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Antioxidant Defense System and Physiological Insights to Drought Stress in *Urtica dioica* L.

Urtica dioica L.'da Kuraklık Stresine İlişkin Antioksidant Savunma Sistemi ve ve Fizyolojik Yaklaşımlar

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Abstract

Urtica dioica L. (stinging nettle), is an herbaceous and dioecious perennial flowering plant, has been used as herbal medicine since ancient times. The aim of the present study was to investigate drought responses and tolerance mechanism of *U. dioica*. For this purpose, growth, water status, osmotic potential, chlorophyll fluorescence, lipid peroxidation (TBARS), hydrogen peroxide (H_2O_2) content and antioxidant enzyme activities were determined under drought stress. Relative growth rate, water content, osmotic potential and chlorophyll fluorescence were significantly reduced with drought treatment. Additionally, drought lead to increase in TBARS and H_2O_2 level. Moreover, the increment in H_2O_2 content under drought was accompanied by increased in superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) activities. On the other hand, drought stress caused a reduction in ascorbate peroxidase (APX) activity, while there was no significant change in peroxidase (POX) activity. These results suggest that *U. dioica* has an efficient drought tolerance, as displayed by enhanced antioxidant enzyme activities. In this study, antioxidant defence and ROS detoxification capacity have been elucidated in *U. dioica* under drought stress.

Keywords: Antioxidant defence system, Drought stress, *Urtica dioica*, Stinging nettle

Özet

Urtica dioica L. (ısırgan otu), otsu, dioik, çok yıllık çiçekli bir bitki olup eski çağlardan beri bitkisel ilaç olarak kullanılmaktadır. Bu çalışmanın amacı, *U. dioica*'nın kuraklığa tepkilerini ve tolerans mekanizmasını araştırmaktır. Bu amaçla, kuraklık stresi altında büyüme, su durumu, ozmotik potansiyel, klorofil floresansı, lipid peroksidasyonu (TBARS), hidrojen peroksit (H_2O_2) içeriği ve antioksidan enzim aktiviteleri belirlenmiştir. Nispi büyüme oranı, su içeriği, ozmotik potansiyel ve klorofil floresansı kuraklık uygulaması ile önemli ölçüde azalmıştır. Ek olarak, kuraklık TBARS ve H_2O_2 seviyesinde artışa yol açmıştır. Ayrıca, kuraklık altında H_2O_2 içeriğindeki artışa süperoksit dismutaz (SOD), katalaz (CAT) ve glutatyon redüktaz (GR) aktivitelerindeki artış eşlik etmiştir. Öte yandan, kuraklık stresi askorbat peroksidaz (APX) aktivitesinde azalmaya neden olurken, peroksidaz (POX) aktivitesinde önemli bir değişiklik meydana getirmemiştir. Bu sonuçlar, *U. dioica*'nın artan antioksidan enzim aktivitelerinin de gösterdiği gibi etkili bir kuraklık toleransına sahip olduğunu göstermektedir. Bu çalışmada, kuraklık stresi altında *U. dioica*'da antioksidan savunma ve ROS detoksifikasyon kapasitesi aydınlatılmıştır.

Anahtar Kelimeler: Antioksidan savunma sistemi, Kuraklık stresi, *Urtica dioica*, Isırgan

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1. Introduction

Plants can be subjected to various abiotic stress factors, including drought, salinity, chemical pollution, and extreme temperatures, which can negatively impact crop yield and quality. Water scarcity has a devastating impact on both humans and the environment, causing drought stress and it is a significant factor that must be addressed. Drought is the main stress factor that adversely affects plant growth and development, resulting in crop losses, and also triggers secondary stresses such as osmotic, ionic and oxidative stresses (Mahajan and Tuteja, 2005; Ahluwalia et al., 2021). Many physiological processes from seed germination to maturity such as membrane integrity, transpiration, water use efficiency, photosynthetic activity, and respiration were affected by drought stress (Fracasso et al., 2016). Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radical are formed during oxidative stress associated with drought (Mattos and Moretti, 2015). At high levels in cells, ROS frequently lead to impaired physiological functions of proteins, lipids and DNA, resulting in cell death (Juan et al., 2021). In plant cells, enzymatic (ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2), peroxidase (POX; EC.1.11.1.7), superoxide dismutase (SOD; EC.1.15.1.1)) and non-enzymatic antioxidants (ascorbic acid, carotenoids, glutathione and tocopherols) are activated to detoxify ROS and protect cellular mechanisms (Mittler, 2002; Gill and Tuteja, 2010; Hasanuzzaman et al., 2020).

