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RESEARCH ARTICLE

ANTIOXIDANT RESPONSES TO DROUGHT STRESS IN PENNYROYAL (*Mentha pulegium* L.)

Funda ULUSU[1*], Kader TÜMER[2], Yakup ULUSU[3]

[1*]Karamanoglu Mehmetbey University, Vocational School of Technical Sciences, Department of Crop and Animal Production, Karaman, fulusu@kmu.edu.tr, ORCID: 0000-0002-0321-2602
[2]Karamanoglu Mehmetbey University, Faculty of Engineering, Department of Bioengineering, Karaman, kadertumer96@gmail.com, ORCID: 0000-0001-6392-0785
[3]Karamanoglu Mehmetbey University, Faculty of Engineering, Department of Bioengineering, Karaman, yakupulusu@kmu.edu.tr, ORCID: 0000-0002-8755-2822

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ABSTRACT

Mentha pulegium L. (Lamiacea) is a valuable medicinal and aromatic plant found in humid and arid bioclimatic regions of Turkey. Drought stress is a growing concern for the future of agriculture, as well as the most common abiotic stress factor affecting the biochemical processes of plants and seriously damaging crop productivity. The aim of the study was to evaluate the effects of drought stress on the activity of enzymatic antioxidants (polyphenol oxidase - PPO, peroxidase - POD, ascorbate peroxidase - ASPX, catalase - CAT) and some ecophysiological (total chlorophyll content, chlorophyll a and b, carotenoid) responses in *M. pulegium* grown in pots under greenhouse conditions. In addition, oxidative stress markers were analysed to determine whether drought stress causes oxidative damage in pennyroyal. The plants were exposed to water stress during the 3rd, 6th and 10th days. All enzymatic antioxidant activities of plants under stress were increased compared to control plants. While there was a significant increase in PPO and POD activities in the first days of drought treatment, the prolongation of the treatment period resulted in a significant decrease in these activities. In addition, drought significantly increased lipid peroxidation (294%), hydrogen peroxide (158%) and proline (3172%) content compared to controls. These results show that drought treatment and duration significantly affect antioxidant enzyme activities, lipid peroxidation, hydrogen peroxide and proline content. DPPH (2-diphenyl-1-picrylhydrazyl), ABTS (2,20'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity, Fe^{+2} (FRAP) reducing activity and total phenolic content analysis were performed to analyse the effects of drought stress on antioxidant properties of pennyroyal plant. In addition, the decrease in photosynthetic pigment content in parallel with the prolongation of the drought period due to oxidative damage shows that this valuable medicinal and aromatic plant has low tolerance to drought.

Keywords; *Mentha pulegium, Drought stress, Enzymatic antioxidants, Proline, MDA*

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1. INTRODUCTION

In nature, plants are frequently exposed to biotic and abiotic stress conditions that cause negative effects in terms of growth, development and productivity. Environmental stressors have a limiting effect on plant growth and food production in many regions. Drought, which is accompanied by changing climatic conditions, is one of the most important abiotic factors limiting plant growth and production [1]. Drought is increasing rapidly on a global scale and affects approximately 55% (~7.8 million km^2) of the total arable land, with the Asian continent having the highest level with 39% (3.03 million km^2) [2]. In this respect, determining the drought stress tolerance mechanisms of plants is a very important ecological goal. In plants exposed to drought stress, many vital biochemical reactions such as stomatal closure at the molecular level, decrease in photosynthesis rate, increase in reactive oxygen species (ROS) are adversely affected, and this generally results in a decrease in crop productivity [3]. These negative effects of drought vary depending on the length, degree, frequency, plant species and growth period (vegetative- generative) of the plant [4].

Plants have developed a complex array of mechanisms to deal with biotic and abiotic stresses. One of the stress defense mechanisms in plants is the defense system, which includes enzymatic antioxidants [5]. It is known as a toxic problem for plants that hydrogen peroxide (H_2O_2), superoxide radicals and hydroxide ions (OH^-) that occur in plants under environmental stress cause ROS accumulation [6]. ROS can cause protein degradation, lipid peroxidation, DNA and cell damage and ultimately cell death [7,8]. In order to improve plant tolerance to environmental stresses, protect homeostasis and reduce the harmful effects of oxidative stress, these ROS are cleaned by some enzymatic such as PPO (polyphenol oxidase), SOD (superoxide dismutase), ASPX (ascorbate peroxidase), POD (peroxidase), CAT (catalase) and non-enzymatic (phenolic compounds, proline and ascorbic acid) antioxidants [9,10].

The degree of increase in antioxidant enzyme activities and amount under drought stress varies considerably drought severity, different plant species and even between two varieties of the same species [11]. In this respect, since the effects of drought stress on plants can vary significantly between species, investigating the genetic and physiological mechanisms that cause this stress difference is an important approach to increase the yield of plants with high medicinal and aromatic value [12,13]. To overcome drought stress, some studies are carried out to increase plant productivity by selecting plant species with high tolerance ability or applying biostimulators [14].

Mentha pulegium L. (Lamiaceae) is a perennial medicinal and aromatic plant with highly valuable essential oil (pulegone, piperitenon) [15] and phenolic compounds (apigenin, luteolin, kaempferol) [16]. In addition to the many pharmacological properties of this plant such as antiparasitic [17], antimicrobial [18], antioxidant [19], antimutagenic [20], it is also frequently used in agricultural food industries. It is known that many phytochemicals are synthesized that protect the plant against some negative environmental factors such as drought, ultraviolet, salt or pathogen experienced during the growth and production of this plant [21]. Therefore, it is important to investigate how this plant, which has an important potential for the pharmaceutical industry with its valuable secondary metabolites, can develop a defense mechanism against abiotic stress factors with enzymatic antioxidants. The effects of drought stress on growth and yields of pennyroyal plants have been reported in some studies [22,23].

