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Effects of Melatonin on *Morus nigra* cv. 'Eksi Kara' Exposed to Drought Stress

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ABSTRACT

Today, drought stress threatens the world seriously. Determining the effects of some exogenous stimulators in acquiring resistance against stress will contribute to agriculture under drought stress. In this regard, we investigated the effects of melatonin (MEL) on *Morus nigra* cv. 'Eksi Kara' (black mulberry) in challenging drought. To reach this object, we reproduced 'Eksi Kara', which is registered in Turkey and has economic importance, in tissue culture by using the meristem culture method. Plants were then transferred in a medium containing polyethylene glycol (PEG) 8000, which causes -1.5 MPa drought stress, and 20 µl MEL has applied. Leaf samples were taken on the 5th, 10th, and 15th days after treatments in groups of plants grown in a different medium (Control, Control+MEL, PEG and PEG+MEL). The changes in the pigment system, relative water content (RWC) and antioxidant system were evaluated comparatively between the groups to assess plants' growth and determine their roles in coping with stress. Our findings showed that RWC decreased in leaves under drought. Exogenous MEL added in MS medium had a mitigation effect on stress. The reduction was detected in the chlorophyll and carotenoid content of leaves. Moreover, MEL+PEG combination improved the chlorophyll level. It was seen that exogenous MEL application promoted the plant defence mechanism of *M. nigra* plants, which exposed to drought stress, by increasing the accumulation of non-enzymatic antioxidants; total glutathione (GSH), total phenolic, proline) and activities of antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD), Glutathione-S-transferase (GST), glutathione reductase (GR), peroxidase (POD), ascorbate peroxidase (APX). This study also indicates that the application of MEL+PEG composition partially prevented membrane lipid peroxidation by decreasing (malondialdehyde) MDA content.

Keywords: Black mulberry, Malondialdehyde, Pigment, Proline, Relative water Content, Total phenolic

1. Introduction

Abiotic stress is the primary cause of yield loss worldwide, and it corresponds to more than 50% of yield loss in high-yield cultivated plants (Wang et al. 2004). Drought is considered one of the significant abiotic stresses, and it alters the average growth balance in plants negatively. It also damages plants' growth and productivity by causing a series of morphological, physiological, biochemical, and molecular changes (Pandey et al. 2017). Researches on obtaining drought-resistant plant varieties are critical in challenging drought.

Morus nigra L. (black mulberry) is in Urticales order Moraceae family and *Morus* genus. *Morus* is widely cultivated, particularly in East, West, Southeast Asia, Southern Europe, the south of North America, the northwest of South America, and some parts of Africa (Datta 2002). Mulberry can be grown in temperate and subtropical climates due to its high ability to adapt to different climatic and soil conditions. Therefore, it has been reported that mulberry will have significant potential bio-energy plants in a rapidly changing global climate under drought conditions (Sekhar et al. 2017).

Mulberry in Turkey is cultivated as a closure and mixed garden or border plant. The fact that urbanization damages the genetic diversity of the mulberry made the protection of varieties critical (Vijayan et al. 2011). Narrowing of agricultural lands and the inverse effect of stress on environmental conditions are reasons for decreased productivity. Thus, it is very substantial to increase productivity in vegetative production, which is achieved by breeding or selecting resistant plants to stress conditions (Arici & Eraslan 2012). Sugar, organic acids, minerals, anthocyanins, and vitamins included in black mulberry make it an essential source of nourishment. Most of the daily calcium, iron, B, and C vitamin requirements can be met by eating black mulberry (Hepsag et al. 2012; Wang et al. 2021). It has also been used in traditional herbal medicine for both animals and humans. It exhibited anti-inflammatory, antimicrobial, anti-diabetic, anti-obesity, and anticancer properties (Lim & Choi 2019;

Erden 2021; Mehta & Kumar 2021). In this regard, it is essential to determine the drought resistance of *M. nigra*, which has medical and economic importance.

Avoidance and tolerance are two primary defence mechanisms that allow the plant to survive under drought stress (Zheng et al. 2017a). Drought tolerance is the ability to keep the plant tissue's physiological and metabolic activities at lower levels when the water potential within the plant decreases (Blum & Ebercon 1981). Plants under drought stress have an effective defence system that uses enzymatic antioxidant systems consisting of antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and Glutathione-S-transferase (GST) as well as a non-enzymatic antioxidant system including some secondary metabolites such as ascorbic acid, GSH, flavonoids, and total phenols to mitigate the deleterious effects of reactive oxygen species (ROS) (Cheng et al. 2018).

Melatonin (MEL) has long been considered an important antioxidant or hormone in animals (Reiter 1991). Recently, it has been proven that endogenous MEL is widely found in bacteria, fungi, animals, and plants (Lin et al. 2019; Qiao et al. 2019). Also, MEL is recognized as a potential growth stimulator in plants (Arnao & Hernández-Ruiz 2019; 2020). MEL acts as the primary regulator by increasing tolerance to biotic and abiotic stresses such as salinity, drought, extreme temperatures, high light intensity, herbicides, ultraviolet radiation (Zhang et al. 2012; Wang et al. 2017; Jahan et al. 2019; Ahammed et al. 2020a; Moustafa-Farag et al. 2020). A compound's qualification as a plant hormone It must be formed within the plant, transported from where it is formed to another location, and be able to manage or regulate various life events in the location where it is transported. It must be able to show these effects even at very low concentrations (Kaynak & Ersoy 1997). MEL can dissolve both in water and lipids and easily reach all intracellular components (Posmyk & Janas 2009). It can effectively protect the cell membrane, organelles, and nucleus from free radicals' devastating effect. Poeggeler et al. (2002) reported that MEL is also a more potent antioxidant than vitamins C, E, and K. According to our literature exploration on the effects of MEL on the antioxidant system, we could not find any study of applications to plants in the form of trees. However, there were many kinds of research conducted with herbaceous plants.

In this study, *M. nigra* cv. 'Eksi Kara', which has economic and medical importance, was reproduced in tissue culture, and groups were formed, in which MEL and drought stress (-1.5 MPa) was applied, *in vitro*. Also, pigment content, RWC, GSH, total phenol, proline content, and antioxidant enzyme activities (CAT, SOD, GST, GR, POD, and APX) were evaluated comparatively in both groups.

