homeostasis and phenolic metabolism, thereby enhancing drought tolerance. Importantly, these findings not only corroborate previous reports on genotype-dependent antioxidant regulation under osmotic stress, but also highlight calcium as a key modulator of non-enzymatic antioxidant capacity under drought conditions.

CAT is a key antioxidant enzyme present in all oxygen-utilizing tissues of living organisms and plays a key role in scavenging reactive oxygen species (ROS) (Ighodaro and Akinloye 2018). Recent studies have further highlighted the central role of CAT in calcium-mediated drought tolerance (Zhao et al. 2024; Mazhar et al. 2022). Multivariate and correlation analyses have shown that CAT activity is positively associated not only with other antioxidant enzymes such as SOD and POD, but also with photosynthetic performance and chlorophyll fluorescence parameters under drought stress. Calcium supplementation under water deficit conditions has been reported to markedly enhance CAT activity, accompanied by reduced MDA accumulation, indicating effective suppression of lipid peroxidation and membrane damage. These findings support the present results, where increased CAT activity under calcium treatments contributed to improved ROS homeostasis. The observation of dose-dependent CAT response suggests that calcium acts as a regulatory signal rather than a simple activator, with optimal concentrations maximizing antioxidant protection while preventing excessive enzyme activation that could disrupt

cellular metabolic balance (Zhao et al. 2024). Plants rely on antioxidant enzymes like CAT to mitigate oxidative stress induced by environmental challenges, such as drought. Studies conducted on corn (Issam et al. 2012; Wang et al. 2009) have determined that exogenous calcium application increases CAT activity. In a study by Xu et al. (2013), plants exposed to drought treated with exogenous calcium showed increased CAT activity (Xu et al. 2013). This increase in antioxidant activity can be said to protect against increased “oxidative” stress agents (ROS) under stress conditions. In a study conducted by Mazhar et al. (2022), calcium oxide-derived applications were made to canola plants exposed to drought stress, and it was reported that CAT activity increased with the relevant application. The responses observed in our study are consistent with previous reports indicating that calcium-mediated enhancement of CAT activity contributes to improved oxidative stress tolerance under drought conditions. The variation in CAT responsiveness further supports the view that antioxidant regulation under calcium supplementation is both dose- and genotype-dependent. Our findings indicate that calcium applications generally increase CAT activity and thus support the plants' defense against oxidative stress. The exceptions observed at high calcium doses suggest a potential threshold where CAT activity may not increase further or may be inhibited. It is thought that the Inan variety, which has a drought-tolerant structure, exhibits greater increases in CAT enzyme activities and has a higher threshold value in stress response formation. It is estimated that the Hilvan pepper variety has lower CAT enzyme activities because it is relatively more sensitive to drought stress compared to Inan. This suggests that drought stress has the potential to cause more damage in the Hilvan variety, and it is thought that Ca applications could be quite effective in mitigating these harmful effects and activating antioxidant defense systems. The concurrent enhancement of CAT activity and reduction of MDA accumulation highlight the role of Ca^{2+} in regulating ROS homeostasis and limiting membrane lipid peroxidation under drought stress. The observed CAT responses under PEG-induced drought and calcium treatments likely result from Ca^{2+} -mediated regulation of ROS signaling. Calcium supplementation may enhance CAT synthesis or activation through Ca^{2+} -dependent signaling pathways, thereby improving enzymatic ROS scavenging capacity. In the Hilvan cultivar, the high CAT activity under PEG and high dose of CaCl_2 suggests that calcium mitigates oxidative damage by directly enhancing ROS detoxification. In Inan, elevated CAT activity under moderate calcium doses reflects more efficient regulation of redox homeostasis, contributing to reduced lipid peroxidation and protection of cellular structures. The threshold effect observed at high calcium concentrations indicates a potential feedback regulation to prevent

overactivation of antioxidant enzymes, which could disrupt cellular metabolic balance.