However, there are few reports on how drought stress may affect parameters such as photosynthetic pigments, antioxidant defense system, protein content and lipid peroxidation.

In this study, we analyzed the effects of three different levels of drought stress (under controlled greenhouse conditions) on the activity of various antioxidant enzymes (PPO, POD, ASPX, CAT), total phenolic compounds (TPC), ABTS, DPPH, FRAP radical scavenging activity and some ecophysiological responses to understand the adaptation of pennyroyal to environmental stresses. The results obtained will guide future studies on the understanding of drought tolerance mechanisms in both *M. pulegium* and other medicinal and aromatic plant species.

2. MATERIAL and METHODS

2.1. Plant Material, Cultivation and Drought Treatments

M. pulegium seed samples were purchased from Zengarden (Turkey), an ecological certified and commercial seed company. Seeds were sterilized with 10% (v/v) sodium hypochlorite (20 min) and washed with distilled water, Then, seeds were sown in pots prepared with half peat-garden soil according to a completely random block design (with three replications). The seedlings that developed after germination were grown in the research greenhouse 20-25°C, 60-65% relative humidity and under natural light conditions. When the *M. pulegium* seedlings reached a sufficient size, the leaves were weighed and frozen in liquid nitrogen and stored at -80 °C until analysis.

2.2. Enzyme Activity Assays

For protein and antioxidant enzyme assays, control (well-watered) and drought-treated Pennyroyal Mint (*M. pulegium*) leaves (0.2 g) were pulverized with liquid nitrogen and extracted in 3 mL buffer containing 50 mM KH_2PO_4 (pH 7.0) buffer, 0.1 mM EDTA and 1% PVPP (w/v). The homogenate was filtered and then centrifuged at 4°C for 15 min at 15000 g. The supernatant was used for CAT, POD, ASPX and PPO activity assay [24].

Catalase (CAT, EC 1.11.1.6) activity was tested in a reaction mixture (final volume: 3 mL) containing 1450 µl of 50 mM KH_2PO_4 buffer (pH:7), 1500 µl of 30% H_2O_2 and 50 µl of plant extract. The disappearance of H_2O_2 was followed at 240 nm (3 min) (Schimadzu UV-1800, Japan) (H_2O_2: 0.036 $\mu mol^{-1}cm^{-1}$) [24].

Peroxidase (POD, EC 1.11.1.7) activity was measured according to the guaiacol method with minor modifications to the procedure of Sharma [25]. The reaction mixture in a total volume of 3 mL contained 970 µl of 50 mM KH_2PO_4 buffer (pH:6), 1000 µl of guaiacol (2-ethoxyphenol), 30% 1000 µl of H_2O_2 and 30 µl of plant extract. The increase in absorbance due to guaiacol oxidation was measured at 470 nm for 3 min.

Ascorbate peroxidase activity (ASPX, EC 1.11.1.11) was analyzed according to the method of Karabal [26] by monitoring a decrease in the absorbance at 290 nm for 3 min. The reaction mixture in a total volume of 3 mL consisted of 1450 µl of 50 mM phosphate buffer (pH: 7), 750 µl of ascorbic acid, 750 µl of 30% H_2O_2 and 50 µl plant extract. The enzyme activity was calculated using the extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1} at 290 nm).

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Polyphenol oxidase activity (PPO, EC 1.10.3.1) was assayed according to the method of Flurkey [27]. The 3 mL substrate mixture contained 50 µl of crude extract, 0.20 M sodium phosphate buffer (pH: 6.5), 25 mM catechol. The increase in absorbance was recorded at 420 nm at 30 °C. Enzyme activity was calculated from the slope of absorbance–time curve. The PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min under assay conditions. Enzyme activities were expressed as enzymatic unit g^{-1} fresh weight (EU g^{-1} FW).

2.3. Determination of Proline Content

The proline (Pro) content (in µg g^{-1} FW of leaf) was determined by the method of Öztürk and Demir [28]. The 0.4 g leaf sample was homogenized in 4% sulfosalicylic acid and the homogenate was filtered through Whatman filter paper. The filtrate (0.5 mL) was 10 times diluted with distilled water. Then, the reaction mixture consisted of 1 mL of diluted sample, 1 mL of 96% glacial acetic acid and 1 mL of acid ninhydrin, which was incubated at 100 °C in a water bath for 1 h. The reaction was stopped by keeping it in an ice bath for 10 min. and 2 mL toluene was added to the reaction mixture and vortexed. After 5 min. of incubation, the absorbance was read at 520 nm. Pro concentration was determined by comparison with a standard graph constructed using known amounts of Pro and expressed as µg Pro g^{-1} fresh weight.

2.4. Oxidative Stress Markers (MDA, H_2O_2)

Malondialdehyde (MDA) content was determined in terms of thiobarbituric acid reactive (TBARS) substances produced according to the Sreenivasulu [29] method. 0.5 g fresh weight (FW) was homogenized with 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10.000 × g for 20 min. and 0.5 mL of supernatant was mixed with 1 mL of 0.5% (w/v) TBA in 20% (w/v) TCA. The mixture was heated at 95°C for 30 min. and then quickly cooled in an ice bath. Then the mixture was centrifuged at 10.000 × g for 5 min., the supernatant measured at 532 nm and optical density (absorbance) was corrected by a nonspecific absorbance read at 600 nm. MDA concentration was calculated using molar extinction coefficient of MDA-TBA product (1.55×105 M^{-1} cm^{-1}) and the results were expressed as µmol MDA g^{-1} FW.

