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Physiological and Antioxidative Responses of Endemic Plant *Seseli resinosum* Freyn & Sint. to Drought Stress

Endemik *Seseli resinosum* Freyn & Sint. Bitkisinin Kuraklık Stresine Fizyolojik ve Antioksidatif Tepkileri

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Abstract

Seseli resinosum Freyn & Sint. is an endemic perennial plant of rocky habitat of the Western Black Sea region of Turkey. To understand drought responses and tolerance mechanism of *Seseli resinosum* Freyn & Sint., relative water content (RWC), chlorophyll fluorescence, proline accumulation, lipid peroxidation (TBARS), hydrogen peroxide (H_2O_2) content and changes in antioxidant enzymes were assayed in polyethylene glycol (PEG) 6000 (5, 10 and 15%) induced drought stress in the present study. Leaf RWC maintained unchanged, while chlorophyll fluorescence reduced with a high PEG level (15%). Additionally, H_2O_2 and proline accumulation were determined with the increase of PEG application, but no increase in TBARS was determined. Moreover, the increment in H_2O_2 content under drought was accompanied by an increase glutathione reductase, catalase and superoxide dismutase activities. On the other hand, PEG-induced drought stress caused a reduction in peroxidase and ascorbate peroxidase activities. These results suggest that endemic *Seseli resinosum* Freyn & Sint. plant has an efficient drought tolerance, as displayed by enhanced antioxidant enzyme activities maintaining water status under drought conditions. In this study, important information about physiological and antioxidative responses of endemic *Seseli resinosum* Freyn & Sint. was revealed for the first time.

Keywords: Antioxidant enzymes, Drought stress, *Seseli resinosum* Freyn & Sint., Hydrogen peroxide

Özet

Seseli resinosum Freyn & Sint. Türkiye'nin Batı Karadeniz bölgesinin kayalık habitatına ait çok yıllık endemik bir bitkidir. Bu çalışmada, *Seseli resinosum* Freyn & Sint.'in kuraklığa olan tepkilerini ve tolerans mekanizmasını anlamak için bağıl su içeriği (RWC), klorofil floresansı, prolin birikimi, lipid peroksidasyonu (TBARS), hidrojen peroksit (H_2O_2) miktarı ve antioksidan enzim miktarındaki değişimleri kuraklık stresini teşvik eden polietilen glikol (PEG) 6000 (%5, 10 ve 15) varlığında analiz edilmiştir. Araştırma sonucunda, yapraktaki RWC değişmeden kalırken, klorofil floresansı yüksek PEG seviyesi (%15) ile azalmıştır. Ayrıca, PEG uygulamasının artmasıyla H_2O_2 ve prolin birikimi gözlenmiş, ancak TBARS miktarında artış belirlenmemiştir. Dahası, kuraklık altındaki H_2O_2 miktarındaki artış, glutatyon redüktaz, katalaz ve süperoksit dismutaz aktivitelerindeki artışa eşlik etmiştir. Diğer taraftan, PEG-teşvikli kuraklık stresi peroksidaz ve askorbat peroksidaz aktivitelerinde azalmaya neden olmuştur. Bu sonuçlar, endemik *Seseli resinosum* Freyn & Sint. bitkisinin, kurak şartlar altında antioksidan enzim aktivitelerindeki artışla su durumunu koruyarak etkili bir kuraklık toleransına sahip olduğunu göstermektedir. Bu çalışmada, endemik *Seseli resinosum* Freyn & Sint.'in fizyolojik ve antioksidatif tepkileri hakkında önemli bilgiler ilk kez ortaya konulmuştur.