Urtica dioica L. (stinging nettle), belonging to Urticaceae family, is an herbaceous, dioecious perennial flowering plant that is widely distributed from Asia, America, Europe, to Africa (Oñate and Munné-Bosch, 2009). Stinging nettle plants are considered as ancient medicinal plant and commonly used food and textile industries for its quality fibers (Bacci et al., 2009; Jaiswal and Lee, 2022). Leaves and roots of *U. dioica* has been used for its ethno-pharmacological features (Upton, 2013; Vajic et al., 2018). The plant has the potential to be used as a fertilizer and for biological pest control (Bán et al., 2010). Due to its dioecious nature, many studies were focused on male and female flowers of this species (Oñate and Munné-Bosch, 2009; Simancas et al., 2016). Although much knowledge is to be obtained with respect to the bioactive compounds and antioxidant activity in stinging nettle, the interactions of antioxidant defense system and ROS detoxifying are still limited and need further explanation for this species under drought stress.

Therefore, because of limited data on the antioxidant defense system power of *U.* dioica, the aim of this study was to examine the physiological and biochemical features under

drought stress in this plant species. For this purpose, APX, CAT, GR, POX and SOD enzyme activities, lipid peroxidation level and hydrogen peroxide content were determined in *U. dioica* under drought stress. In addition, the relative growth, water content of the leaves, chlorophyll fluorescence and osmotic potential were measured for this aim.

2. Material and Method

2.1. Plant Material and Growth Conditions

A controlled greenhouse experiment (27/22 °C, day/night, 16/8 h; 70%, relative humidity) was performed with stinging nettle (*Urtica dioica* L.). Seeds of *U. dioica* were obtained from nature and cleaned by rinsing them with deionized water to eliminate any dirt or debris. After sterilization with 5% NaOCl, seeds were rinsed with deionized water to remove blench. Then, sterilized seeds were planted in 16-cm pots filled with peat+perlite+torf (1:1:1). Drought stress treatments were started when plants were three-month old and leaving plants without water was considered as a drought treatment. After three-week drought stress period, plants were harvested. Mature leaves of *U. dioica* were used for analyses and harvested leaves immediately frozen in liquid nitrogen (-196 °C) and stored at -80 °C for further analyses.

2.2. Relative Growth Rate, Relative Water Content, Osmotic Potential and Chlorophyll Fluorescence

Before drought period started (on day 0), 10 random plants from control and drought plants were harvested to determine the relative growth rate (RGR). When drought period ended (on day 21), another 10 random plants from control and drought plants were harvested to be used for the growth analyses.

Ten leaves from control and drought-stressed plants during the harvest were weighed and fresh weights (FW) were recorded. After that, leaves were put in water for at least 8 h and turgid weights (TW) of leaves were determined. Then, leaves were dried (70°C; 72 h) and dry weights were obtained. Relative water content (RWC) was calculated as ((FW-DW)/(TW-DW)) x 100.

Leaf osmotic potential (Ψ_s) was measured after harvest using an osmometer (Wescor Vapro Pressure Osmometer 5600). Ten leaves from control and drought-stressed plants were extracted with a glass rod and then centrifuged. The readings for Ψ_s from the instrument were measured according to Santa-Cruz et al. (2002).

Ten leaves from control and drought-stressed plants were used for chlorophyll fluorescence (Fv/Fm) analyses. After the leaves adapted to the dark at least 20 min, Fv/Fm was measured with Plant Efficiency Analyzer of Hansatech (UK).

2.3. Lipid Peroxidation and Hydrogen Peroxide Content

Thiobarbituric acid reactive substances (TBARS) were used to determine lipid peroxidation levels in control and drought-stressed Urtica dioica (Heath and Packer, 1968). Briefly, fresh leaves were extracted in trichloroacetic acid (TCA). They were then centrifuged at 12000 g for 15 min at 4 °C. Supernatant was mixed with 20% TCA and 0.5% TBA. After 30 min at 95°C, the samples were cooled. Absorbance at 532 and 600 nm was recorded for TBARS.