The content of H_2O_2 was measured according to the method of Velikova [30] as follows: 0.5 g leaf tissue was homogenized with 5 ml of 0.1% (w/v) TCA. The mixture was centrifuged at 12.000 × g for 15 min. 0.5 mL of the resulting supernatant was mixed with 0.5 mL of 50 mM potassium phosphate (pH: 7) buffer and 1 mL of 1 M KI. The absorbance of the reaction mixture was measured at 390 nm.

2.5. Measurement of Photosynthetic Pigment Contents

Chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll contents were calculated according to Arnon [31] (Eş. 1, 2, 3) total carotenoid content according to Witham [32] (Eş. 4) and reported as mg of each pigment per g leaf FW. Leaf tissue samples (4 g) were homogenized with 15 mL of 80% (v/v) of acetone and ground for 1h on an ice bath. Then, the samples were centrifuged at 3000 × g for 5 min at 4 °C. Chlorophyll in the supernatant was measured immediately at wavelengths of 450, 645 and 663 nm to avoid interference from pigment degradation. Photosynthetic pigment amounts were calculated according to the following equations.

$$\text{Chlorophyll a (mg } g^{-1} \text{ FW)} = 12.7\ A_{663} - 2.69\ A_{645} \quad (1)$$

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Chlorophyll b (mg g^{-1} FW) = 22.9 A_{645} – 4.68 A_{663} (2)

Total chlorophyll (mg g^{-1} FW) = 20.2 A_{645} + 8.02 A_{663} (3)

Total caroteonids (mg g^{-1} FW) = 4.07 A_{450} - (0.0435 Chl a amount + 0.3367 Chl b amount) (4)

2.6. Total Protein Determination

The analysis of total protein was based on the procedure of Bradford [33]. 0.25 g of leaf tissue was homogenized with 2.5 mL of 50 mM KH_2PO_4 (pH:7) and the homogenate was centrifuged at 15.000 g for 20 min. at +4 °C. 2.5 mL of Coomassie Brilliant Blue G-250 was added to 20 µl supernatant and vortexed. After incubation (10 min), samples were measured at 595 nm. Bovine serum albumin (BSA; Sigma A7906) was used as the standard protein for the total protein amount calculation in the leaves.

2.7. Analysis of Total Phenolic Compounds and Antioxidant Properties

DPPH (2-diphenyl-1-picrylhydrazyl), ABTS (2,20'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity, Fe^{+2} (FRAP) reducing activity and total phenolic content analysis were performed to analyze the effects of drought stress on antioxidant properties of pennyroyal plant. For the analysis, 0.3 g of plant leaf sample was ground in liquid nitrogen and suspended in 5 mL of 80% methanol. After this suspension was mixed gently on a magnetic stirrer for 24 hours, it was centrifuged at 3000 rpm for 10 min., filtered through cellulose, and the supernatant was used for analysis.

Total phenolic content was determined by comparison with the gallic acid standard. For analysis, 250 µL of 10% Folin-Ciocalteu reagent and 500 µL of 1M Na_2CO_3 were added to 250 µL of extract and the final volume was made up to 3 mL with distilled water. After the prepared reaction mixtures were incubated in the dark for 20 min., absorbances were recorded at 765 nm. The total amounts of phenolic substances in the samples were calculated using the standard graph prepared with gallic acid [24].

In order to determine the ABTS radical scavenging activity, firstly, the 7mM stock ABTS solution was incubated with 2.45 mM $K_2S_2O_8$ for 12 hours at room temperature in the dark to produce radicals. Then, 50 µL of sample was mixed with 2 mL of ABTS radical solution and incubated for about 10 min., and then its absorbance at 734 nm was recorded. ABTS removal activity was calculated using the difference between the initial absorbance and the absorbance at the end of the reaction. ABTS radical scavenging activity calculated by the graph prepared using Trolox in the range of 0.1 to 0.8 mM as a standard, was expressed in µmol g^{-1} FW [34].

In order to determine the DPPH radical scavenging activity, 127 µM DPPH was prepared in methanol. Then, the reaction mixture consisting of 100 µL of pennyroyal extract, 400 µL of methanol and 1 mL of DPPH solution was prepared. After incubation at room temperature for 15 min for the reaction to take place, absorbances were recorded at 515 nm. DPPH removal activity calculated using the calibration curve prepared with standard Trolox was expressed as µmol g^{-1} FW [35].

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The FRAP method is an antioxidant activity determination method based on the reduction of ferric ions to ferrous ions. To perform this analysis, a working solution containing 40 mM HCl in 300 mM pH 3.6 acetate buffer, and 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) prepared in 20 mM $FeCl_3$ x $6H_2O$ solution was prepared. 20 µL of sample extract was added to 3 mL of working solution and incubated in the dark for 30 min. The color change (ferrous tripyridyl-triazine complex) resulting from the reaction was recorded at 593 nm. FRAP radical scavenging activity calculated by the calibration graph obtained by using the $Fe_2(SO_4)_3$ solution prepared in the range of 0.005-0.5 mM as a standard was expressed as µmol g^{-1} FW [36].

2.7. Statistical Analysis

The results shown in the figures were evaluated as the mean of three replicates (n = 3) for each group. Data were analyzed and processed using SPSS Standard Version package program. The significance of differences between control and treatment groups was statistically evaluated by one-way ANOVA ($p<0.05$ as significant level) [37]. Figures were illustrated using GraphPad Prism 8.4.3.