Anahtar Kelimeler: Antioksidan enzimler, Kuraklık stresi, *Seseli resinosum* Freyn & Sint., Hidrojen peroksit

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1. Introduction

Seseli resinosum Freyn & Sint., belonging to Apiaceae family, is a perennial and endemic species that is widely distributed in the Western Black Sea region of Turkey (Davis et al., 1988; Duman et al., 2000). Because of its anti-inflammation effects, various vegetative and generative parts of this species have been used in traditional medicine (Kaya et al., 2003). Kupeli et al. (2006) reported that the seeds of *Seseli resinosum* Freyn & Sint. had anthelmintic, carminative, stomachic and stimulant features. Moreover, secondary metabolites such as coumarins (Tosun et al., 2006), essential oils (Dogan et al., 2006), anomalin and deltoin (Tosun et al., 2007) were isolated from *Seseli resinosum* Freyn & Sint. However, the impact of undesirable environmental conditions in *Seseli resinosum* Freyn & Sint. have not been still conducted.

Plants are exposed to many abiotic stress factors such as drought, salinity, chemical pollution, high and low temperature, which reduce the amount and quality of crops. Water scarcity is one of these factor having devastating impact on humans and environment and cause drought stress. Drought is the primary factor, which negatively affect plant growth and development and cause crop losses, also trigger secondary stress factors such as osmotic, ionic and oxidative stress (Mahajan and Tuteja, 2005). 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). Oxidative stress accompanying drought stress causes the formation of ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) (Mattos and Moretti, 2015). So, antioxidant enzymes (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 (glutathione, ascorbic acid, carotenoids and tocopherols) are activated for detoxifying of ROS to protect plant cellular mechanisms (Mittler, 2002; Gill and Tuteja 2010; Hasanuzzaman et al., 2020).

Therefore, no data is available on the antioxidant defense system power of *Seseli resinosum* Freyn & Sint., the aim of this study was to examine the physiological and biochemical features under drought stress. For this purpose, the relative water content, chlorophyll fluorescence, proline accumulation, lipid peroxidation and hydrogen peroxide content and antioxidant enzyme activities such as APX, CAT, GR, POX and SOD of this species were determined under drought.

2. Material and Method

2.1. Growth Conditions and Treatment Applied

Seseli resinosum Freyn & Sint. seeds were collected from the plant's natural habitat on disturbed ground in an open rocky area located in Gölyaka, Düzce Province (Latitude 40º44′08″E, Longitude 31º03′28″N) (Fig. 1). The seeds were surface sterilized with 5% NaOCl and rinsed with dI-H_2O for removing the bleach. Then, 16-cm pots filled with peat + perlite + river sand, was considered 1:1:1, and the seeds were sown into those pots. The seedlings were grown in a controlled greenhouse at 27/22 °C (day/night; 16/8 h) at relative humidity of 70%. After four months, drought stress treatments were started. The drought stress groups consisted of a control and 5, 10, and 15% polyethylene glycol (PEG) 6000-treated plants. The experimental design comprised a randomized block with three replicates, and each replication had ten seedlings (30 seedling for each individual treatment). After 21-day drought period, harvest period started. The 3rd and 4th fully grown leaves were took and immediately frozen in liquid nitrogen and stored at -86 °C until further analysis.

Figure 1. *Seseli resinosum* Freyn & Sint. (Photo; Aydin, H.)

2.2. Relative Water Content (RWC) and Chlorophyll Fluorescence (Fv/Fm)

Seven leaves from each group during the harvest were weighed and fresh weights were recorded. For turgid weight determination, leaves were put in water for at least 10 h. After that, turgid leaves were dried for 72 h at 70°C and dry weights were obtained. The following formula was utilized for the calculation of RWC of leaves:

RWC (%) = ((Fresh weight-Dry weight)/(Turgid weight-Dry weight)) x 100

Chlorophyll fluorescence was measured according to the manufacturer's instructions. Seven leaves from each group were used for analyses. After the leaves adapted to the dark, Fv/Fm was measured with Plant Efficiency Analyzer of Hansatech (UK).

2.3. Lipid Peroxidation, H_2O_2 and Proline Content

Lipid peroxidation (TBARS) level were determined according to the method of Heath and Packer (1968). Fresh leaves were extracted in trichloroacetic acid (TCA; 0.1%) and then centrifuged at 12000 g for 15 min at 4°C. Supernatant was mixed with 20% TCA with 0.5% thiobarbituric acid. After 30 min at 95°C, samples were cooled. The absorbance for TBARS was recorded at 532 and 600 nm.