2. Material and Methods

2.1. Plant growth in tissue culture

The *M. nigra* cv. 'Eksi Kara' explants used in the research were obtained from the National Mulberry Gene Resources parcel located in in the Republic of Turkey, Ministry of Agriculture and Forestry, Malatya Apricot Research Institute. 'Eksi Kara' is the only *M. nigra* variety registered in the Seed Registration and Certification Center, and its fruits are economically and pharmacologically important. Explants were taken from nodal buds on one-year shoots in June, July, and August in 2017-2019. Explants were reproduced in MS medium (Murashige & Skoog 1962, M0222 Duchefa) after surface sterilization. In the first phase of culture, 0.75 mg L^{-1} benzyl amino purine (BAP) was used, then 1 mg.L^{-1} BAP for shoot propagation, and 1.5 mg.L^{-1} indole butyric acid for rooting.

2.2. Drought and melatonin application

Plants were grown over a period of 5 months prior to application. Following applications were made to the plants rooted *in vitro* tissue culture: **Control:** Liquid (agar-free) MS medium was used. **Control+MEL:** The plants rooted under *in vitro* conditions were transferred to a liquid MS medium containing 20 µM MEL. The MEL dose was chosen as a result of preliminary evaluations based on reference studies (Korkmaz et al. 2016; Han et al. 2017; Zheng et al. 2017b). **PEG group:** Plants were transferred to a liquid MS medium containing PEG 8000, a polymer that retains water, to provide the drought condition. To reach -1.5 MPa drought, 355 g.L^{-1} PEG 8000 was added to the liquid MS medium according to the formula developed by Michel (1983). **PEG+MEL group:** Plants were transferred to liquid MS medium containing 20 µM MEL and 355 g L^{-1} PEG 8000.

$$PEG = \frac{4 - \sqrt{(5.16\,\Psi\,T - 560\,\Psi + 16)}}{(2.58\;T - 280)} \times 1000$$

Ψ= -1.5 MPa T= 25 ºC

2.3. Samples for analysis

The leaves to be used in the analysis were taken on the 5th, 10th, and 15th days after the applications. Samples were frozen in liquid nitrogen and preserved at -40 °C to be used for analysis. They were studied as soon as possible to eliminate the risk of loss of enzymatic activity. Fresh leaves were used without freezing to evaluate the relative water content.

2.4. Determination of relative water content

Fresh weights (FW) of the leaf samples were determined. Then, these leaves were kept in distilled water for 4 hours, and their turgor weights (TW) were determined. After drying for 48 hours in a 70 °C drying oven, the dry weights (DW) of samples were weighed. (Sanchez et al. 2004; Demiral & Turkan 2005).

2.5. Determination of total chlorophyll and carotenoid content

The method of De Kok & Graham (1980) was used for pigment content. In the first step, 1 g sample taken from the leaf was homogenized in 50 mL acetone (100% Merck) and kept at +4 °C for 24 hours. Homogenates were then centrifuged, and their absorbance values were calculated according to Lichtenthaler & Welburn (1983) with spectrophotometric reading (Biochrom Libra S22) at 662, 645, 470 nm.

2.6. Extraction for enzyme analysis

To analyze enzyme, 0.5 g leaves were homogenized with 2.5 mL 0.1 M, pH 7.5, Tris-HCl buffer, 2.5 mL 0.1mM EDTA and 0.5 mL 1% PVP. The homogenate was centrifuged at +4 °C and 18.000 rpm for 30 minutes. Then, the supernatant was stored at -40 °C until analysis (Andrews et al. 2005). Enzyme values were calculated in terms of specific activity by dividing by total protein.

2.7. CAT activity

The method of Luck (1963) was used to determine CAT enzyme activity. CAT enzyme activity was defined as the absorbance change obtained in 1 minute by reading at 240 nm in the spectrophotometer (molar extinction coefficient for H_2O_2 is 0.0396 $cm^2.\mu mol^{-1}$).

2.8. SOD activity

SOD enzyme activity determination was made using the method of McCord & Fridovich (1969). The SOD activity was measured spectrophotometrically at 550 nm.

2.9. GST activity

The method of Habig et al. (1974) used to assign GST enzyme activity. Enzyme activity was determined as 344 nm in the spectrophotometer (the extinction coefficient of CDNB is 9.6 $mM^{-1}.cm^{-1}$).

2.10. GR activity

Determination of GR activity was made according to Carlberg & Mannervik (1985) method. Enzyme activity was measured in the spectrophotometer at 340 nm wavelength. NADPH was calculated with an extinction coefficient of 6.2 $mM^{-1}.cm^{-1}$.

2.11. GSH content

Assignation of total glutathione content was made according to the method of Akerboom & Sies (1981). One-minute absorbance change was determined at 412 nm.

2.12. POD activity

The method of Peters et al. (1989) and MacAdam et al. (1992) was performed for POD activity determined with modification. The change in enzyme activity in the first minute was measured at the wavelength of 436 nm (Guaicol's extinction coefficient is 26.6 $mM^{-1}.cm^{-1}$).

2.13. APX activity

The methods of Nakano & Asada (1981) and Cakmak (1994) were used for APX activity determination. Enzyme activity was determined at 290 nm in the spectrophotometer, and APX activity was calculated with an extinction coefficient of 2.8 $mM^{-1}.cm^{-1}$.

2.14. Determination of MDA content

MDA analysis was made according to the method of Heath & Packer (1968). The absorbance of the supernatant was measured at 532 and 600 nm. The content of MDA was calculated with an extinction coefficient of 155 $mM^{-1}.cm^{-1}$.

2.15. Determination of total phenolic

Slinkard & Singleton's (1977) and Chandler & Dodds' (1983) method used to determine the total phenolic determination. It was measured at 760 nm in the spectrophotometer, and a standard curve was prepared with the gallic acid solution.

2.16. Determination of proline content

Proline determination was made spectrophotometrically by the acid-ninhydrin method according to Bates et al. (1973). The absorbance was measured at a wavelength of 520 nm. The same method was repeated using proline, and a standard graphic was created.

2.17. Total protein content

Total protein content was determined using the method of Bradford (1976). 50 µL of homogenate prepared from leaves was added onto 1000 µL Bradford reagent. It is measured in a spectrophotometer at a wavelength of 595 nm.

2.18. Statistical analysis

SPSS (Statistical Program in Social Sciences) V.25 software was used to evaluate this study's data. The data's compliance with normal distribution was checked with the Shapiro – Wilk test, and the homogeneity of variances was controlled with the LEVENE test at the 5% threshold. Duncan's multiple range test (Duncan 1955) was performed for paired comparisons of groups, and variance analysis (ANOVA) was made. Differences between the means were showed with different letters (P>0.05). Statistically same groups were showed with the same letters (P>0.05). Lower case letters show the comparison between averages in days (within groups), upper case letters show the comparison between groups. Each stage (5th, 10th, and 15th days) was compared within itself with Duncan's multiple range test.