Phenylalanine ammonia-lyase (PAL) is an important enzyme that catalyzes the first stable step in the phenylpropanoid pathway and leads to the production of various phenolic compounds that play a significant role in plant defense (Aluko et al. 2025; Yadav et al. 2020). PAL also interacts with phytohormones under stress conditions, including drought (Ibrahim 2014). The enzyme deaminates phenylalanine to trans-cinnamate, providing carbon flux from the shikimate pathway to secondary metabolites in higher plants (MacDonald and D'Cunha 2007). PAL activity generally increases under drought stress and with calcium treatment, but the magnitude of this increase can vary between plant species and treatment conditions (Ibrahim 2014; Ibrahim et al. 2016). The observed increases in PAL activity indicate that plants exhibit an adaptive response to drought and highlight the role of calcium in regulating secondary metabolism and defense mechanisms. A study suggest that the phenylpropanoid pathway plays a central role in plant adaptation to drought stress through the coordinated regulation of PAL and downstream flavonoid biosynthesis enzymes (Jan et al. 2024). Under water deficit conditions, activation of PAL is frequently associated with enhanced expression of key pathway enzymes such as CHS, CHI, F3H, and ANS, resulting in increased accumulation of flavonoids and anthocyanins with strong antioxidant capacity. These secondary metabolites function as efficient ROS scavengers and contribute to osmotic adjustment and cellular protection during drought stress (Jan et al. 2024). The calcium-induced modulation of PAL activity observed here is consistent with reports demonstrating that stress-responsive activation of the PAL pathway strengthens antioxidant defense systems under drought (Yar et al. 2025; Jan et al. 2024). Our study consistent with calcium-compatible *Trigonella foenum-graecum* (Yar et al. 2025) and *Avena sativa* (Bibi et al. 2022), suggesting that calcium supplementation may support the phenylpropanoid pathway and enhance plant resilience under water-limited conditions. Our study aligns with previous reports demonstrating that calcium-dependent regulation of PAL activity contributes to enhanced phenylpropanoid metabolism under drought stress, supporting the view that calcium-mediated modulation of secondary metabolism is a key component of plant adaptive responses to water deficit. In Hilvan, enhanced PAL activity under low-calcium treatments may stimulate phenolic biosynthesis as a compensatory response to drought-induced oxidative stress, whereas in the Inan cultivar, higher calcium concentrations more effectively trigger PAL activity, leading to increased phenolic accumulation and antioxidant protection. These cultivar-specific responses indicate that calcium functions not only as a structural stabilizer but also as a signaling molecule modulating secondary

metabolism, thereby reinforcing cellular defense mechanisms and maintaining redox balance under osmotic stress.

Total phenolic content (TPC) is an important secondary metabolite associated with plant defense mechanisms and antioxidant capacity. It is thought that increases in total phenolic content may positively affect the plant's secondary metabolism by modulating calcium, possibly through PAL activity and related pathways, thereby contributing to the reduction of the harmful effects of stress under drought conditions. In this regard, Oh et al. (2009) reported that PAL activity increases under drought stress and is associated with elevated phenolic compound levels. Similarly, Madany and Khalil (2017) found that sunflowers under drought stress exhibited higher phenolic content and that calcium-treated plants, contained higher phenolic compound levels compared to the control group. Studies have also been conducted on different plant species, such as *Solanum lycopersicum* (Birgin et al. 2021) and *Trigonella foenum-graecum* (Begum et al. 2024), and the results were found to be similar. Studies have demonstrated that calcium supplementation enhances phenolic accumulation under drought stress in a concentration-dependent manner (Birgin et al. 2021; Begum et al. 2024). In a CaCl_2 -based experiment, drought stress alone increased phenolic levels, while moderate calcium concentrations further promoted phenolic accumulation. This pattern suggests that optimal calcium levels are required to effectively activate phenolic biosynthesis pathways under stress conditions (Gusain et al. 2025). In our study results show that calcium treatments, either alone or in combination with PEG, increased phenolic accumulation in both varieties. Elevated total phenolic content in calcium-treated plants indicates that Ca^{2+} may activate the phenylpropanoid pathway, supporting the synthesis of antioxidant secondary metabolites that contribute to ROS detoxification and overall stress resilience. Calcium signaling may upregulate PAL and other key enzymes, facilitating the flow of carbon into phenolic biosynthesis. These results confirm that calcium plays an important role in increasing phenolic metabolism and antioxidant capacity in stressed plants. The responses observed in our study are consistent with previous reports indicating that calcium-mediated regulation of phenolic metabolism plays a central role in enhancing antioxidant capacity under drought stress. The alignment between our findings and the existing literature supports the involvement of Ca^{2+} -dependent activation of the phenylpropanoid pathway as a key mechanism contributing to drought tolerance. In Hilvan, moderate calcium doses effectively enhanced phenolic accumulation to buffer oxidative stress, whereas in Inan, higher calcium concentrations were more efficient in activating this pathway, reflecting cultivar-specific responsiveness. These results suggest that Ca^{2+} functions not only as a structural stabilizer but also

as a signaling molecule that coordinates secondary metabolism, reinforcing antioxidant defenses and promoting stress resilience under drought conditions.