3. RESULTS and DISCUSSION

3.1. Antioxidant Enzyme Activities

Enzyme (CAT, ASPX, POD, PPO) activities were measured in pennyroyal leaves under different drought stress and compared with the values of well-watered plants. The CAT activity of pennyroyal plants under drought stress increased by 122% at the end of the 3rd day compared to the control, and this increase was fourfold at the end of the 6th day. Although the CAT level increased in parallel with the continuation of drought stress, a rapid decrease occurred after 10 days due to oxidative damage. The increase in this enzyme activity during the early drought period can be evaluated as a physiological response to activating the antioxidant defense system and providing hemostasis in the pennyroyal plant. The absence of a statistically significant increase in H_2O_2 levels, which is the substrate of the catalase enzyme, in the 3rd and 6th day drought applications can be considered as a response of the plant to the increase in CAT activity (Figure 1). However, the decrease in CAT activity on the 10th day is an indication that the plants cannot tolerate the adverse effects of drought. In previous studies, it was determined that in moderate and severe drought conditions, drought stress significantly increased the CAT activities of *M. pulegium* compared to the control [38]. Similar results have been reported in *Cicer arietinum* L. [39,40], *Oryza sativa* L. [41], olive trees [42]. Again, like our study, Chakraborty and Pradhan [43] reported that CAT activity increased on the 3rd day of 5 different wheat cultivars, in which drought stress was applied for 3rd, 6th and 9th days. However, the continuation of drought stress in the following days, it was determined that the CAT activities in the same cultivars fell below the control values. This suggests that the applied drought stress is above the tolerance capacity of the cultivars in question.

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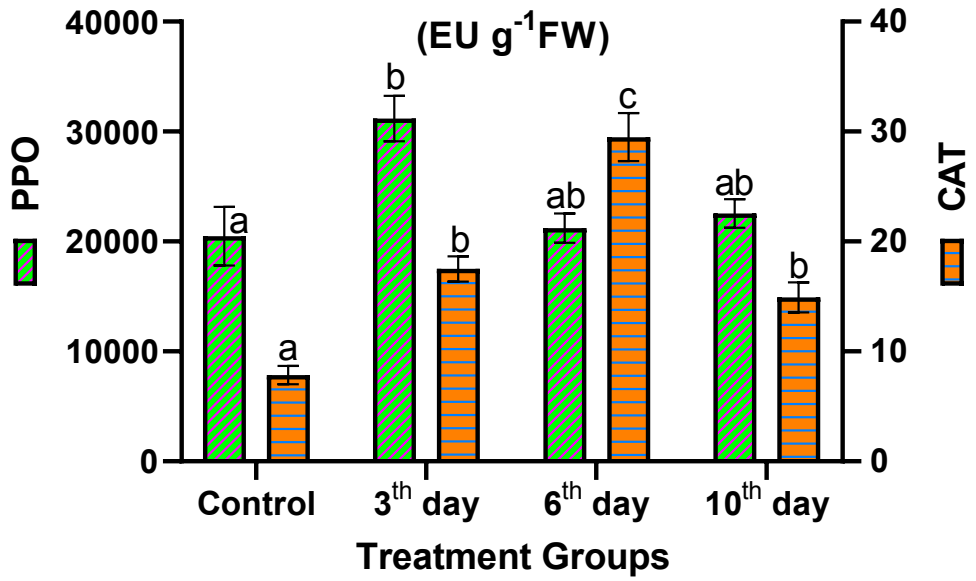

Figure 1. Effect of drought treatment on activity of PPO and CAT enzymes in leaves of *M. Pulegium.*

ASPX activity did not differ significantly between the first two drought treatments and control, but this activity was two times higher than control after ten days (Figure 2). In other words, while drought stress in the early stage did not affect ASPX activity in pennyroyal, the activity of this antioxidant enzyme increased significantly with the prolongation of stress. In previous studies, sweet pepper plants showed high enzyme activities against 30% and 60% drought stress, and ASPX activities increased 1.29 and 1.69-fold, respectively [44]. Again, Naderi [45] reported similar results in examining wheat genotypes under different drought stress conditions. ASPX are enzymes responsible for the H_20_2-dependent oxidation of ascorbate in plants in general [46]. Increases in CAT and ASPX activities because of drought treatments can be characterized as a defensive response related to excessive ROS production and damage, possibly resulting from drought stress.

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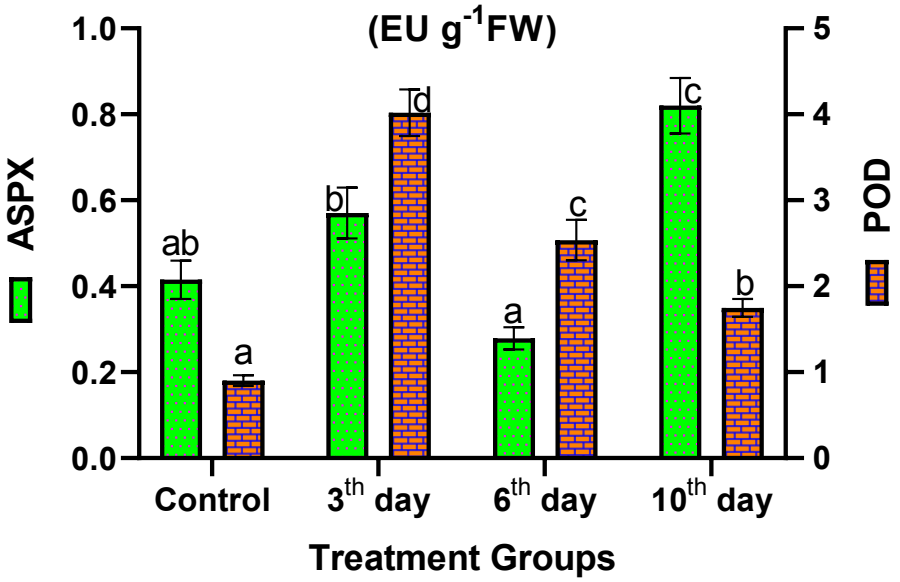

