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Research Article

Responses of *Helichrysum arenarium* (L.) Moench Distributing in the Sivas Region to Drought Stress via the Antioxidant Defense System

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ABSTRACT

Helichrysum species are recognized for their therapeutic properties as aromatic herbs that have been utilized for millennia in both conventional and alternative medicine. In the germination and early growth stages of *Helichrysum arenarium* (L.) Moench (immortelle) seeds obtained from the Sivas region were attempted to ascertain the morphological, physiological, and antioxidant defence system-based responses to drought stress. Immortelle seeds grown *in vitro* under PEG-6000 (%0-30 PEG) were exposed to drought stress. Germination test results showed that seeds could not germinate in 20% and 30% PEG medium and 15% PEG medium significantly inhibited growth in the early stages of seedling development. The physiological and biochemical responses of plants in PEG-6000 (0% (control), 5% and 10% PEG) medium during the early stages of seedling development were examined over a two-week period. The growth parameters decreased by 5% and 10% PEG medium. In addition to leaf water content (LCW), catalase (CAT) and glutathione reductase (GR) activities; APX, H_2O_2, SOD and TBARS values also increased. According to the seventh day data, CAT and GR activity decreased, SOD activity did not change. This observation indicates rapid responses in drought-tolerant plants such as *H. arenarium*, highlighting the necessity for further investigation within a seven-day period.

1. Introduction

Helichrysum species are xerophytic plants that are used in traditional folk medicine as well as in modern medicine [1]. The genus *Helichrysum* is systematically accepted as a member of the Asteraceae family. This genus is known to include more than 500 species worldwide, and the regions where it spreads are generally South Africa, the Mediterranean basin and Australia. Of these distribution areas, 245 are in South Africa, and 16 are in Europe [2, 3]. It has been determined that 34 immortelle species are spreading in Türkiye and 17 of these species are endemic to Türkiye [4].

Drought is an important stress factor. This is because the physiological processes of plants depend on water. A decrease in water absorbtion causes a reduction in plant development and yield values [1]. Drought stress can cause specific and nonspecific damage in plants by affecting water levels in cells, tissues, and organs [5]. The growth and development of plants depend on their environment. The stress response mechanisms developed by plants can be divided into four groups: morphological, physiological, chemical, and molecular mechanisms [6]. The antioxidant defense mechanism is expressed as one of the response mechanisms to drought stress [7].

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Oxidative stress is the main factor that causes damage when a profound incompatibility between reactive oxygen species (ROS) production and the antioxidant defence system occurs. Moreover, oxidative stress has lately been categorized into subtypes: oxidative stress (eustress) that arises under physiological settings and oxidative stress (distress) that has detrimental effects on macromolecules [8]. When plants are exposed to drought stress for an extended period, they are exposed to oxidative damage because of the excessive production of these four forms called ROS, such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-), H_2O_2 and hydroxyl radical (HO*) [9].

Plants have implemented many defence mechanisms to protect cells against the harmful effects of excessive ROS. These are enzymatic and nonenzymatic antioxidant defence mechanisms [7]. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione S-transferase (GST) etc. are examples of plant enzymatic defence systems. Glutathione, ascorbate, tocopherol, flavonoids, alkaloids, carotenoids, and nonprotein amino acids are examples of nonenzymatic antioxidants [10].

The aim of this study was to determine the changes in germination of *H. arenarium* seeds collected from Sivas region (Türkiye) under drought stress and the responses in plant growth, physiological and biochemical levels during the growth period.

2. General Methods

Helichrysum arenarium (L.) Moench (immortelle) seeds were collected from Sivas (40°8'48.7716" North and 37°14' 34.5660" East) (Figure 1).

2.1.Growth conditions and drought stress

The surface-sterilized seeds with 1% sodium hypochlorice were left to germinate vertically in plastic sterile Petri dishes. Polyethylene glycol-6000 (PEG-6000) was used to cause drought stress. Plants were grown in six different environments (0% (control), 5, 10, 15, 20 and 30% PEG) with at least six repetitions of fifty seeds in each Petri dish containing Murashige and Skoog media [11]. Petri dishes were wrapped with strech film to prevent moisture loss in all germination trials, and in the same way, seeds were taken to the plant growth room in all trials for seven days.