H_2O_2 level were determined according to the method of Liu et al. (2000). Fresh leaves were extracted in TCA (1%) and then centrifuged at 12000 g for 15 min at 4°C. $TiCl_4$ solution prepared with H_2SO_4 (20%) was mixed with supernatant. 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.

The accumulation levels of free proline were determined according to the method of Bates et al. (1973). Acid-ninhydrin method was used and leaf samples were homogenized in sulphosalycylic acid. Then the supernatant of this extract was mixed with equal amounts of acid-ninhydrin and glacial acetic acid solutions. The proline contents were determined using a standard curve prepared on a UV-VIS spectrophotometer and the absorbance values were recorded at 520 nm.

2.4. Antioxidant Enzyme Assays

Fresh leaves were ground with liquid nitrogen and 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. Supernatants were used for protein and enzyme activity assays. Estimation of protein from extracts was carried out by bovine serum albumin method (Bradford, 1976).

The procedure of Beauchamp and Fridovich (1971) was used for the activity of SOD. The reaction mixture contained phosphate buffer (50 mM; pH 7.0), 13 mM methionine, 0.1 mM EDTA, 0.075 mM nitro blue tetrazolium (NBT) and 2 µM riboflavin. The absorbance was recorded at 560 nm. One unit of the activity was defined as the quantity of enzyme required to produce 50% inhibition of NBT. The procedure of Mika and Lüthje (2003) was used for the activity of POX. Sodium acetate (25 mM; pH 5.0), 10 mM guaiacol and 10 mm H_2O_2 were used for the reaction mixture. The absorbance was recorded at 470 nm. One unit of the activity was defined as the amount required to decompose 1 µmol H_2O_2 per min^{-1}. The procedure of Aebi (1984) was utilized for the activity of CAT. Phosphate buffer (50 mM; pH 7.0) and 10 mM H_2O_2 were used for the reaction mixture. The absorbance was recorded

at 240 nm. One unit of CAT activity was defined as the amount needed to decompose 1 µmol H_2O_2 per min^{-1}. The procedure of Nakano and Asada (1981) was used for the APX activity. Phosphate buffer (50 mM; pH 7.0), 250 µM ascorbate and 5 mM H_2O_2 were used for the reaction mixture. The absorbance was recorded at 290 nm. One unit of APX was defined as the amount needed to oxidize 1 µmol ascorbate per min^{-1}. The procedure of Foyer and Halliwell (1976) was utilized for the GR activity. Tris-HCl buffer (50 mM; pH 7.6), 5 mM NADPH and 10 mM oxidized glutathione were used for the reaction mixture. The absorbance was recorded at 340 nm. One unit of GR was defined as the amount required to reduce 1 µmol oxidized glutathione per min^{-1}.

2.5. Statistical Analysis

Statistical analyses for all data obtained in this study were carried out using the analysis of variance and the significant differences among all treatments were compared using Duncan's Multiple Range test at the $P < 0.05$ probability level. The SPSS 22.0 (IBM™) software was used for all the analyses. The results were expressed as means and error bars were used to show standard error of the mean (± SEM).

3. Results and Discussion

This study was mainly objected to evaluate the antioxidant defense system power of *Seseli resinosum* Freyn & Sint. Previous studies about this endemic species have focused on its composition of secondary metabolites isolated from aerial parts and roots. Essential oil composition (Dogan et al., 2006), coumarins (Tosun et al., 2006) and anti-inflammatory properties (Khan et al., 2014) of *Seseli resinosum* Freyn & Sint. were reported. However, ROS detoxifying and antioxidant defense system interactions are still need further explanation for this species under drought stress. So, in the present study, antioxidant defense system in terms of physiological and biochemical approaches under drought was studied in *Seseli resinosum* Freyn & Sint.