Figure 2. Effect of drought treatment on activity of ASPX and POD enzymes in leaves of *M. pulegium.*

Another antioxidant enzyme group that has a significant effect on preventing the harmful effects of ROS derivatives such as H_2O_2, which is formed as a result of plant metabolic reactions, is known as POD. POD activity in all drought-treated plants in the current study was higher than in control. In addition, POD activity reached its highest level in the early drought period (day 3) but started to decrease in the long term due to the negative effects of prolonged stress conditions on plant metabolism (such as H_2O_2 level, which can be kept under control thanks to increases in CAT activity). POD activity decreased ~40% on day 6 and ~60% on day 10 compared to day 3 but increased 2.7-fold and 1.8-fold compared to control, respectively (Figure 2). In physiological studies carried out by different research groups regarding POD activity, it was determined that this activity of *M. pulegium* exposed to drought stress decreased compared to the control [23], while exogenous application of penconazole to pennyroyal increased the same enzyme activity [47]. Again, *Brassica napus* L. showed low POD activity against drought stress [48], Chugh [49] reported an increase in POD activities of drought-resistant maize varieties under 72 h drought stress, while this enzyme activity decreased in drought-sensitive maize varieties. Similarly, it was determined that peroxidase activity in the leaves of drought-resistant and sensitive soybean varieties exposed to drought stress increased significantly in resistant varieties and decreased in susceptible varieties [50]. The susceptibility or tolerance ability of plants to drought stress is associated with enzymatic antioxidant responses synthesized in the biochemical process. As a result of the studies, drought-tolerant varieties try to minimize the damage that may be caused by oxidative stress (reducing H_2O_2 content, eliminating MDA, maintaining cell membrane integrity, etc.) because of physiological drought by increasing antioxidant enzyme activity

while under water stress [51]. Prolonged drought in plants can promote the accumulation of ROS, which causes cell damage or death and suppresses POD activity. The fact that peroxidases oxidize various substrates in the presence of H_2O_2 and take part in a hydroxylic cycle that can lead to the formation of ROS shows that these enzymes can work in two directions [52,53].

PPO activity in plants under drought stress, increased 1.5 times on day 3 compared to control, but at the end of day 6 and day 10, this rate decreased to 1.05 and 1.1 times, respectively (Figure 1). It has been reported that the drought stress applied by Khazaei and Estaji [44] to *Capsicum annuum* L. plant caused a significant increase in PPO activity compared to normal conditions, and the application of abscisic acid significantly increased the PPO activities in all drought levels. Again, drought stress applied in two drought tolerant (Bolani) and sensitive (Sistan) wheat varieties increased the PPO activity, and even severe drought stress caused this enzyme activity to reach the maximum level with 4.9 and 2.3 times compared to the control, respectively [45]. During stress, the increase in PPO activity, in addition to scavenging ROS and mitigating cell damage, is extremely important for plants' tolerance to oxidative stress [54,55]. In addition, thanks to the increase in PPO activity in plants exposed to stress, some harmful effects (reducing the amount of H_2O_2 and maintaining membrane integrity, etc.) that may occur can be minimized [44].

Plants with high antioxidant activities can be used as a source of natural medicine and therefore can be called medicinal plants [56]. These antioxidant properties in pennyroyal seedlings show that this plant has the potential for further use as a nutraceutical in the field of pharmacology.

3.2. Proline Content

The proline content in plants increased in parallel with the prolongation of the trial period in drought treatment ($p < 0.05$). On the 3^{rd}, 6^{th} and 10^{th} days of drought, the amount of proline in the plant increased by ~194%, 3172%, 479% compared to the control treatment, respectively (Figure 3). The pennyroyal plant was able to cope with drought stress at an early stage thanks to its antioxidant defense systems, but prolonged stress conditions caused almost 32 times more proline accumulation in the plant. Similarly, in other studies, it was noted that proline accumulation increased significantly in rice [57,58], cotton [59], tall fescue [60] and wheat genotypes [61] with increasing drought stress. Proline is very important for actively dividing cells, as it ensures the maintenance of sustainable growth (vegetative and generative) in plants under long-term environmental stress conditions. Therefore, accumulation of proline, which is used as a stress marker, in plant cells under biotic and abiotic stress conditions (heavy metal accumulation, pathogens, drought, salinity, UV, etc.) is associated with stress avoidance mechanisms [62-66]. In addition, proline, as a ROS scavenger and a molecule with the capacity to regulate many cellular homeostasis, is estimated to protect against cellular damage caused by stress factors such as salinity and drought [67, 68]. Plant tissues have the ability to accumulate proline, which is a powerful osmoprotectant, and to rapidly decompose it when necessary. The conversion of pyrroline-5-carboxylate (P5C) to proline during proline synthesis may cause an increase in the amount of ROS derivatives, followed by apoptosis and programmed cell death. For this reason, proline accumulated during a stress period is reduced to provide an energy source for growth and development after the stress is relieved, thus trying to prevent it from showing toxic effects [69, 70].