3. Results

3.1. Relative water content

It was found that RWC decreased in the application of PEG to *M. nigra* leaves compared to the Control (P<0.05). The highest RWC was 64.11% in the Control+MEL group, while the lowest RWC was 21.11% in the PEG group. Moreover, there was a substantial decrease in RWC in different PEG group days (P<0.05) (Figure 1).

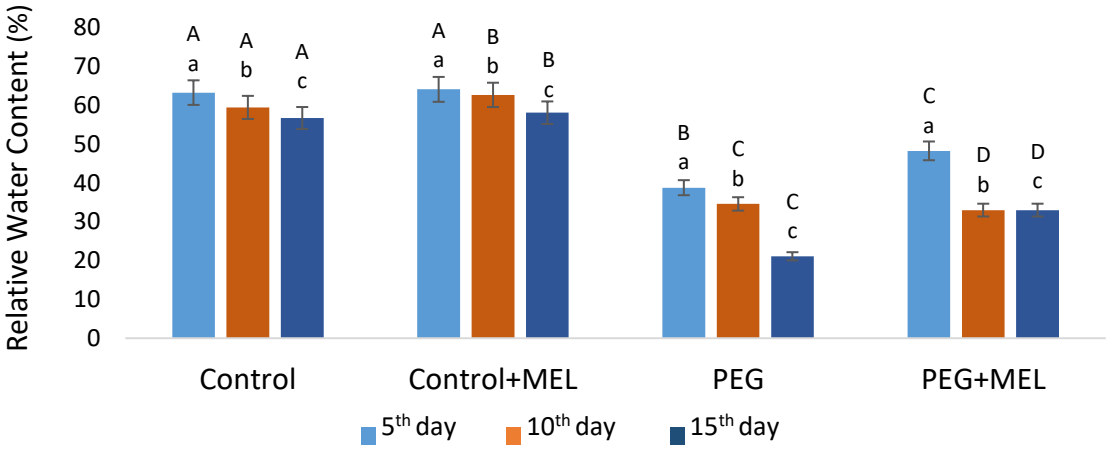

Figure 1- Relative water content changes in *M. nigra* leaves depending on groups and days

3.2. Total chlorophyll and carotenoid content

The total chlorophyll content was lower in all treatment groups than in the Control (P<0.05). The highest total chlorophyll content was 11.43 µg.g^{-1} in the control group, while the lowest was 4.54 µg.g^{-1} in the PEG group. Higher total chlorophyll content was observed in the PEG+MEL group compared to the PEG group. These differences were found to be statistically significant (P<0.05) (Figure 2). The carotenoid content was lower in all treatment groups than in the control group (P<0.05). The highest carotenoid content was observed as 2.57 µg.g^{-1} in the Control+MEL group, while the lowest was 1.46 µg.g^{-1} in PEG+MEL group. PEG+MEL group were determined lower than the PEG group in terms of carotenoid content (P<0.05) (Figure 3).

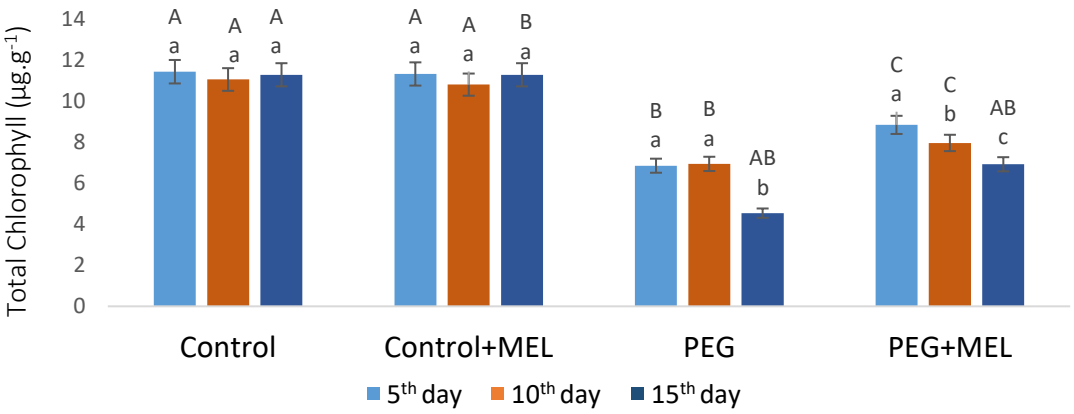

Figure 2- Variation of total chlorophyll content of M. nigra leaves depending on groups and days

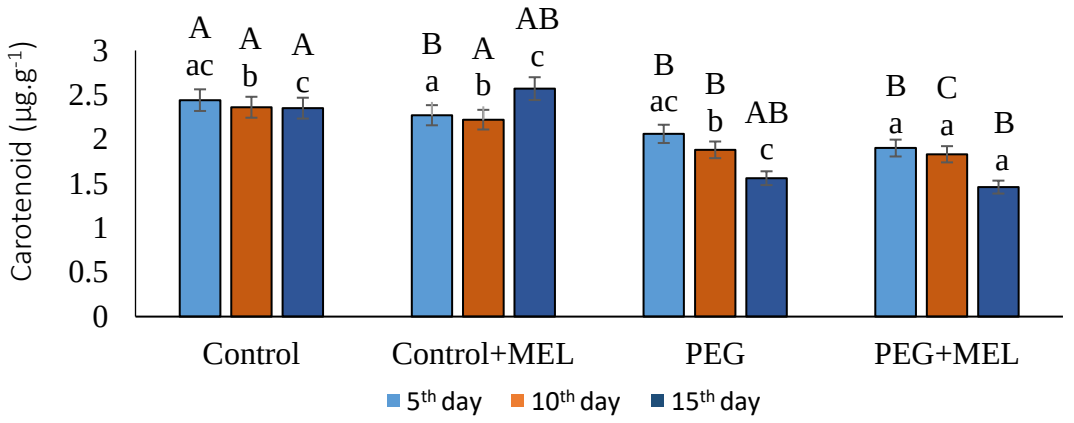

Figure 3- Variation of carotenoid content of M. nigra leaves depending on groups and days

3.3. CAT activity

CAT activity increased in all groups treated than the Control group (P<0.05). The highest enhancement in PEG+MEL group was found by 40% on the 10th day and 57% on the 15th day than the PEG group (P<0.05). On the other hand, CAT activity improved by 19% on the 5th day and 39% on the 10th day in Control+MEL group compared to the Control group (P<0.05) (Figure 4).