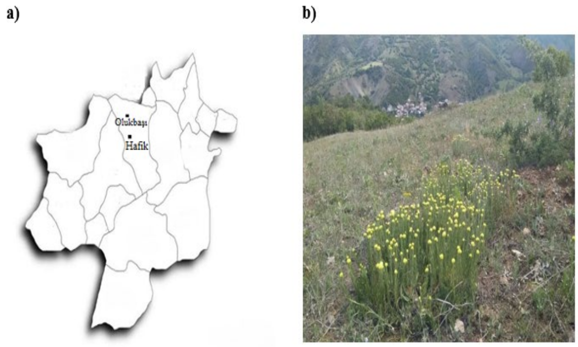

Figure 1. Map showing the location of *H. arenarium* seed collection in a) Sivas-Olukbaşı village and b) natural distribution area

In the early stages of seedling development to determine the responses of plants to three PEG-6000 applications (0% PEG (control), 5% PEG, 10% PEG), the plants were sampled on the 7^{th} and 14^{th} days. Due to restricted plant development, the 15% PEG treatment was not included in biochemical measurements.

The plants were grown in a controlled plant growth room at 25±2°C for 16 hours under illumination by LED lamps with a light intensity of 250 µmol $m^{-2}s^{-1}$ at 60±5% humidity.

For biochemical analyses, plant leaves were subjected to deep freezing on the sampling day and stored at -18°C until the day of analysis.

2.2.Plant growth parameters

Ten randomly selected 7- and 14-day-old plants at each replication were divided into roots and shoots. First, root length (RL) and shoot length (SL) were measured with a ruler (cm) and were weighed for fresh weight (RFW and SDW). After, the materials were dried in an oven at 70°C for 24 hours to determine their dry weights (RDW and SDW) (mg).

2.3.Determination of leaf water content (LWC)

The fresh weights (Wf) (mg) of the plants treated with PEG-6000 on the 7th and 14th days were determined by weighing them as whole plants. Then, these leaves were dried at 104°C for 2 hours and then at 80°C for 72 hours. After this, their dry weight (Wd) was determined. The LWC was calculated according to formula 1 [12].

$$LWC\ (\%) = \frac{Wf - Wd}{Wf} x100 \qquad (1)$$

2.4.Determination of H_2O_2 content

Leaves (200 mg) were homogenised with 1.5 ml 100 mM potasium phosphate (KPO_4) buffer (pH 6.8). The homogenate was centrifuged at 13000 rpm for 30 min at 4°C. The supernatant is mixed with 2.5 ml of peroxide reagent and incubated for 10 minutes at 30°C. And then, the reaction is stopped by adding 1 M perchloric acid. The amount of leaf H_2O_2 was determined spectrophotometrically at 436 nm. The H_2O_2 level of the samples was determined according to the H_2O_2 standard curve [13].

2.5.Determination of lipid peroxidation level

Leaves (200 mg) were homogenised with 0.1% trichloroacetic acid (TCA) solution, and the homogenised extracts were centrifuged at 10000 rpm for 5 min at 4°C. Supernatant was mixed with TCA and tiyobarbituric acid (TBA) solution and then mixture was incubated for 30 min at 95°C. After the second centrifugation, the thiobarbituric acid reactive substances (TBARS) content was calculated using the spectrophotometric absorbance values at 532 and 600 nm of the formed supernatant [14].

2.6.Determination of total protein content

Leaves (200 mg) were homogenised with 1 ml of 50 mM Na-P buffer containing 1 mM $EDTANa_2$. H_2O and extraction buffer (pH 7.8) at 4°C. The extracts obtained after homogenisation (30 min at 13000 rpm at 4°C) were centrifuged. The supernatant was used for total protein, SOD, CAT and GR analyses. After centrifuge process, 1 ml of reagent was added to 50 µl of supernatant in new centrifuge tubes and kept in the dark for 5 minutes. After the staining process, the samples were read against the blank in a UV-Vis spectrophotometer at 595 nm. A protein standard graph was created to determine the protein content of the samples. The graph was made using bovine serum albumin, and calculations were made on this graph [15]. The determined amount of protein (mg/g tissue) was used to calculate enzyme activities.