Proline is an osmolyte that plays an important role in maintaining cellular osmotic balance under drought stress. Xu et al. (2013) reported that proline content increased in *Zoysia* under drought stress, but proline accumulation decreased significantly with exogenous CaCl_2 treatment (5–10 mM), especially at high concentrations. Similarly, Jaleel et al. (2007) indicated that CaCl_2 application under drought stress reduced proline levels by decreasing proline-synthesizing enzymes while increasing proline-degrading enzymes. Gusain et al. (2025) applied exogenous calcium to plants exposed to drought stress and observed increases and decreases in proline levels with this application. Consistent with previous reports, drought stress strongly induced proline accumulation as a classical osmotic adjustment response. However, calcium supplementation attenuated this response, suggesting that Ca^{2+} reduces stress severity perception and limits excessive osmolyte accumulation by stabilizing cellular redox and osmotic homeostasis. Such genotype-dependent modulation of proline metabolism indicates that calcium indicates drought responses rather than simply amplifying stress-induced proline synthesis (Licaj et al. 2023). These results indicate that plants accumulate proline to regulate their osmotic potential and protect the tertiary structures of proteins under drought conditions. Calcium is thought to modulate proline metabolism by potentially affecting the balance between proline synthesis and degradation, thereby stabilizing osmotic regulation during stress. The suppression of excessive proline accumulation in calcium-treated plants suggests a reduced perception of stress severity and a shift toward a more stabilized physiological state. Proline accumulation under drought appears to be tightly regulated by Ca^{2+} -dependent signaling pathways. Calcium may modulate the activity of key enzymes such as Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) for proline biosynthesis and proline dehydrogenase (ProDH) for degradation. These findings are consistent with our results, which concluded that high-calcium PEG applications reduced proline accumulation compared to PEG alone.

The patterns observed in our study are in strong agreement with previous reports indicating that calcium-mediated regulation of proline metabolism is a key component of drought stress adaptation. The consistency between our findings and the literature supports the view that Ca^{2+} alleviates osmotic adjustment by limiting excessive proline accumulation, thereby contributing to improved stress tolerance in a genotype-dependent manner. In Hilvan, PEG-induced drought strongly upregulated proline synthesis, whereas calcium supplementation, especially at higher doses, appears to activate proline degradation or suppress overproduction,

helping maintain osmotic homeostasis without over-accumulating osmolytes. In Inan, proline levels were less responsive to calcium, suggesting that this cultivar relies more on enzymatic antioxidants and phenolic pathways for stress mitigation. These findings indicate that calcium not only stabilizes cellular osmotic potential but also adjust osmolyte metabolism, promoting a balanced physiological state under drought stress. This in vitro study shows that the Inan variety is more resistant to drought stress, while the Hilvan variety is more sensitive to drought.

It has been reported that plants exposed to drought stress exhibit impairments in their ROS balance mechanisms and antioxidant defense systems (Cherono et al. 2021; Ju et al. 2018). Previous studies have shown that MDA content is positively correlated with the degree of damage observed in the plasma membrane. MDA is a widely used lipid peroxidation marker that reflects oxidative damage in plant cells under stress conditions (Kawade et al. 2025; Turker 2025). Huang et al. (2018) reported that MDA content significantly increased in *Arabidopsis thaliana* under drought stress. Similarly, Xu et al. (2013) observed that MDA levels increased under drought, but were lower in plants treated with CaCl_2 compared to plants exposed to drought, suggesting that calcium plays a protective role against oxidative damage. Issam et al. (2012) also confirmed that CaCl_2 applications reduced MDA accumulation, thereby alleviating and delaying lipid peroxidation caused by drought stress. Similar results were obtained in another study, where calcium application to *Rosa* spp plants subjected to drought stress resulted in reductions in lipid peroxidation (Zhao et al. 2024). Our study, MDA content increased under drought (PEG) treatment in the Hilvan variety, while it decreased in all other treatments. The observations of our study are in agreement with previous reports demonstrating that calcium-mediated regulation of lipid peroxidation is a key mechanism in drought stress tolerance. The consistency between our findings and the literature supports the role of Ca^{2+} in reducing oxidative membrane damage and maintaining cellular integrity under drought conditions, particularly through coordinated antioxidant and membrane-stabilizing processes. Especially Hilvan pepper variety showed a higher MDA content than the Inan variety, even in the control group, is thought to be due to its weaker or thinner membrane structure. The observed reductions in MDA levels under calcium treatment suggest that Ca^{2+} enhances membrane stability by mitigating ROS-induced lipid peroxidation, likely through both direct scavenging of free radicals and upregulation of antioxidant enzymes. The modulation of MDA levels by calcium under drought stress likely involves multiple interconnected pathways. Calcium stabilizes cellular membranes by binding to phospholipids and pectins, enhancing membrane rigidity and reducing susceptibility to reactive