Drought stress primarily causes a decline in plant water content (Shivakrishna et al., 2018) and growth (Mårtensson et al., 2017; Sun et al., 2020; Kaya, 2021). Growth of *Seseli resinosum* Freyn & Sint. in terms of leaf length, fresh and dry weight was reduced under drought as compared to non-stressed plants and it was reported in our previous study (Aydin et al., 2020). This reduction can be also seen as morphologically in Figure 2. Similar to our results, the findings for tomato (Rady et al., 2020), wheat (Hassan et al., 2020) and pepper (Kaya, 2021) support our remarks in terms of drought-induced reduction in plant growth. A

possible reason of reduction in growth might be related the reduction of water uptake and loss of turgor under drought stress (Ings et al., 2013). However, *Seseli resinosum* Freyn & Sint. maintained leaf relative water content (RWC) under drought (Figure 3A). *Seseli resinosum* Freyn & Sint. may have preserved the leaf water status under drought by synthesizing osmolytes that can easily replace water in the cytoplasm. In our study, chlorophyll fluorescence value, expressed as Fv/Fm, was measured to elucidate the effects of drought stress on the photosystem II (PSII) apparatus. Fv/Fm of this endemic species was reduced by 3.8% as compared control plants at 15% concentrations of PEG (Figure 3B). The reduction observed in the photosynthetic efficiency of *Seseli resinosum* Freyn & Sint. might also mean a reduction in stomatal conductivity. Thus, it can be associated with a reduction in CO_2 uptake through PSII activity and stomatal conductivity (Seeman and Critchley, 1985). Another possible reason for the decrease in the photosynthesis efficiency of *Seseli resinosum* Freyn & Sint. may be the decrease in leaf growth parameters and the corresponding decrease in the number of chloroplasts per unit area.

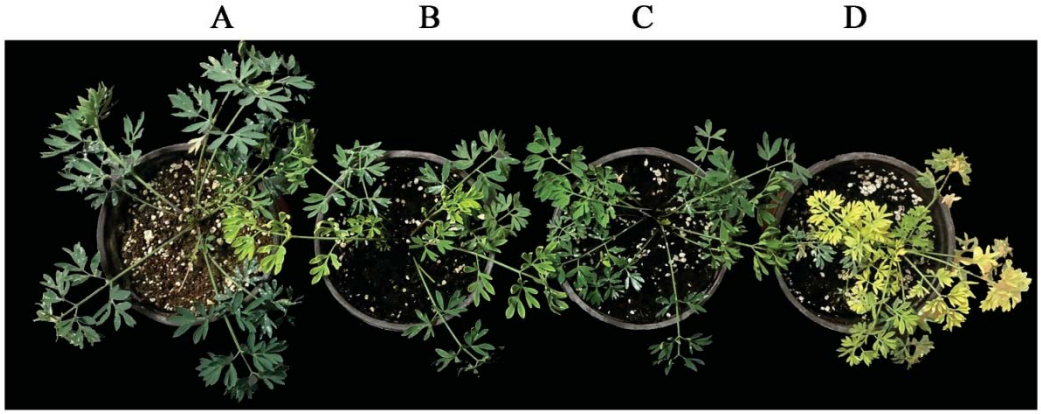

Figure 2. Effects of PEG-induced drought on morphology of *Seseli resinosum* Freyn & Sint. (**A:** Control, **B:** 5%, **C:** 10%, **D:** 15%)