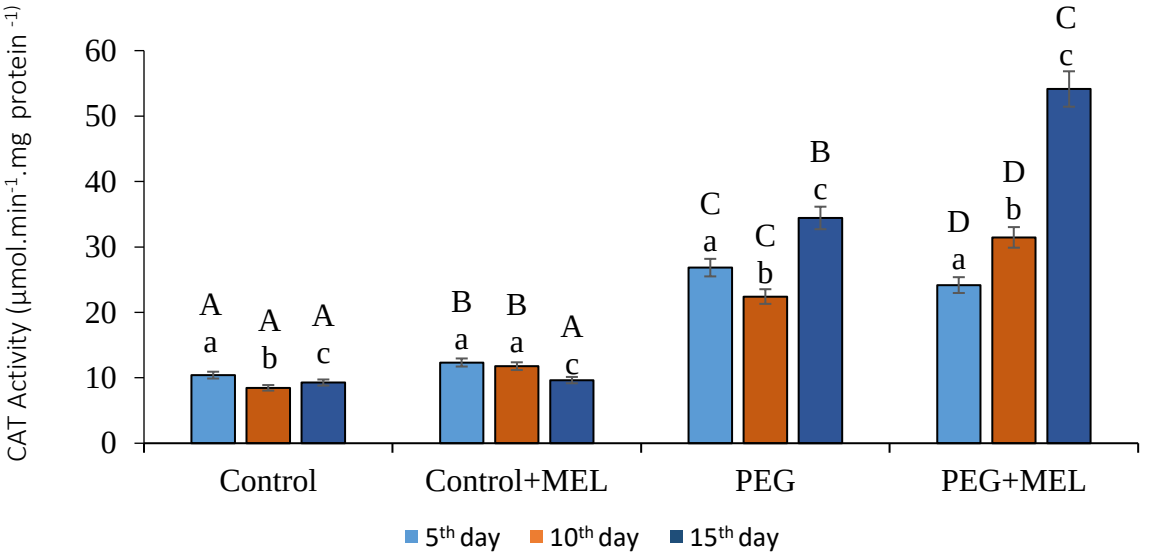

Figure 4- Variation of CAT activity in *M. nigra* leaves depending on groups and days

3.4. SOD activity

We determined improvement in SOD activity in all treatments, excluding the Control group (P<0.05). The highest SOD activity was found in PEG+MEL group as 97.06 µmol.min^{-1}.mg protein^{-1}. We also observed that SOD activity increased 100% on the 10th day and 92% on the 15th day in the PEG+MEL group compared to the PEG group (P<0.05). Also, MEL supplementation to the Control group enhanced SOD activity by 52% on the 10th day and 384% on the 15th day (P<0.05) (Figure 5).

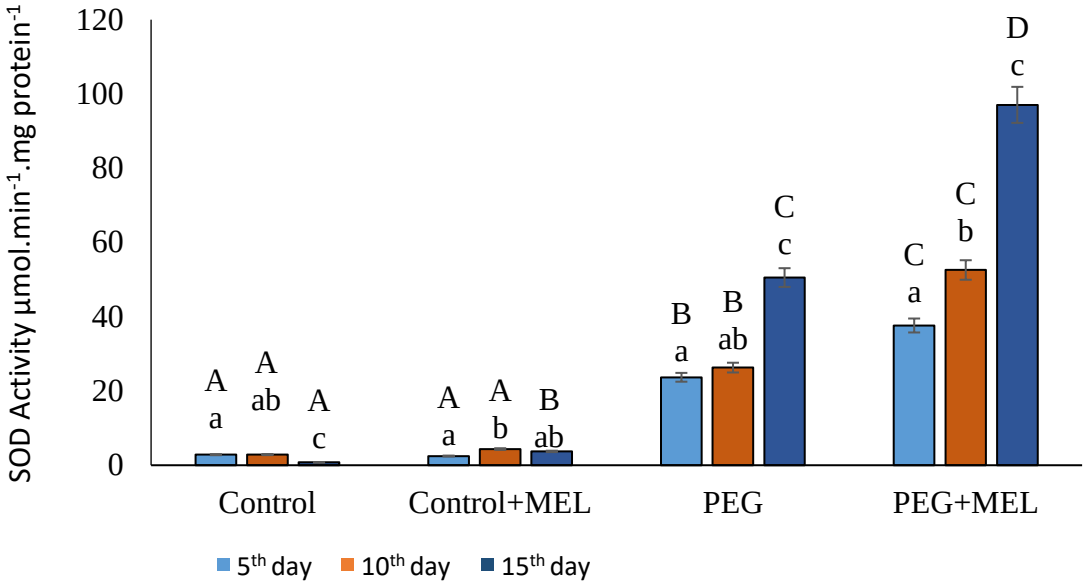

Figure 5- Variation of SOD activity in M. nigra leaves depending on groups and days

3.5. GST activity

It was determined that the GST activity increased in all the treatment groups compared to the Control (P<0.05). The highest GST activity was 66.57 µmol.min^{-1}.mg protein^{-1} in PEG+MEL group, and increasing by 125% was found compared to the 15th day of the PEG group (Figure 6).

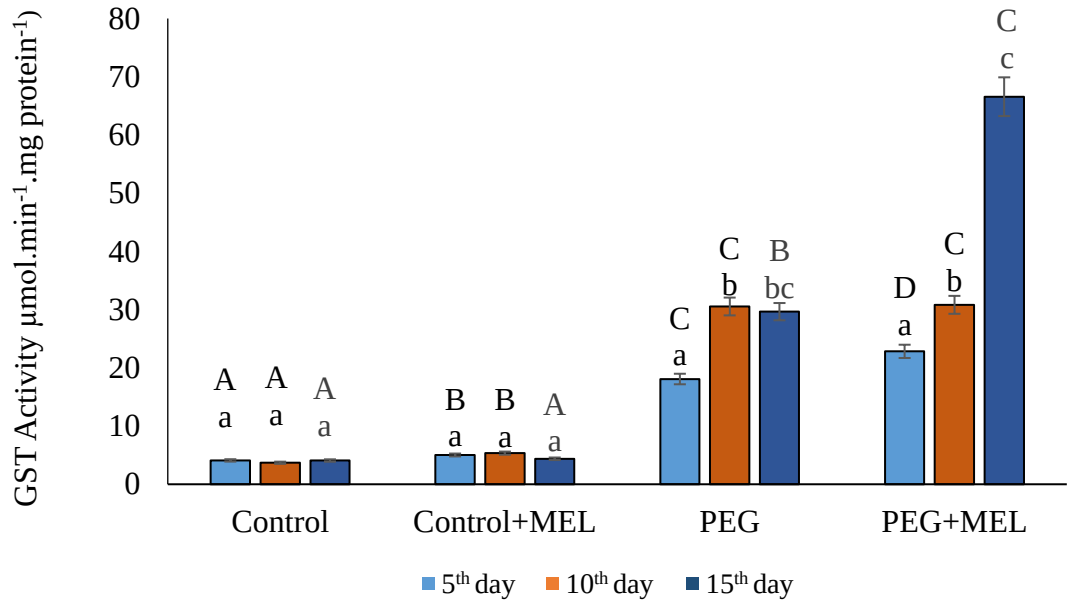

Figure 6- Variation of GST activity in M. nigra leaves depending on groups and days