oxygen species (ROS)-induced lipid peroxidation. Additionally, Ca^{2+} signaling can activate antioxidant enzymes such as catalase, superoxide dismutase, and peroxidases, which directly scavenge ROS before they attack membrane lipids. In Hilvan, the pronounced increase in MDA under PEG suggests a weaker endogenous antioxidant defense, whereas Inan exhibits more efficient ROS detoxification, consistent with higher CAT and PAL activities observed in this cultivar. Calcium supplementation appears to adjust these defense mechanisms, lowering oxidative damage and preserving membrane integrity. These observations indicate that Ca^{2+} not only acts as a structural stabilizer but also as a signaling mediator, coordinating enzymatic and non-enzymatic antioxidant responses to mitigate drought-induced lipid peroxidation.

Phenotypic responses to drought stress and calcium supplementation have been widely used as integrative indicators of plant stress tolerance. Visual traits such as shoot vigor, leaf turgor, and chlorosis provide rapid insight into the extent of osmotic and oxidative stress experienced by plants under water deficit conditions. Previous studies have demonstrated that PEG-induced drought stress commonly results in reduced shoot growth, loss of turgor, and chlorophyll degradation, whereas calcium supplementation helps preserve shoot architecture and leaf integrity by stabilizing cellular membranes and maintaining water relations. Calcium has been shown to support phenotypic stability under drought by enhancing cell wall cohesion, regulating stomatal function, and improving osmotic adjustment. Through these mechanisms, Ca^{2+} contributes to the maintenance of shoot structure and leaf density, even under prolonged osmotic stress. Genotypic differences in phenotypic resilience under drought conditions are also well documented, with drought-tolerant cultivars typically exhibiting milder visual symptoms and better preservation of shoot integrity than sensitive genotypes. Overall, phenotypic evaluations provide strong qualitative evidence that calcium supplementation can buffer drought-induced morphological stress, while inherent genetic tolerance plays a decisive role in determining the extent of visible damage under osmotic stress conditions. Distinct phenotypic responses were observed in the Hilvan and Inan cultivars following drought and calcium treatments. Calcium applications alone supported a more compact shoot architecture and overall improved visual plant integrity relative to PEG-treated plants. High-dose calcium did not exhibit a visually suppressive effect on shoot morphology. Under PEG + calcium treatments, the shoot system remained intact, turgor and leaf density were maintained, and calcium appeared to effectively buffer PEG-induced phenotypic stress. The Inan cultivar exhibited consistently greater vigor under all treatments when compared to Hilvan. Control plants presented strong turgor and well-developed

leaf tissue. PEG application led to mild chlorosis restricted primarily to older leaves, with overall shoot structure largely maintained. Calcium-treated Inan plants showed enhanced leaf integrity and compact shoot morphology. Under PEG + calcium treatments, the shoot system remained intact, turgor and leaf density were maintained, and calcium appeared to effectively buffer PEG-induced phenotypic stress. Collectively, the phenotypic evaluations indicate that the Inan cultivar displays superior morphological resilience and greater visual tolerance to osmotic stress compared to the Hilvan cultivar, while exogenous calcium applications contribute to the maintenance of shoot integrity in both cultivars under PEG-induced drought conditions. The phenotypic trends observed in the present study are consistent with previous reports indicating that calcium application alleviates drought-induced morphological damage and supports shoot integrity under osmotic stress. The agreement between our visual assessments and the existing literature reinforces the role of Ca^{2+} in maintaining plant structural stability, while also highlighting the contribution of cultivar-dependent drought tolerance to phenotypic resilience.