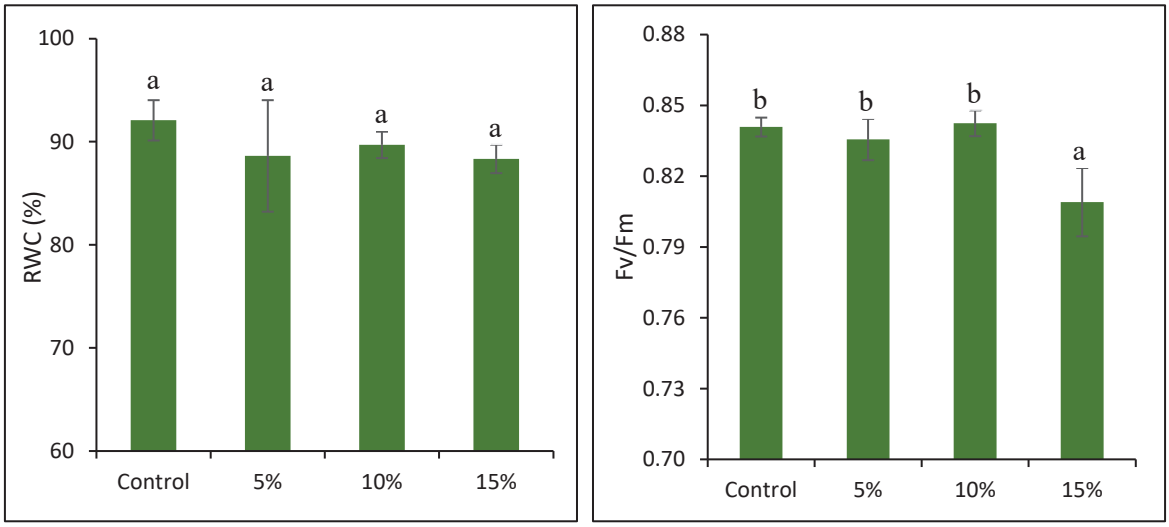

Figure 3. Effects of PEG-induced drought on relative water content (RWC, A) and chlorophyll fluorescence (Fv/Fm, B) of *Seseli resinosum* Freyn & Sint. Data represent the mean ± standard deviation (n = 6). The same letters within each column are not significantly different at $P < 0.05$.

Drought stress lead to the generation of ROS. Among the ROS, H_2O_2 shows the most destructive effect on plants (Kaiser, 1979) and excessive accumulation of H_2O_2 which caused an increase in TBARS content as an indicator of oxidative damage in membrane lipids (Amoah et al., 2019; Killi et al., 2020). In our study, TBARS content didn't increase in *Seseli resinosum* Freyn & Sint. leaves under drought stress (Figure 4A), while H_2O_2 content increased 6.7-, 16- and 16.2-fold, respectively, at 5, 10 and 15% PEG6000 treatment as compared to non-treated control plants. Similar to our results, high accumulation H_2O_2 were detected in *Oryza sativa* (Basu et al., 2010), *Solanum lycopersicum* (Rady et al., 2020) and *Triticum aestivum* (Hassan et al., 2020) under drought. Moreover, proline accumulation is one of the main effect of drought stress to take more water from growth medium (Sadak et al., 2019). In addition, proline as an osmolyte play a role in cell protection against ROS accumulation under stress conditions (Verbruggen and Hermans, 2008). In the present study, drought-induced proline accumulation to preserve the water content within the plant was also detected in *Seseli resinosum* Freyn & Sint. leaves, in accordance with previous studies (Ashraf and Foolad, 2007; Jungklang et al., 2017; Kaya, 2021). Proline content increased by 2.5-fold at 15% PEG treatment as compared to control.