3.6. GR activity

GR activity increased in all treatment groups compared to the Control ($P<0.05$). The highest GR activity was found in PEG+MEL group as 124.48 µmol.min^{-1}.mg protein^{-1}. Compared to the PEG group, GR activity in PEG+MEL group decreased 12% on the 5th day, while increased 28% on the 10th day and 55% on the 15th day ($P<0.05$) (Figure 7).

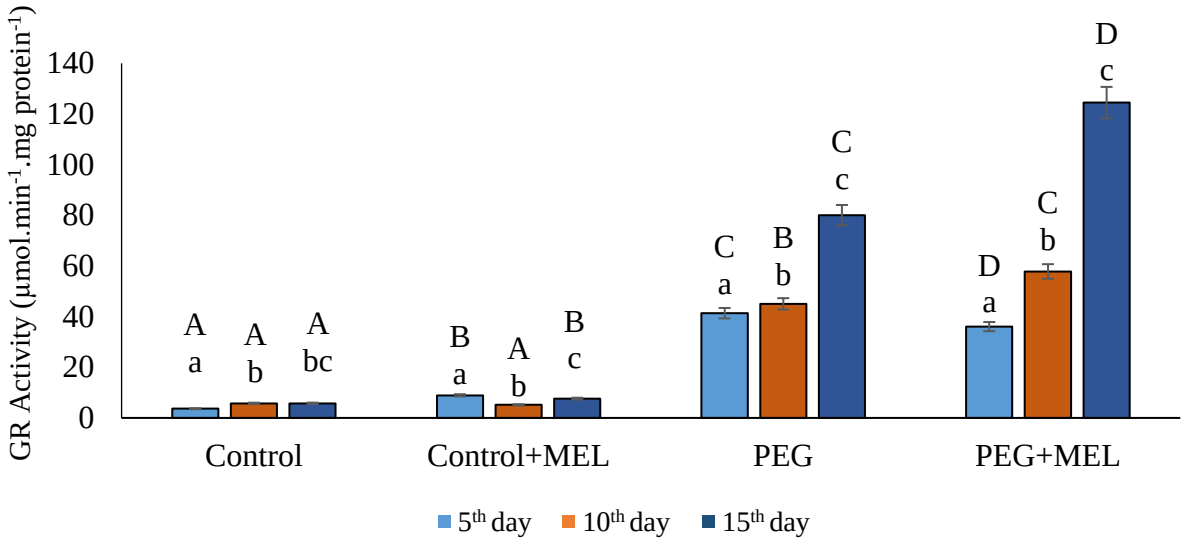

Figure 7- Variation of GR activity in *M. nigra* leaves depending on groups and days

3.7. GSH content

It was seen that treatment groups, excluding Control, positively impacted GSH content ($P<0.05$). The highest GSH content detected in the PEG+MEL group was 2.34 µmol.min^{-1}.mg protein^{-1}. It was found that the PEG+MEL group improved, compared to the PEG group, 38% on the 10th day and 93% on the 15th day ($P<0.05$) (Figure 8).

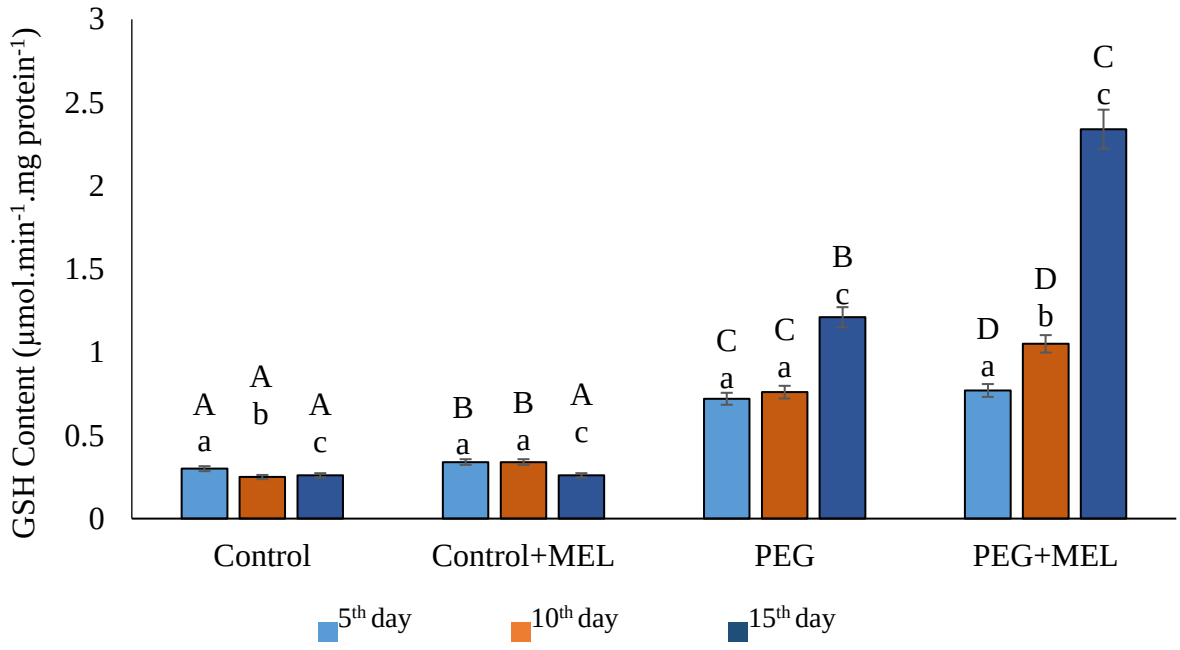

Figure 8- Variation of GSH content in *M. nigra* leaves depending on groups and days

3.8. POD activity

Our findings showed that PEG application increased POD activity in *M. nigra* leaves compared Control group on all days ($P<0.05$). The highest POD activity was observed in the PEG+MEL group with 3.92 µmol.min^{-1}.mg protein^{-1}. The PEG+MEL group enhanced POD activity by 48% on the 5th and 10th days and by 92% on the 15th day when compared to the PEG group ($P<0.05$) (Figure 9).