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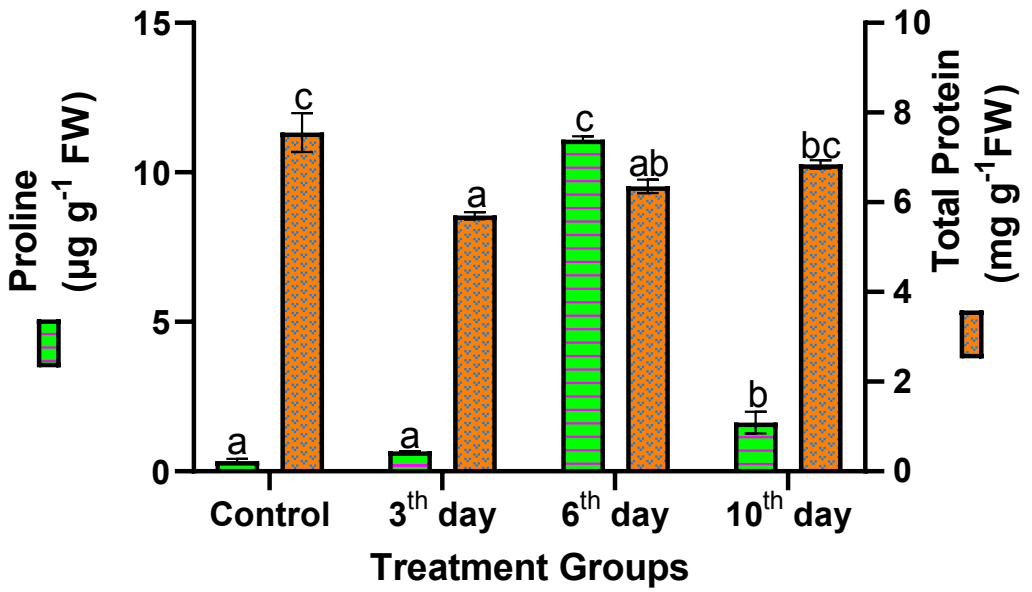

Figure 3. Effect of drought treatment on proline and total protein contents in leaves of *M. Pulegium.*

3.3. Changes in H_2O_2 and MDA Content

H_2O_2 and MDA content, two important indicators of oxidative stress, were measured to determine oxidative damage in *M. pulegium* exposed to different drought periods. In the study, H_2O_2 and MDA content of plants under drought stress showed a positive correlation with each other (Figure 4). The H_2O_2 content after 3, 6, and 10 days of drought increased 1.01, 1.21 and 1.32 times compared to the control plants, respectively (Figure 4). In addition, this treatment caused a significant increase in the MDA content of the plants. After 3, 6, and 10 days, MDA content increased 1.61, 2.92 and 2.61 times, respectively, and this increase was statistically significant ($p<0.05$) (Figure 4). The results obtained in H_2O_2 and MDA content are similar to Azad [23] and Siswoyo [71] studies.

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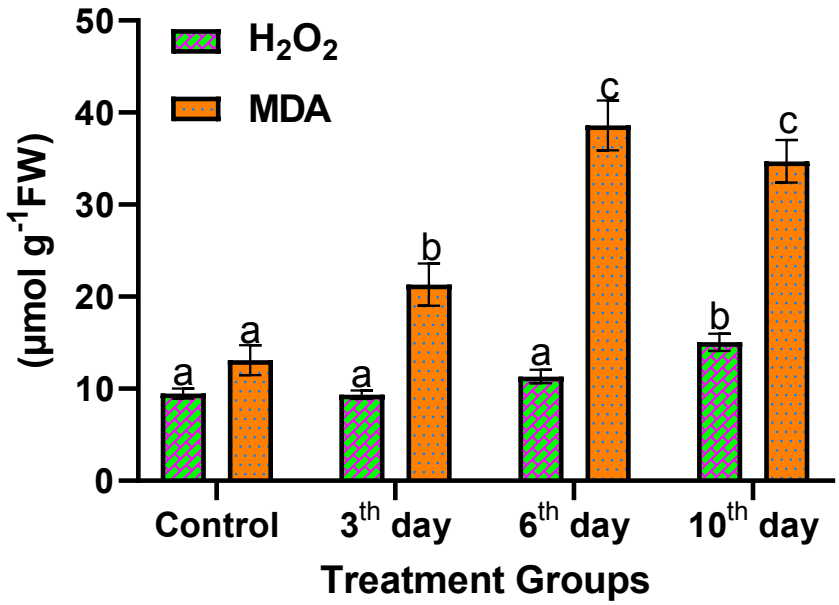

Figure 4. Effect of drought treatment on H_2O_2 and MDA contents in leaves of *M. pulegium.*

One of the most important problems that occur in plants under stress conditions is lipid peroxidation, which leads to deterioration of membrane stability. Despite the increase in the activities of antioxidant defense systems, the unavoidable increase in the amount of ROS derivatives such as H_2O_2 causes damage to the biological membrane structure. In cases where the antioxidant defense system cannot prevent the harmful effects of ROS, which attacks the double bonds in the phospholipid layer containing unsaturated fatty acids, a complex structure called malondialdehyde (MDA) emerges with the deterioration of lipids in the membranes [25]. Therefore, determining the amount of MDA allows us to have an idea about a measure of tissue and cell membrane damage. The data obtained show that a high level of cellular damage occurs in parallel with the long stress period with the accumulation of ROS and lipid peroxidation in *M. pulegium*, which is experiencing drought stress. It is also known that this deterioration in biological membranes releases the precursor compounds used in the synthesis of herbal stimulants and hormones that activate the antioxidant defense system in plants. These messenger structures, called phytohormone/plant growth regulators (PGR) and acting as signal molecules in cells, regulate the responses of plants to physiological conditions and can function even at very low concentrations. For example, jasmonic acid (JA), a signaling molecule, is currently considered as an endogenous phytohormone that regulates plant reproduction and growth, nutrient storage. Besides this important role in plant growth, JA also facilitates the adaptation of plants to different environmental stresses [72]. The release of precursors for the synthesis of plant defense hormones such as JA, due to the deterioration of the membranes, can be considered important in terms of combating stress conditions when considered from a different perspective.