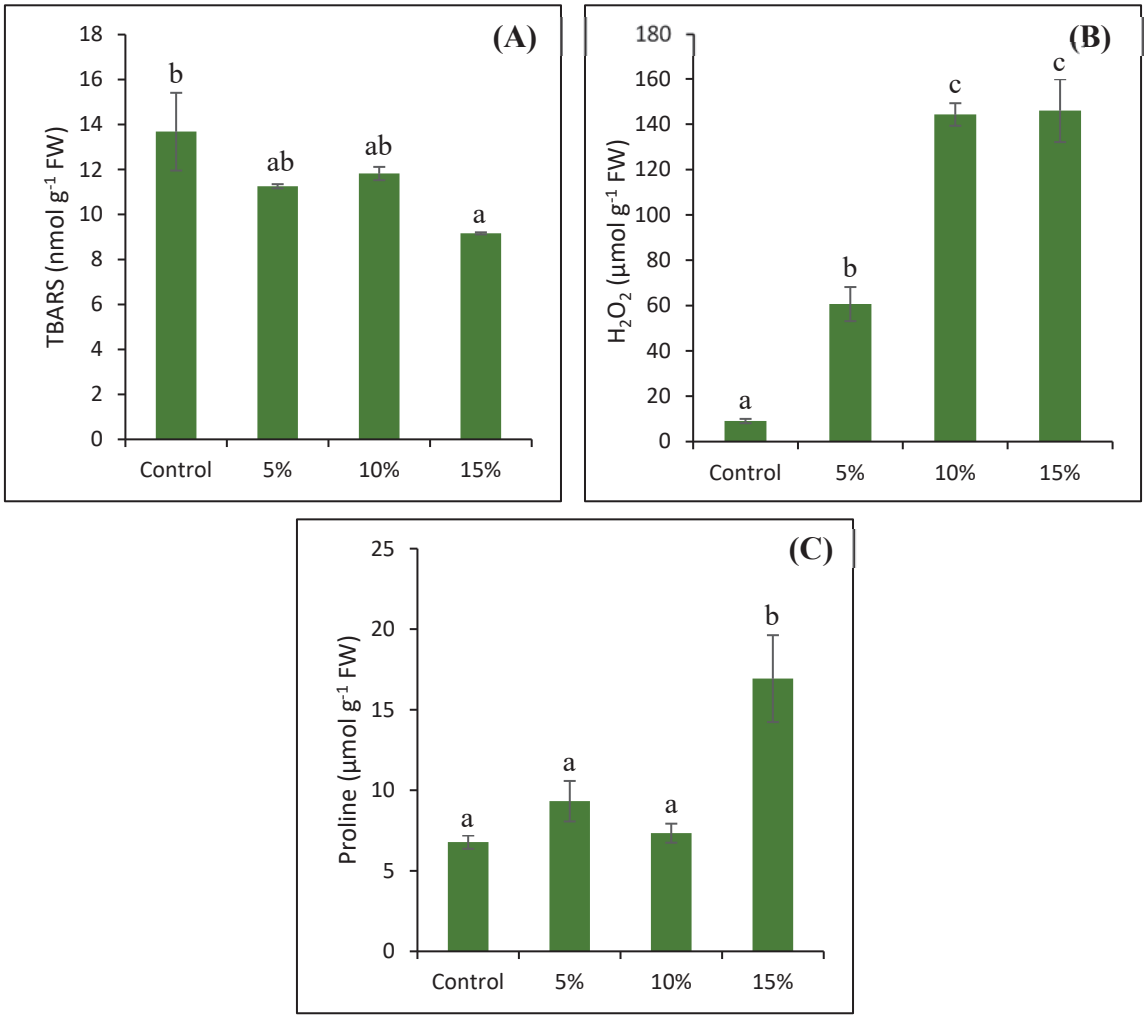

Figure 4. Effects of PEG-induced drought on lipid peroxidation (TBARS, A), hydrogen peroxide (H_2O_2, B) and proline (C) of *Seseli resinosum* Freyn & Sint. Data represent the mean ± standard deviation (n = 6). The same letters within each column are not significantly different at $P < 0.05$.

Drought stress limits gas exchange in plants and excessive ROS production is observed in chloroplasts and peroxisomes. This increase in ROS also promotes the enzymatic and non-enzymatic antioxidant defense system. APX, CAT and SOD enzymes are the main ROS scavenging enzymes in the defense process keeping plant cells in oxidative balance (Mittler, 2002). The decrease in the efficiency of CO_2 fixation with stress causes both the deterioration of the balance between light and carbon reactions in chloroplasts, and an increase in photorespiration with O_2 binding by RuBisCO instead of CO_2. In chloroplasts, this situation is tried to be eliminated with antioxidant enzymes such as SOD and APX which is known as the water-water cycle (Rizhsky et al., 2003).