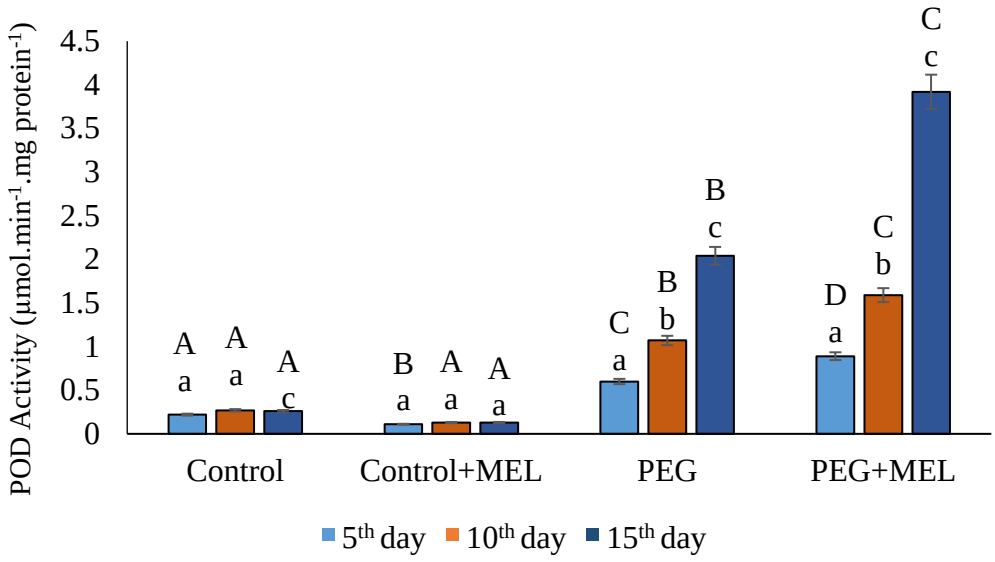

Figure 9- Variation of POD activity in *M. nigra* leaves depending on groups and days

3.9. APX activity

It was seen that APX activity in *M. nigra* leaves increased along with PEG treatment compared to the Control group on all days ($P<0.05$). The highest APX activity was found in the PEG+MEL group with 317.59 µmol.min^{-1}.mg protein^{-1}. In this context, it was observed that improvement was 84% on the 10th and 15th days in PEG+MEL group compared to the PEG group ($P<0.05$) (Figure 10).

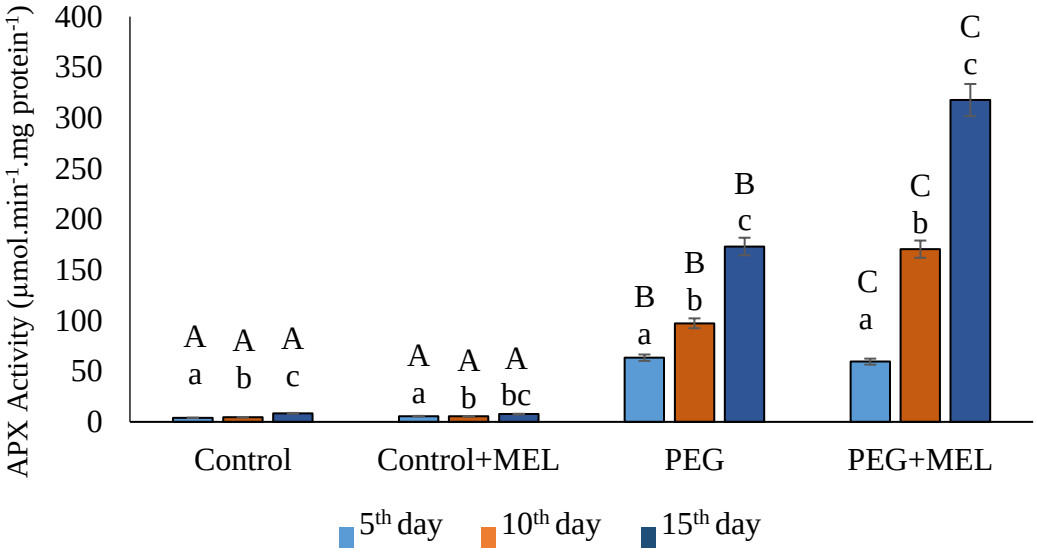

Figure 10- Variation of APX activity in *M. nigra* leaves depending on groups and days

3.10. MDA content

When compared with the Control group, it was determined that the MDA content of *M. nigra* leaves increased on all days with PEG application (P<0.05). On the other hand, the highest MDA content was examined as 27.75 µmol MDA.g^{-1} FW in the PEG group. (P>0.05) (Figure 11).

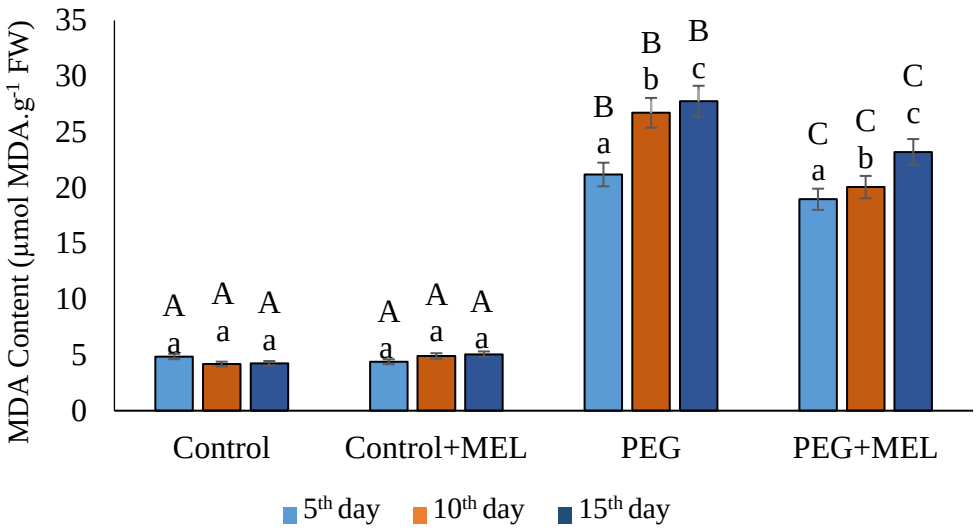

Figure 11- Variation of MDA content in M. nigra leaves depending on groups and days

3.11. Total phenolic content

The highest total phenolic content was found as 13.5 µg.g $^{-1}$ FW in the PEG+MEL group. On the other hand, it was observed that PEG+MEL group enhanced total phenolic content by 17% on the 5th day, 12% on the 10th day, and 53% on the 15th day compared with the PEG group (P<0.05). Analysis results indicate that there was no difference between the Control+MEL and Control group in terms of total phenolic content (P>0.05) (Figure 12).