Fresh leaves were extracted in TCA and then centrifuged at 12000 g for 15 min at 4 °C to determine the hydrogen peroxide (H_2O_2) content of control and drought-stressed plants (Liu et al., 2000). The supernatant was mixed with a $TiCl_4$ solution prepared with 20% H_2SO_4 after centrifugation. The H_2O_2 content was determined using a standard curve prepared on a UV-VIS spectrophotometer and the absorbance was recorded at 410 nm.

2.4. Antioxidant Enzyme Assays

Fresh leaves from control and drought-stressed *Urtica dioica* were extracted ice-cold phosphate buffer (50 mM; pH 7.0) consisting 1 mM EDTA and polyvinylpyrrolidone (1%). 2 mM ascorbate was added to the buffer for APX activity assay. Samples were centrifuged at 14000 g for 30 min. Protein and enzyme activity assays were performed on the supernatants. Protein was estimated from the extracts using the bovine serum albumin method (Bradford, 1976).

The activity of SOD was measured according to the procedure described by Beauchamp and Fridovich (1971). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 13 mM methionine, 0.1 mM EDTA, 0.075 mM nitroblue tetrazolium (NBT) and 2 μM riboflavin. The activity was measured by recording the absorbance at 560 nm. The amount of enzyme required to produce 50% inhibition of NBT was defined as one unit of activity. The POX activity was carried out using the procedure described by Mika and Lüthje (2003). The reaction mixture consisted of 25 mM sodium acetate (pH 5.0), 10 mM guaiacol, and 10 mM H_2O_2. Absorbance was measured at 470 nm and the activity was defined as one unit decomposing 1 μmol H_2O_2 in 1 min. The CAT activity was measured using the Aebi (1984) procedure. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0) and 10 mM H_2O_2. Absorbance was recorded at 240 nm and the amount required to decompose

1 µmol H_2O_2 in 1 minute was defined as 1 unit of CAT activity. The APX activity was measured using the procedure of Nakano and Asada (1981). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 250 µM ascorbate, and 5 mM H_2O_2. Absorbance was recorded at 290 nm and the amount required to oxidise 1 µmol ascorbate in 1 minute was defined as one unit of APX. The GR activity was determined using the procedure of Foyer and Halliwell (1976). The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.6), 5 mM NADPH, and 10 mM oxidized glutathione (GSSG). Absorbance was measured at 340 nm and the unit of GR was defined as the amount required to reduce 1 µmol GSSG in 1 min.

2.5. Statistical Analysis

Statistical analyses were conducted using analysis of variance. Significant differences among treatments were compared using Duncan's Multiple Range test at the $P < 0.05$ probability level. All analyses were performed using SPSS 22.0 (IBM™) software. Results were presented as means with standard error of the mean (± SEM) shown using error bars. The experimental design consisted of a randomized block with three replicates. Each replication included seven seedlings, resulting in a total of 21 seedlings for each individual treatment.

3. Results and Discussion

Drought adversely affected physiological and biochemical processes in *Urtica dioica*. In the current study, *U. dioica* plants exhibited noteworthy adaptations in response to drought stress. When faced with drought, relative growth rate, leaf water content, osmotic potential and chlorophyll fluorescence reduced in stinging nettle (Figure 1). Drought stress significantly decreased growth in *U. dioica* by 76.5%. Similar to present results, drought-induced reduction was recorded in tomato (Rady et al., 2020), wheat (Hassan et al., 2020) and pepper (Kaya, 2021) in plant growth. The inhibition of plant growth under drought stress was mainly due to a reduction in plant water content. Reduced water uptake and loss of turgor under drought stress could be a possible reason for the reduction in growth (Ings et al., 2013). RWC of *U. dioica* supports our remarks in terms of plant growth under drought. There was a slight decrease (5.3%) in leaf RWC (Figure 1B). RWC is positively correlated with drought tolerance (Askari and Ehsanzadeh, 2015). In our study, the greater RWC was recorded as 72% in drought-treated *U. dioica*. Stinging nettle may have maintained leaf water status under drought conditions through the synthesis of osmolytes that can easily replace water in the cytoplasm. In general, plant biomass is most directly related to plant

photosynthesis (Heyneke and Fernie, 2018). In this study, chlorophyll fluorescence value was measured to elucidate the effects of drought stress on the photosystem II apparatus. Chlorophyll fluorescence of *U. dioica* was reduced by 1.8% as compared to control plants under drought stress (Figure 1C). The decrease in photosynthetic efficiency may be due to a reduction in leaf growth parameters, resulting in a decrease in the number of chloroplasts per unit area. Moreover, drought stress also significantly decreased leaf osmotic potential by 17.6% (Figure 1D).