2.7.Determination of SOD activity

The SOD reaction mixture (50 mM Na-P buffer (pH 7.8), 0.1 M L-methionine, 1 mM nitro blue tetrazolium (NBT), and 0.1 mM $EDTA.Na_2$ and 0.2 mM riboflavin) was used during the analysis. The procedure is based on the understanding that colour change occurs at different supernatant volume (0 to 200 µl) and 3 ml reaction mixture for 10 min at 25°C under 300 µmol $m^{-2}s^{-1}$ light. The colour changes were determined by a spectrophotometer at 560 nm. The enzyme activity was defined as enzyme units (U) mg $protein^{-1}$ [16].

2.8.Determination of GR activity

The supernatant and GR reaction mixture (25 mM Na-P buffer (pH: 7.8), GSSG buffer and $NADPH.Na_4$ buffer) were measured for 90 sec at 340 nm in a spectrophotometer. One enzyme unit is the amount of oxidised glutathione (µmol ml^{-1}) per minute. The specific enzyme activity was determined as U mg $protein^{-1}$ [17].

2.9.Determination of CAT activity

A CAT reaction mixture containing 1 mM EDTA, 0.05 M Na-P buffer (pH 7.0), dH_2O and 3% H_2O_2 was used. A decrease in H_2O_2 was observed by reading the spectrophotometer at 240 nm for 90 sec against the blank. The amount of µmol H_2O_2 consumed per minute was determined as one enzyme unit, and the specific enzyme activity was defined as U mg $protein^{-1}$ [18].

2.10. Determination of APX activity

Two hundred milligrams of plant sample were homogenised with 50 mM Na-P buffer containing 2 mM ascorbic acid and 1 mM

$EDTANa_2.2H_2O$ at 4°C. Following centrifugation (30 min at 13000 rpm at 4°C), the supernatant was reacted with the reaction mixture (50 mM Na-P buffer (pH 7.0), 5 mM ascorbate, 1 mM $EDTA.Na_2$ and 1,2 mM H_2O_2), and the activity was measured by spectrophotometry at 290 nm. The specific enzyme activity was determined as U mg protein^{-1} [19].

2.11. Statistical analysis

This study was carried out in three replications according to the experimental design of the divided plots, which were split into random plots. All physiological and biochemical data were analysed using one-way analysis of variance (ANOVA) and the SPSS 18.0 statistical package program. The differences between the means were compared with Tukey test at *P≤0.05*. The results are presented in charts and figures as the mean ± standard error.

3. Results and Discussion

Helichrysnum spp. seeds have a very low germination rate. Various pre-treatment techniques have been used in many studies to increase seed germination rate [20]. This study examines the antioxidant defense system's role in *H. arenarium*'s tolerance to drought stress, as existing literature lacks information on this topic. Drought has a negative effect on plant growth and development. Figure 2a shows that the control group had the highest seed germination rate (55%), and the 15% PEG group had the lowest germination rate (15%) due to increasing PEG concentration. However, there was no statistically significant difference in germination between 10% and 15% PEG treatments. In this study, it was found that PEG treatment significantly decreased the germination rate.

PEG applications inhibited growth, except for RFW and RDW values on day 7 (Figure 2b-g). Overall, shoot development was more suppressed than root development (Figure 2c, 2e, and 2g).