3.12. Proline content

Our findings indicate that proline content was higher in all treatment groups than in the Control group (P<0.05). The highest proline content was found as 115.11 µg.g^{-1} FW in PEG+MEL group. Exogenous MEL application to the PEG group improved proline content by 32% on the 5th day, 20% on the 10th day, and 17% on the 15th day (P<0.05) (Figure 13).

4. Discussion

Drought has become the most critical problem limiting agricultural production. Moreover, it ranks first according to evaluating the adverse effects of natural disasters in the world (Marchin et al. 2020). In this context, to find solutions to yield losses caused by drought, understanding plants' mechanisms that could adapt to deficient water conditions is crucial. Studies on different plant species have shown that PEG can successfully provide drought stress conditions to plants (Zhu et al. 2005; Rouhi et al. 2006; Caruso et al. 2008; Chen et al. 2010; Ipek 2015). Also, PEG has a substantial impact on plants' water uptake by affecting its environment's osmotic potential. Hence, the intensity of use of PEG causes different levels of drought stress.

The soil's water potential is, on average, -1.5 MPa at the permanent wilting point (Kocacaliskan 2008). Leaf water content reflects the ability of the plant leaves to maintain water balance. On the other hand, RWC in plant leaves is reduced as soil moisture decreases (Marshall et al. 2000; Chen et al. 2012). It has been reported by Korkmaz et al. (2016) that chilling injury stress caused a diminishment in RWC in the leaves of pepper seedlings while MEL treatment enhanced it. In this study, although RWC was observed to be lower in the PEG group compared to the Control group, PEG+MEL composition improved RWC. Also, MEL was potent in protecting RWC and water potential in *M. nigra* exposed to drought (Figure 1).

According to Santos (2004), decreasing chlorophyll biosynthesis or increase in chlorophyll fragmentation is the cause of degradation in chlorophyll content in plants. On the other hand, Fracheboud et al. (2004) stated that drought stress might reduce chlorophyll and carotenoid content. They also reported that it is closely related to the carbon exchange ratio. Drought prevents photosynthesis by causing inconsistency in the light-harvesting complex, which triggers oxidative stress (Smirnoff 1993). Our

findings showed that PEG treatment in *M. nigra* negatively affected the pigment system. The enhancement of the total chlorophyll content in the PEG+MEL group indicates that MEL positively affects the pigment system (Figure 2).

Carotenoids are a photoprotective agent used to mitigate the deleterious effects of light and oxygen. They protect all photosynthetic organisms against light damage by transforming the light into heat or detoxifying ROTs, which leads to a reduction in lipid peroxidation (Collins 2001). Sharma et al. (2020) determined that MEL's application to *Carya cathayensis* (Chinese hickory) plants under drought stress conditions regulates metabolic pathways such as phenylpropanoid, chlorophyll, and carotenoid biosynthesis, and carbon fixation. Our findings regarding carotenoid content indicate that the decrease in PEG and PEG+MEL groups' carotenoid content may be due to the consumption of carotenoids in the plant during challenges with drought stress (Figure 3).

Free radical-induced peroxidation of lipid membranes is both the reflection and measurement of stress-induced damage at the cellular level (Jain et al. 2001). MDA is the end-product of membrane lipid peroxidation, and its accumulation demonstrates that plant cells have high levels of lipid peroxides (Zhao & Tan 2005; Upadhyaya et al. 2007; Liu et al. 2014; Ju et al. 2018). On the other hand, plant resistance can be assessed by measuring the MDA content (Gao et al. 2020). According to our findings, the MDA content was the highest in the PEG group's drought stress compared to the Control group. It was also observed that MEL+PEG group, interestingly, had lower MDA content compared to the PEG group. In this context, it is thought that MEL prevented membrane lipid peroxidation, while drought stress increased MDA content as well as the simultaneous application of MEL and PEG decreased MDA content (Figure 11). According to some studies, the MDA content decreased in groups that applied melatonin under stress (Li et al. 2019; Imran et al. 2021). The obtained findings indicate that MEL might alleviate abiotic stress-triggered ROS accumulation and cell membrane damage.

CAT is one of the antioxidant enzymes that affect cell life as it contributes to coping with ROS, which forms in oxidative stress (Volkert et al. 1994). In the study of Huihui et al. (2020), it was reported that SOD and CAT activity was increased in leaves of mulberry (*M. alba* L.) seedlings exposed to NaCl stress, and O_2 accumulation was not observed. Liu et al. (2015) reported that exogenous MEL application (0.1 mM) to the roots of tomato (*Lycopersicum esculentum*) under drought stress conditions promoted CAT, SOD, POD enzyme activities, and AsA levels. It has been stated that this status is related to the alleviation of damage in the membranes and thus the enhancement in drought tolerance. We observed that our findings are coherent with this statement (Figures 4, 5, 9).

It has been reported that SOD plays a role in the antioxidant defence mechanism of plants during oxidative stress and increases the activity of this enzyme as a reaction to stress (Jacoby et al. 2010). Plant GSTs detoxify ROSs, which form as consequences of various stresses, and can reduce peroxides along with GSH (Gill & Tuteja 2010). Kaya & Doğanlar (2019) reported that proline, GSH, and MDA contents and APX, GST, and GR activities increased in peppers (*Capsicum annuum*) exposed to pendimethalin and drought stress. They also found that exogenous MEL application reduced the plants' MDA content compared to control plants, while the proline content, GSH, and antioxidant enzymes (APX, GST, and GR) activities increased. Oxidized glutathione and NADPH transform GSH again with the reaction catalyzed by GR reductase (GR), and GR is the only enzyme that catalyzes this recovery (Karuppanapandian et al. 2011; Guller et al. 2020). On the other hand, increasing GR activity in plants promotes GSH accumulation. Thus, resistance and tolerance can be formed in plants against oxidative stresses (Shereefa & Kumaraswamy 2016). GR is the only enzyme that catalyzes the recovery of reduced glutathione from oxidized glutathione using NADPH as a reducing agent. In the research conducted by Xia et al. (2020), it is reported that exogenous MEL application to seedlings grown under drought stress led to the enhancement of ascorbic acid-glutathione (AsA-GSH) cycle, carotenoid biosynthesis, and antioxidant enzymes activities. It has been stated that the increase in POD activity under drought stress conditions is associated with the drought tolerance of the plant and that high POD activity will strengthen drought stress tolerance in the plant (Sairam & Saxena 2000). In another study, Campos et al. (2019) reported that MEL application (300 µM) increased the enzymatic and non-enzymatic antioxidants activity and reduced lipid peroxidation in coffee plants under water stress. Our findings indicate that SOD, GST, GR, POD, and APX enzyme activities and GSH content were increased in the PEG group compared to the Control group. Moreover, these parameters raised more in PEG+MEL group (Figures 5-10). We observed that MEL enhanced these enzymes, which are prominent in defence, and it demonstrates that MEL has a positive effect on the antioxidant system. These findings are in accordance with the literature.