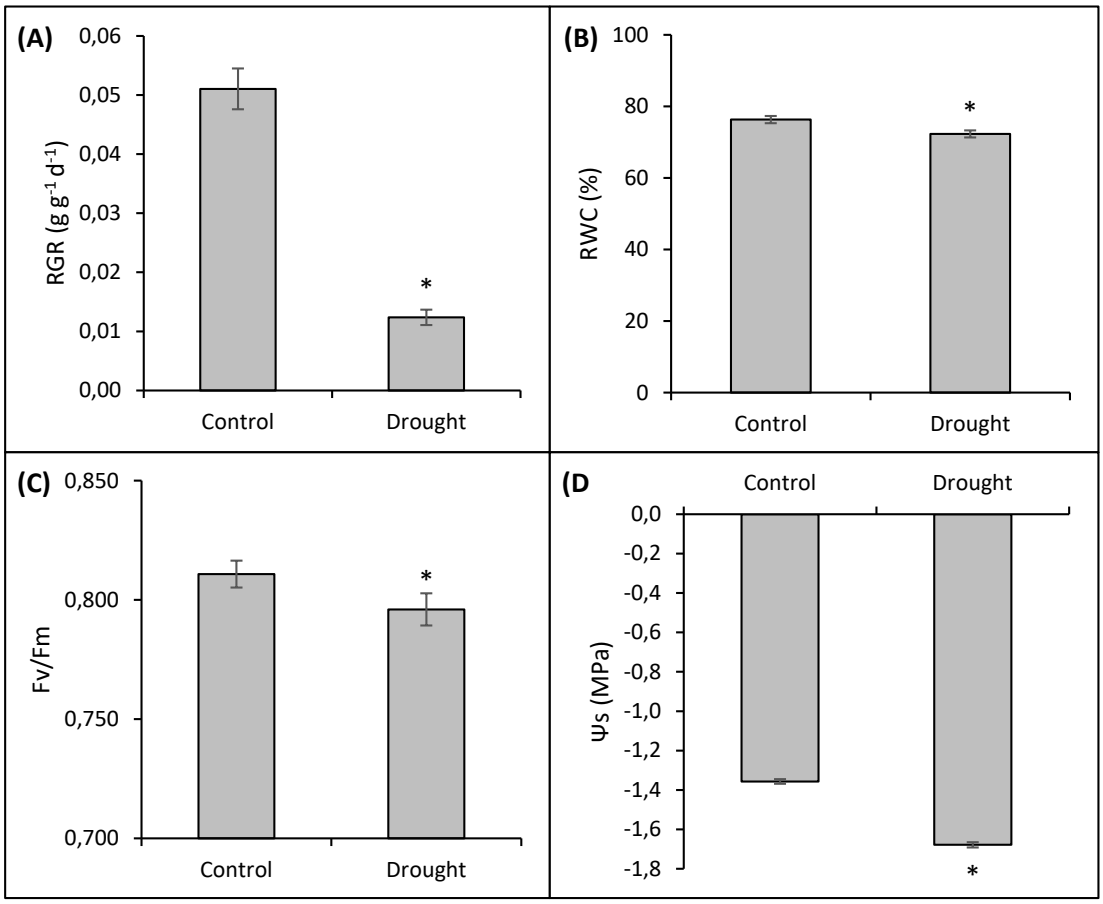

Figure 1. Effects of drought stress on relative growth (RGR, A), relative water content (RWC, B), chlorophyll fluorescence (Fv/Fm, C) and osmotic potential (Ψ_s, D) of *Urtica dioica*. Data represent the mean ± standard deviation. The asterisk is used to represent a statistically significant result at $P < 0.05$.