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3.4. Chlorophyll (chlorophyll a, b and total chlorophyll) and Carotenoid Content

It has been reported in many studies that biotic and abiotic stress conditions reduce the photosynthetic efficiency of plants and therefore the hemostasis of the plant is impaired. Chl a, Chl b, total chlorophyll (total Chl) and carotenoid (Car) analysis were carried out to determine how the pigment degradations, which decrease the photosynthesis efficiency such as chlorosis and break the plant's defense power against stress conditions, change with drought. In parallel with the prolongation of drought stress in pennyroyal plants, the pigment content decreased significantly. Even, Chl a, Chl b, total Chl and Car contents decreased by 69%, 52%, 62% and 65%, respectively, compared to the control after 10 days of drought application. These results showed that the water stress experienced had to reduce the rate of photosynthesis due to the loss of chlorophyll in plants. The results obtained from the present study showed that the chlorophyll content of the pennyroyal plant decreased as a result of the suppression or degradation of chlorophyll biosynthesis by drought stress (Figure 5). Oxidative stress in plants causes disruption of chloroplast and can lead to a decrease in chlorophyll content [73]. Therefore, the results obtained can be considered as a distinctive symptom of oxidative stress [74]. Similar to our results, the gradual increase in drought stress resulted in decreased pigment contents in chickpeas [75, 76], maize [77], and peanuts [78].

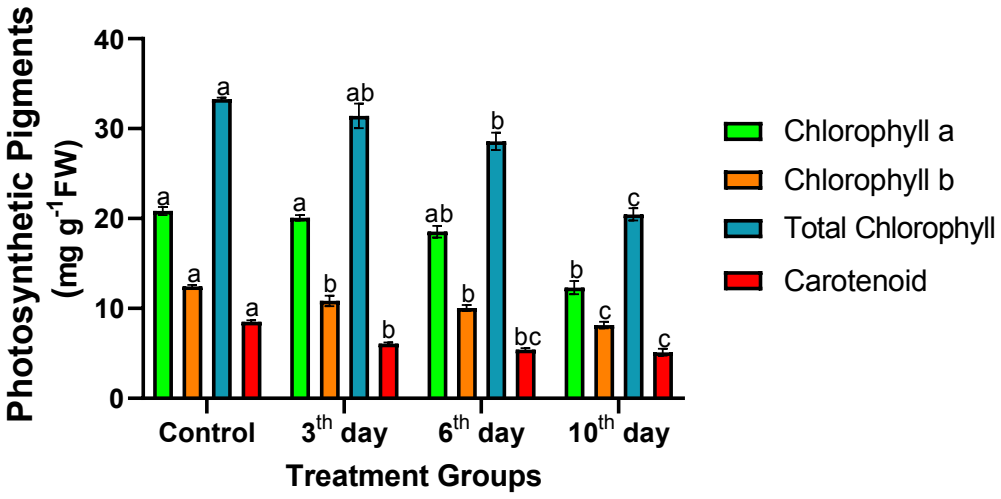

Figure 5. Effect of drought treatment on photosynthetic pigment contents in leaves of *M. pulegium.*

3.5. Total Protein Content

There was a gradual decrease in total protein content of the watermelon plant due to water stress. Variations of total protein content in leaves of *M. pulegium* plants subjected to drought stress for 10 days are shown in the Figure 3. With the onset of drought stress, the total protein content decreased by about 30%, while this decrease was more limited in the following days. Due to water stress, the total protein content initially decreased compared to the control and increased again in parallel with the increases in the synthesis of antioxidant enzymes triggered by the continuation of these adverse

conditions. In addition, the increased viscosity due to water loss in the cell cytoplasm under prolonged drought stress can be considered as the reason for the increase in the amount of protein per tissue compared to the early period. While there was a significant decrease in the total protein content of the drought-sensitive (Bahar) wheat variety among the 2 different wheat cultivars tested for drought stress, this decrease was slight in the drought-resistant (Kavir) variety [79]. Again, the total protein content of *Zea mays* L. [80], *Pistacia vera* L. [81] and *Levisticum officinale* Koch. [82] plants decreased under drought stress, which is consistent with our results.

3.6. Total Phenolic Compounds and Antioxidant Properties

Plants activate their antioxidant defense systems against biotic and abiotic stress conditions. This situation usually manifests itself in the form of an increase in antioxidant enzyme activities until plant homeostasis deteriorates in parallel with the increase in stress conditions. Herbal antioxidant defense systems first cope with stress conditions by increasing the activity of antioxidant enzymes, eliminating free oxygen radicals or converting them to less harmful molecules. In the present study, there is an increase in the activity of PPO, CAT, APX and POD enzymes in parallel with increasing drought stress. In addition to enzymatic antioxidants in plants, there are also non-enzymatic antioxidants such as ascorbic acid, phenolic compounds and carotenoids. With the increase in stress conditions, enzymes that eliminate the damages of peroxides such as CAT, APX, GPX first come into play and try to prevent the damages. In this study, besides enzymatic antioxidants, DPPH, FRAP, ABTS radical scavening activity and TPC amount were analyzed in pennyroyal plant. As can be seen from Figure 6, 7, while there was no significant change in these activities during the early drought period, the relative decrease in stress markers parallel to the increase in the activity of both enzymatic antioxidants during prolonged drought stress conditions; In addition, plant hemostasis, which started to deteriorate under 10 days of drought stress, caused a decrease in these activities.