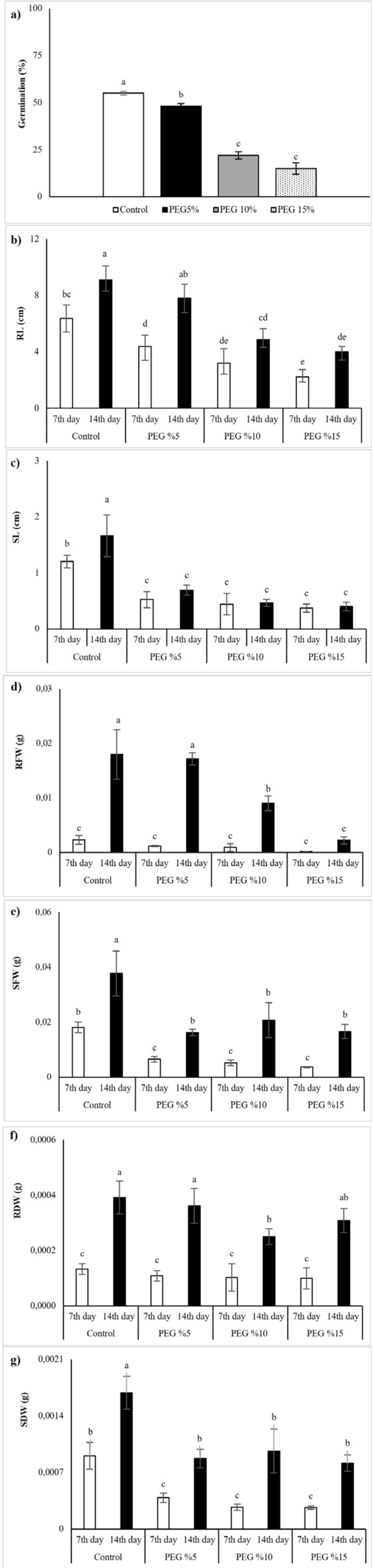

Figure 2. Changes in the early developmental stages of *H. arenarium* (a: Germination; b: RL, c: SL, d: RFW, e: SFW, f: RDW, g: SDW) under drought stress

The decline in water balance directly impacts plant metabolic activities. The LWC is one of the most common physiological parameters for determining plant drought tolerance and limits photosynthesis efficiency and biomass productivity in plants [12]. Physio-biochemical processes manifest themselves before the reduction in plant growth and development brought on by drought [21]. In this study, while there was a decrease in the percentage of 5% PEG applied to the immortelle plants on the 7th day of LWC compared to that in the control group. With the prolongation of the stress period, there was a decrease in the LWC compared to that in the control group due to the increase in PEG concentration (5%-10%) on the 14th day (*P>0,05*) (Figure 3a).

Excessive amounts of H_2O_2 in the cell cause oxidative damage [22], and TBARS byproduct of polyunsaturated fatty acid oxidation has been identified as one of the main stress-related effects in plants and is one of the key indicators of stress on membranes, the main target of stress [23]. In this study, enzyme activities of the immortelle plant were higher 7 days after PEG application compared to the 14th day. However, there was no significant change in LWC during the drought period (Figure 3a-g). The statistically significant difference in H_2O_2 on the 7th and 14th days was detected among the immortelle plant grown under control, 5% PEG, and 10% PEG treatments (Figure 3b).

The change in the amount of H_2O_2 increased in the immortelle plant due to PEG application. It was determined that the amount of TBARS in the control group plants increased day by day, while in the plants grown in 5% PEG medium, there was a decrease in the opposite direction (*P<0.05*). It was determined that there was no significant change in the TBARS values of the plants growing in 10% PEG medium on the 7th and 14th days. A significant change in the amount of TBARS was detected on the 7th and 14th days of immortelle plant growth under control, 5% PEG, and 10% PEG treatments (Figure 3c).

The formation of oxygen radicals can be exacerbated under environmental stress, and the SOD enzyme plays an essential role in plant stress tolerance [24]. In this study, a significant increase in SOD activity was detected in drought-stressed plants on the 7th day (Figure 3d). H_2O_2 transforms into a hydroxyl radical and causes cell damage. CAT is an antioxidant defence system enzyme that converts H_2O_2 into H_2O and O_2 [8,10]. CAT is critical in maintaining redox balance during oxidative stress [25]. This study determined that CAT activity decreased in the immortelle plants under drought stress due to the increase in PEG concentration (Figure 3e). APX plays an essential role in drought stress tolerance, contributing to H_2O_2 detoxification at the maximum level [26]. It has been determined that APX activity increases due to the increase in PEG concentration in the immortelle plant. However, increase in APX activity was not progressive (Figure 3f). GR is localised only in the mesophyll cells of leaves. Conversely, several antioxidant enzymes (APX and SOD) are exclusive to bundle sheath cells, while CAT is roughly split evenly between the two cell types. Glutathione is a multifunctional metabolite in plant defence. Enzyme-catalyzed reactions provide reduced glutathione for detoxifying H_2O_2 in the ascorbate-glutathione cycle [27]. In this study, the GR activity was low on day 14 and stayed constant, although it declined on day 7 as the PEG content increased (Figure 3g).