Drought can induce oxidative stress in plants, resulting in increased levels of hydrogen peroxide (H_2O_2) and thiobarbituric acids reactive substances (TBARS). H_2O_2, a type of reactive oxygen species, has been shown to have a destructive effect on plants (Kaiser, 1979). Excessive accumulation of H_2O_2 can lead to increased TBARS in plant cells, which is indicative of oxidative damage to membrane lipids (Amoah et al., 2019; Killi et al., 2020). In this study, we found that drought stress significantly increased H_2O_2 and TBARS

levels in *U. dioica* by 33% and 25.3%, respectively, compared to control conditions (Figure 2A and B). Our results are consistent with previous studies that have reported high accumulation of H_2O_2 and TBARS content in *Oryza sativa* (Basu et al., 2010), *Solanum lycopersicum* (Rady et al., 2020), and *Triticum aestivum* (Hassan et al., 2020) under drought stress.

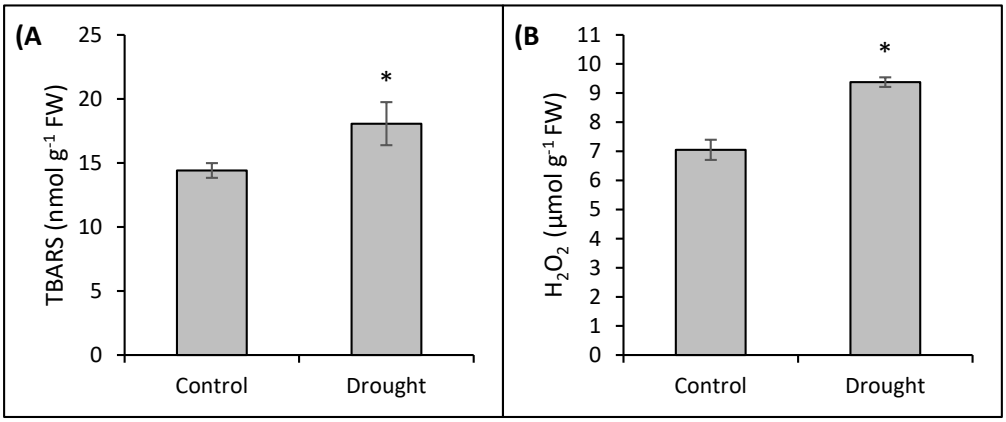

Figure 2. Effects of drought stress on lipid peroxidation (TBARS, A) and hydrogen peroxide (H_2O_2, B) of *Urtica dioica*. Data represent the mean ± standard deviation. The asterisk is used to represent a statistically significant result at $P < 0.05$.

Plants can suffer a variety of negative effects from drought stress, including osmotic and oxidative stress. As a result of drought, excessive production of ROS occur in chloroplasts and peroxisomes. Plants have antioxidant defence mechanisms to regulate the accumulation of ROS in plant cells. In order to determine whether drought affects the antioxidant defense system, antioxidant enzyme activities such as SOD, POX, CAT, APX and GR were examined in the current study. These enzymes protect plants from oxidative stress damage in adverse environments by reducing ROS levels in cells (Liu et al., 2014). SOD is a crucial enzyme that converts $O_2^{\cdot-}$ to H_2O_2 in the cell, reducing the likelihood of $^{\cdot}OH$ formation (Gill et al., 2015). Antioxidant enzymes must scavenge stress or dismutation-generated H_2O_2 (Mittler, 2002; Mittler et al., 2004). However, the activity of superoxide dismutase (SOD) not only scavenges superoxide to produce H_2O_2, but also contributes to the production of H_2O_2 in several compartments of plant cells through glycolate oxidase activity in peroxisomes, β-oxidation of fatty acids in glyoxysomes, and NADPH oxidase enzyme activity (Mittler et al., 2002; Hasanuzzaman et al., 2020). In our study, while SOD, CAT and GR activities were increased, APX activity was reduced (Figure 3). SOD and CAT were increased by 26% and 78.6%, respectively, as compared to control. Moreover, APX activity was reduced by 15.4%. The study suggests that an increase in H_2O_2 content due to drought may be linked to a decrease in APX activity and an increase in SOD activity. On the other