SOD is a key enzyme that catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 in the cell and reduces the possibility of $^{\cdot}OH$ formation (Gill et al, 2015). H_2O_2 generated by stress or

dismutation activity must be scavenged antioxidant defense system enzymes (Mittler, 2002; Ozfidan-Konakci et al., 2015). However, SOD activity is not only the source of H_2O_2 by scavenging of superoxide, but also glycolate oxidase activity in peroxisomes, β-oxidation of fatty acids in glyoxysomes, NADPH oxidase enzyme activity also lead to produce H_2O_2 in several compartments of plant cells (Mittler et al., 2002; Hasanuzzaman et al., 2020). In our study, the greatest increase in SOD activity in *Seseli resinosum* Freyn & Sint. was determined by 43.8% at 15% PEG treatment, while the SOD activity showed a slight decrease by 13.8 and 17.5% at 5 and 10% PEG treatment, respectively, as compared to the control (Figure 5A). Similar to our findings, drought stress enhance the SOD activities of various species such as alfalfa (Wang et al., 2009), tomato (Torre-González et al., 2017), and *Amaranthus tricolor* (Sarker and Oba, 2018). SOD enzyme activity increases might be one of the reason of the strong defence in drought-treated *Seseli resinosum* Freyn & Sint. plants. Besides the increase in SOD activity, drought stress caused a decrease in POX and APX activities, while it lead to increase in CAT and GR activities in *Seseli resinosum* Freyn & Sint. leaves (Figure 5). POX activity decreased by 25.5, 58.8 and 11.8% at 5, 10 and 15% PEG treatment, respectively, as compared to control (Figure 5B). APX catalyzes the scavenging of H_2O_2 using ascorbate as an electron donor (Asada and Takahashi, 1987). Similar to POX activity, APX activity in *Seseli resinosum* Freyn & Sint. also decreased, but this reduction was more pronounced (44.1%) at 15% PEG treatment (Figure 5D). Drought-induced increase in H_2O_2 content in this study can be possible with the decrease in POX and APX activities due to the increase in SOD activity. The CAT catalyzes the conversion of H_2O_2 into water and localized in peroxisomes (Mittler et al., 2004). In our study, CAT activity in *Seseli resinosum* Freyn & Sint. plant decreased by 32.7% with 5% PEG application, and increased by 42.3% with 10% PEG application under drought stress, while no statistically significant change was detected at 15% PEG application, as compared with the control (Figure 5C). Moreover, like CAT, GR activity increased by 25%, 2.5-fold and 2-fold at 5, 10 and 15% PEG treatment, respectively, as compared to control plants (Figure 5E). Similar results related to enhanced activity of CAT and GR and improved protection against oxidative stress were obtained in *Amaranthus tricolor* (Sarker and Oba, 2018), *Brassica napus* (Ayyaz et al., 2021), and pepper (Kaya, 2021) under drought stress. Moreover, high SOD, CAT and GR activities in *Seseli resinosum* Freyn & Sint. leaves seems to be sufficient to catalyze the destruction of H_2O_2 as shown by decreased lipid peroxidation with the low amount of TBARS under PEG-induced drought stress. Similarly, more efficient

antioxidative defence system between lower TBARS accumulation was found in wheat (Abid et al., 2018) and rapeseed (Ayyaz et al., 2021) supporting our findings.