In this study, the germination capacity of immortelle seeds was low and osmotic stress via PEG application inhibited the germination percentages and plant growth. However, *H. microphyllum* subsp. *tyrrhenicum* was characterised by high germination percentages and did not require any dormancy-breaking treatment [28]. Some external applications, such as smoke water in H. odoratissimum [20] and gibberellic acid in Lavandula angustifolia [29], can accelerate the seed germination and growth of immortelle plants.

In physio-biochemical results, the CAT activity declines and the increases in H_2O_2 and TBARS content on the seventh day are comparable to those observed in Silybum marianum plants grown under water restriction for two weeks [30]. The findings of Bayat and Moghadam [31] conflict with this study's CAT, APX, and GR results. According to the authors, *Salvia nemorosa* is under water stress for ten days of enhanced CAT and POD activities. The rise was more significant in tolerant cultivars than in sensitive cultivars.

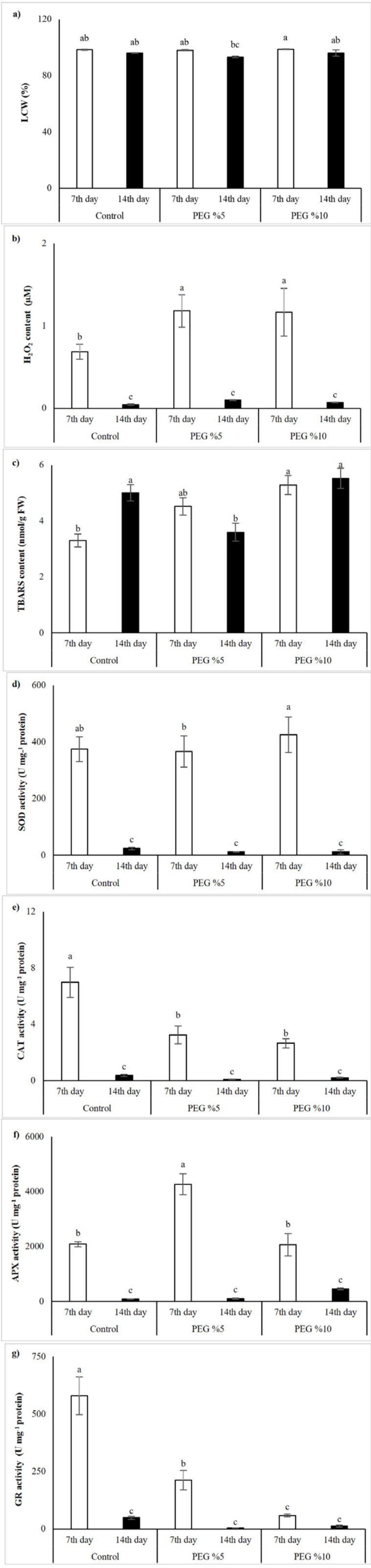

Figure 3. Changes in physio-biochemical parameters of *H. arenarium* (a: LCW, b: H_2O_2, c: TBARS, d: SOD, e: CAT, f: APX, g: GR) under drought stress

4. Conclusion

This study shows for the first time that *H. arenarium* exhibits increased antioxidant defense enzyme activities under drought stress. After seven days of drought treatment, enzyme activities were higher than fourteen days. This suggests quick responses in drought-tolerant plants like *H. arenarium*, indicating the need for further investigation before seven days. Morphological development was not significantly affected by these biochemical processes. The study provides valuable insights for future research on enhancing the stress tolerance threshold of *H. arenarium*.

Article Information Form

Authors Notes

This study was produced from Melda (Ateş) Şenyurt'’s master thesis titled “Antioxidant Defense System Based Responses to Drought Stress of Helichrysum arenarium (L.) Moench (Sandy Everlasting) Distributing in Sivas (Hafik, Olukbaşı) Region”.

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Authors' Contribution

MŞ collected seeds from the field, performed laboratory studies, and prepared the initial draft of the manuscript. SD supervised the master's thesis, analyzed the data, and reviewed the manuscript.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by authors.

Artificial Intelligence Statement

No artificial intelligence tools were used while writing this article.

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