hand, POX activity did not show any significant change when compared to non-drought-treated control plants. Among antioxidant enzymes, enzyme activity increases were more pronounced in GR. When compared to control plants, GR activity was increased by 2.1-fold. In line with our findings, drought stress increased the SOD activity in various species such as alfalfa (Wang et al., 2009), tomato (Torre-González et al., 2017), and *Amaranthus tricolor* (Sarker and Oba, 2018). The increase in SOD enzyme activity might be one of the reasons for the strong defense in drought-treated *U. dioica*. CAT catalyses the conversion of H_2O_2 to water and is localised in peroxisomes (Mittler et al., 2004). Similar results were found in *Amaranthus tricolor* (Sarker and Oba, 2018), *Brassica napus* (Ayyaz et al., 2021), and pepper (Kaya, 2021) under drought stress, indicating enhanced activity of CAT and GR and improved protection against oxidative stress. A number of studies have shown a positive correlation between drought stress tolerance and the levels of antioxidant enzyme activities in the tissues of different plant species (Fan et al., 2022; Kaya and Shabala, 2023). Furthermore, it appears that the high activities of SOD, CAT, and GR in *U. dioica* leaves are adequate for maintaining the water status of the leaves.

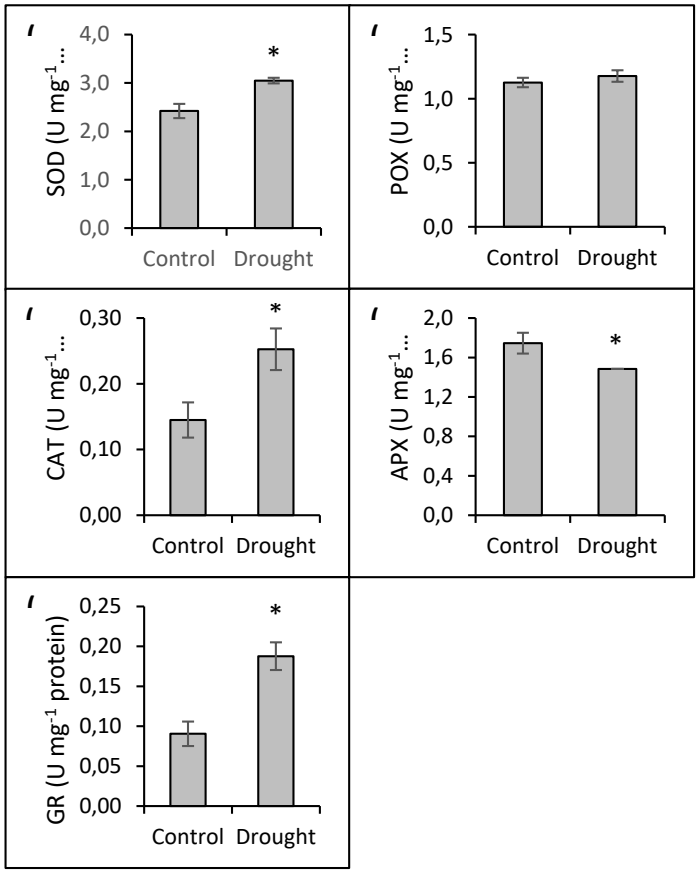

Figure 3. Effects of drought stress on superoxide dismutase (SOD, A), guaiacol peroxidase (POX, B), catalase (CAT, C), ascorbate peroxidase (APX, D) and glutathione reductase (GR, E) of *Urtica dioica*. Data represent the mean ± standard deviation. The asterisk is used to represent a statistically significant result at $P < 0.05$.

4. Conclusion

In our study, we found that drought stress had significant effects on the physiological and biochemical processes of *Urtica dioica*. Specifically, we observed a reduction in leaf relative growth rate, water content, osmotic potential, and chlorophyll fluorescence under drought conditions. Additionally, we noted an increase in H_2O_2 and TBARS levels, as well as an enhancement in SOD, CAT, and GR enzyme activities and a reduction in APX activities. The results indicate that the *Urtica dioica* plant exhibits efficient drought tolerance, as evidenced by increased antioxidant enzyme activities despite a slight decrease in leaf water status and chlorophyll fluorescence during drought. Future studies should investigate the involvement of non-enzymatic antioxidants, phytohormones, and other signal molecules in *Urtica dioica* under drought stress.

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