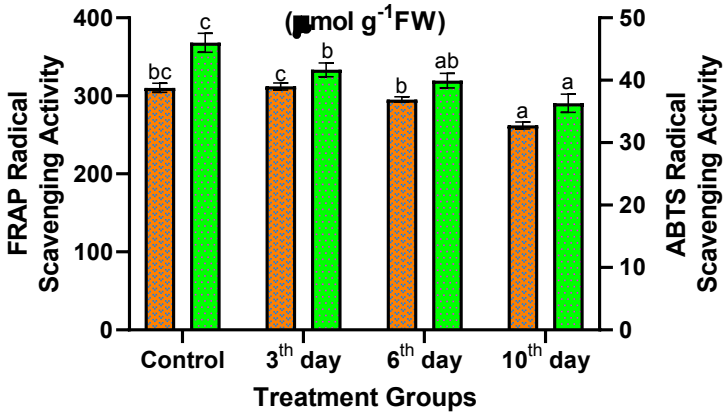

Figure 6. Effect of drought treatment on FRAP and ABTS radical scavenging activity in leaves of *M. pulegium.*

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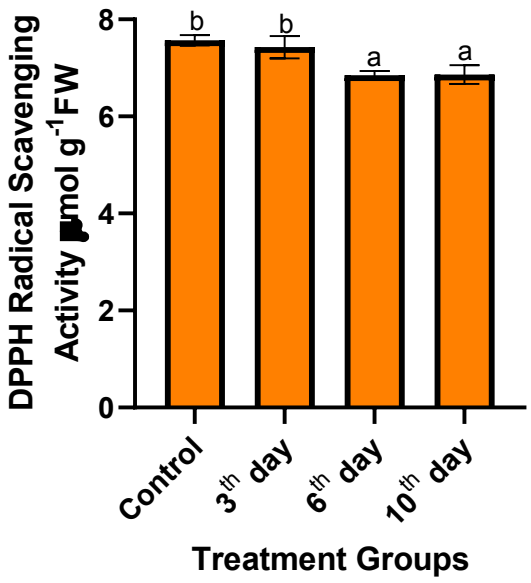

Figure 7. Effect of drought treatment on DPPH radical scavenging activity in leaves of *M. pulegium.*

The amount of TPC changed with drought conditions, especially during the long drought period. TPC content started to decrease from the 6th day, and this decrease became statistically significant on the 10th drought day (Figure 8). There are many studies conducted on plants such as wheat, pepper and *Amaranthus*, in which abiotic stress conditions cause the TPC content to increase and decrease [83-85]. In these studies, it has been reported that stress conditions may produce different results in plants with different genotypes. In our study, although a relative increase was observed in proline and antioxidant enzyme activities with long-term drought stress, the reason for the long-term decrease in total phenolic content may be due to the insufficient prevention of lipid peroxidation and stabilization of cell membranes [45].

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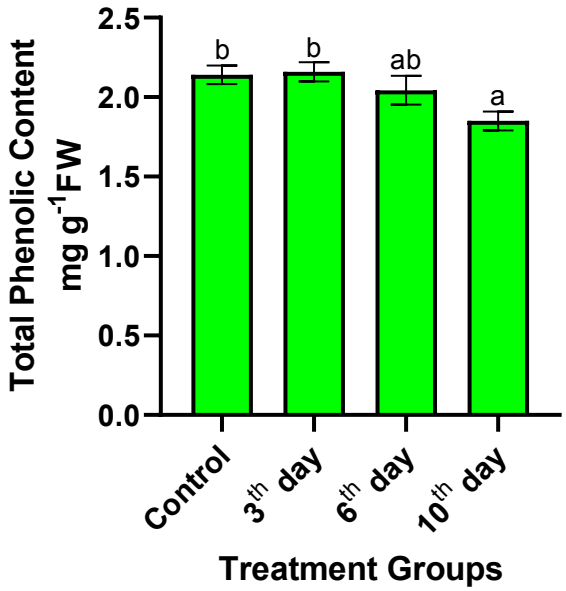

Figure 8. Effect of drought treatment on total phenolic content in leaves of *M. pulegium.*

Three different tests, DPPH, FRAP and ABTS removal activity, were performed as antioxidant activity test in the pennyroyal plant exposed to drought stress. No significant change was observed in all three activity tests, especially in the early drought period. Here, it can be thought that the increase in the activities of enzymatic antioxidants has a more dominant effect on creating normal physiological conditions in the plant. However, increased stress conditions caused a decrease in all three activities during the prolonged drought period.

4. CONCLUSION

Overall, drought stress caused an increase in antioxidant enzyme activities of *M. pulegium*, while prolongation of water stress caused a decrease in these enzyme activities. The significant decrease after an increase in CAT activity was compensated by an increase in ASPX and PPO activities. The significant positive correlation of PPO with ASPX and proline activities confirmed its important role in ROS scavenging. In terms of changes in lipid peroxidation (MDA content) and H_2O_2 amounts, it has been determined that the antioxidant defense system can slow down ROS production and membrane damage to a certain level. In addition, a rapid increase was observed in the amount of proline in the early stress period, but following this, proline levels decreased through the activation of the defense system triggered by the induced PGRs (plant growth regulators). Water stress increased chlorosis and caused a decrease in the amount of all photosynthetic pigments. Our results show that the antioxidant abilities in the medicinal and aromatic plant *M. pulegium* act as a defense mechanism

against drought stress. The use of these natural antioxidant sources as additives in food and cosmetics is increasing day by day, and therefore, the information obtained about the adaptive mechanisms of medicinal and aromatic plants exposed to drought, which poses a global threat, continues to attract attention from both an agricultural ecology and an economic point of view.

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The authors declare that they have no conflict of interest.

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