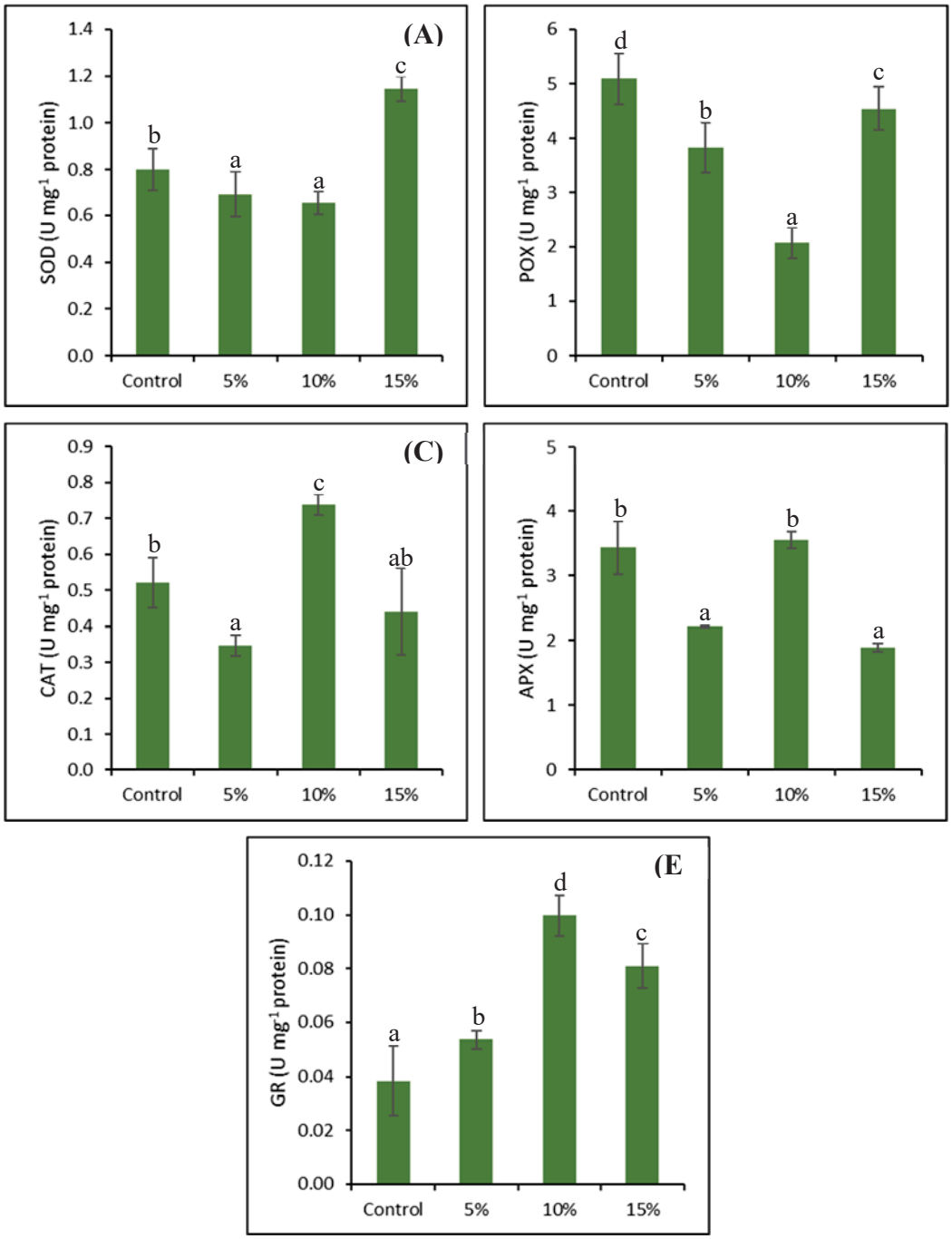

Figure 5. Effects of PEG-induced drought on superoxide dismutase (SOD, A), peroxidase (POX, B), catalase (CAT, C), ascorbate peroxidase (APX, D) and glutathione reductase (GR, E) of *Seseli resinosum* Freyn & Sint. Data represent the mean ± standard deviation (n = 6). The same letters within each column are not significantly different at $P < 0.05$.

4. Conclusion

Overall, in our study, polyethylene glycol (PEG) 6000-induced drought stress caused responses in physiological and biochemical processes in *Seseli resinosum* Freyn & Sint. were obtained. Leaf relative water content remained unchanged, while chlorophyll fluorescence was significantly reduced with 15% PEG. H_2O_2 and proline accumulation were increased. Moreover, enhancement in SOD, CAT and GR enzyme activities and reduction in POX and APX activities were determined under drought stress. These results suggest that endemic *Seseli resinosum* Freyn & Sint. plant have an efficient drought tolerance, as displayed by enhanced antioxidant enzyme activities with maintaining water status and lowering lipid peroxidation under drought. In the future, the participation of non-enzymatic antioxidants, phytohormones or other signal molecules required to be studied in *Seseli resinosum* Freyn & Sint. under drought stress.

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