When all measured morphological, physiological, biochemical, and phenotypic parameters were evaluated collectively, a clearly variety-dependent response to PEG-induced drought stress was observed. Across growth traits, photosynthetic pigment stability, oxidative damage indicators (MDA), osmolyte accumulation (proline), protein content, antioxidant enzyme activities (CAT, PAL), non-enzymatic antioxidant capacity (DPPH), total phenolic content, and visible plant integrity, the Inan cultivar consistently exhibited a more stable and coordinated stress response compared to the Hilvan cultivar. Hilvan plants showed more pronounced changes in stress-induced fluctuations, particularly pigment degradation, lipid peroxidation, and growth inhibition, while Inan plants maintained metabolic balance under drought conditions, suffered less cellular damage, and provided more effective antioxidant regulation. Exogenous calcium application alleviated stress responses in both cultivars; however, its protective effects were more pronounced and sustained in the Hilvan cultivar across multiple parameters. These integrated responses indicate that the Inan cultivar possesses a higher overall tolerance to *in vitro* PEG-induced drought stress than the Hilvan cultivar under the experimental conditions of this study. The differential responses of the Hilvan and Inan pepper cultivars to PEG-induced drought stress indicate distinct levels of inherent drought tolerance and calcium responsiveness. PEG treatment triggered pronounced oxidative and physiological disturbances in Hilvan, as evidenced by severe reductions in shoot growth, chlorophyll content, protein levels, and increased MDA accumulation, reflecting enhanced membrane lipid peroxidation. In contrast, Inan

maintained relatively stable growth and metabolic activity under drought stress, suggesting a more efficient intrinsic stress-adaptation capacity. Calcium application notably modulated these responses by activating calcium-dependent signaling pathways, which enhanced antioxidant defense mechanisms, including CAT and PAL activities, and promoted phenolic compound accumulation. These responses collectively contributed to improved redox homeostasis and reduced cellular damage. In the drought-sensitive Hilvan cultivar, calcium application played a compensatory role by partially restoring physiological balance and limiting oxidative injury. Conversely, in the more drought-tolerant Inan cultivar, calcium acted primarily as a reinforcing factor, further strengthening existing defense mechanisms rather than reversing severe damage. This cultivar-dependent variation demonstrates that calcium application is particularly effective in mitigating stress-induced dysfunctions in sensitive genotypes, while also enhancing stress resilience in tolerant cultivars through the regulation of antioxidant metabolism and membrane stability.

All these findings indicate that calcium applications play a crucial role in mitigating the negative effects of drought stress in both the Hilvan and Inan cultivars. Calcium enhanced the metabolic balance and adaptive responses of plants by stabilizing root and shoot growth, protecting photosynthetic pigments, supporting protein and proline levels, and increasing the activities of the antioxidant system. These results indicate that calcium plays a role in promoting plant defense mechanisms against drought stress (in the initiation of response signals) and show that optimal dosages are critically important.

Conclusions

Drought stress is a complex type of stress that seriously affects plant growth and yield. Responses to drought stress vary depending on the plant species, variety, age, duration, and severity of the stress. This study determined the different morphological and biochemical responses of Hilvan and Inan pepper varieties grown under *in vitro* conditions to PEG-induced drought stress and exogenous calcium application. The results of the study indicate that the Hilvan and Inan pepper varieties were negatively affected by drought stress and that exogenous calcium applications were effective in increasing drought tolerance in both pepper varieties, positively affecting growth while reducing oxidative damage. Our results show that Hilvan is more sensitive to PEG stress and, consequently, responds at the morphological, biochemical, and physiological levels through modulation by calcium applications. In contrast, the Inan pepper variety was found to be more tolerant than Hilvan and exhibited

a balanced adaptation by preserving shoot structure and increasing root biomass through calcium applications, the effect of the relevant stress factor was reduced. Biochemically, calcium generally increased total phenolics, PAL, and CAT activities while reducing MDA and proline accumulation in both varieties. However, it is thought that the regulation of antioxidant and osmoprotective pathways, depending on the variety, also plays a role in these responses. Exogenous calcium treatments can be used to support plant growth under water-deficient conditions and in agricultural production. This study also suggests that exogenous calcium treatments can be used as a lower-cost and easier-to-apply alternative method compared to genetic modifications and breeding efforts. Furthermore, the findings obtained from this study will shed light on more comprehensive studies to be conducted in the future.

Acknowledgements This study was supported by Niğde Ömer Halisdemir University Scientific Research Projects Coordination Unit (BAP) under project number FMT 2022/5-LÜTEP, and we would like to express our gratitude for their support.

Author Contributions Nurevsan Gundogdu and Huseyin Turker contributed to the study design, literature review, writing of the article, and laboratory work. Bengu Turkyilmaz Unal contributed to the planning of the study, laboratory work, and statistical analysis. All authors collaborated on the final version of the article.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK). Open access funding is provided by Springer Nature Open Access Agreement for Türkiye (TÜBİTAK). Niğde Ömer Halisdemir University, Scientific Research Projects Coordination Unit, Project No. FMT 2022/5-LÜTEP has provided support for this project.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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