Flavonoids and phenolic acids function as scavengers of free radicals and play a role in chelating ROS-producing metals by the Fenton reaction (Zhang et al. 2016). Gao et al. (2020), who experimented on two ornamental plants (*Adonis amurensis* and *A. pseudoamurensis*), reported that drought stress conditions improved flavonoid and total phenol content remarkably. Researchers also stated that plants mitigated damage caused by drought stress through soluble sugar and proline accumulation. Our findings indicate that drought stress increased the total phenolic content in *M. nigra* by 53% in PEG+MEL treatment than in the PEG group (Figure 12). Literature results suggest that exogenous MEL application in drought stress conditions contributes to plants' defence mechanism in stress tolerance by enhancing the phenolic content.

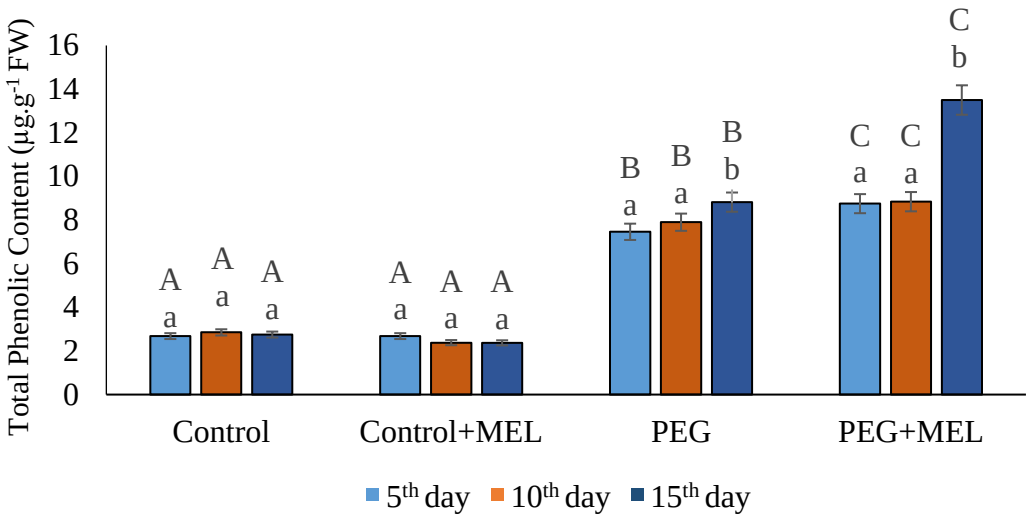

Figure 12- Variation of total phenolic content in *M. nigra* leaves depending on groups and days

Proline accumulation in plants preserves the moisture in the tissue, assists the absorption of water from the environment, and supports the cells to maintain their normal physiological and biochemical activities. Many studies have confirmed this finding in different plants under drought stress conditions (Karimi et al. 2012; Rostami & Rahami 2013; Bolat et al. 2014; Ipek 2015). Besides, many researchers reported soluble sugar and protein increment also seen under drought stress conditions as a protective mechanism of plants (Sarker & Oba 2018). Relative to the results of the study conducted by Ding et al. (2017), it was observed that exogenous MEL application to the leaves of tomato seedlings increased antioxidant enzyme activity, sugar, and proline content. Our findings demonstrated a similar trend in the *M. nigra* plant, and proline was higher, especially in the MEL group (Figure 13).

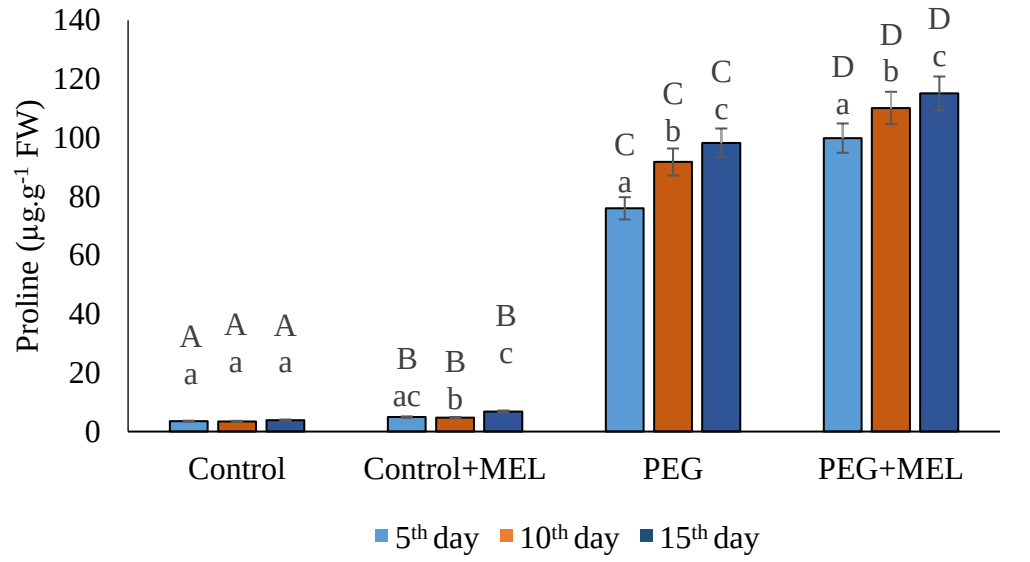

Figure 13- Variation of proline content in *M. nigra* leaves depending on groups and days

5. Conclusions

This study determined that MEL mitigates drought stress damage in *M. nigra* plants and could use as a protector. Moreover, our findings have shown that MEL positively impacts the pigment system, RWC, MDA content, non-enzymatic (GSH, proline, carotenoid), and enzymatic antioxidants (SOD, CAT, GST, GR, POD, APX). Hence, this study suggests significant findings to *in vitro* and *in vivo* research in the future.

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