INVESTIGATION OF THE EFFECT OF MELATONIN PRIMING AGAINST SALT STRESS IN THREE DIFFERENT LENTIL CULTIVARS

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Abstract. Salinity is recognized worldwide as one of the most influential environmental stresses that greatly reduces crop yield and quality. More than 20% of the world's arable land faces the adverse effects of salt stress and these salt-prone areas are constantly increasing due to both natural and anthropogenic activities. However, in the last 20 years, this problem has become much more severe in arid and semi-arid regions due to increased demand for irrigation water. Salt stress is an important environmental stress that affects plant growth and development. It increases intracellular osmotic pressure and can cause sodium to accumulate to toxic levels. Melatonin is a pleiotropic signaling molecule that reduces the negative effects of abiotic stresses and enhances the growth and physiological function of many plant species. Many recent studies have demonstrated the important role of melatonin in plant function, especially in the regulation of crop yield. However, the mechanism by which melatonin regulates plant growth and crop yield under abiotic stress conditions has not yet been fully elucidated. In this study, lentil seeds of Kafkas, Çiftçi and Ozbek varieties were pretreated with melatonin and then their responses to salt stress were tested. Firstly, germination percentages of the varieties were obtained and their responses to different concentrations of NaCl were tested. The results showed that the most resistant variety was Çiftçi and the most sensitive variety was Özbek. Then, melatonin pretreatment was applied and germination responses of the seeds were examined and it was observed that melatonin application increased the germination percentages compared to the control. The results of chlorophyll analysis showed that melatonin pretreatment had a positive effect on chlorophyll content under salt stress. Our biochemical analysis showed that melatonin application increases plant resistance to stress compared to the control, even if it differs between varieties.
Keywords: *antioxidant activities, Lens culinaris, melatonin priming, NaCl, salt stress*

Introduction

Plants show optimal growth under conditions that are most suitable for them. Although plants can continue to grow in response to daily and seasonal changes due to the flexibility of normal metabolism, exposure to unexpected conditions continuously or intermittently can result in diseases, damage, or physiological changes that affect their growth and survival (Shao et al., 2008). Increasing salinity, alongside the growing food demand of human population, is one of the environmental factors that significantly restrict productive agriculture by endangering the production of food crops (Botella et al., 2005). Increased soil salinity due to global warming and the misuse of arable lands is one of the most critical abiotic stresses negatively impacting plant growth and development. Salt stress impedes plant growth and development by causing osmotic and ionic stress (Parida and Das, 2005). The initial stage of osmotic stress occurs when salt concentration increases in the root rhizosphere, leading to a

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reduction in available water and resulting in a condition termed “physiological drought” (Tuteja, 2007). Reduced water availability leads to decreased cell expansion and slowed shoot development. During the subsequent ionic stress phase, increased Na and Cl ions compete with essential nutrients like K+, Ca2+, and NO3-, causing nutrient deficiency in plants (Hu and Schmidhalter, 2005). Salt stress directly impacts plants by creating osmotic and ionic stress, while its indirect (secondary) effects manifest through structural damage and the synthesis of toxic compounds resulting from these stress factors. Major secondary effects caused by NaCl include the synthesis of reactive oxygen species (ROS) that damage DNA, proteins, chlorophyll, and membrane functions; inhibition of photosynthesis; metabolic toxicity; inhibition of K + uptake; and cell death (Botella et al., 2005; Hong et al., 2009). The effects of salt stress on plants vary depending on the plant species, the type and amount of salt applied, and the duration of exposure. Plants exhibit diverse responses to salt stress based on genotypic differences (Dajic, 2006). These varying growth responses to salinity are not only observed between different plant species but also among different varieties of the same species (Munns, 2002).

Salt stress induces numerous adverse effects at morphological, physiological, cellular, and molecular levels. To mitigate the negative impacts of salt stress, plant growth regulators that provide stress tolerance are employed (Ashraf and Foolad, 2007). Studies on the exogenous application of substances like jasmonic acid, salicylic acid, and silicate, which increase tolerance to salt stress, have been increasing in recent years (Talebi et al., 2018; Idrees et al., 2012; Mohammed et al., 2018). Melatonin (N-acetyl-5-methoxytryptamine) (MT) is an indoleamine discovered in bovine pineal glands in 1958 (Lerner et al., 1958) and found in plants in 1995 (Dubbels et al., 1995; Hattori et al., 1995). It is synthesized from the amino acid tryptophan (Ke et al., 2018). MT is known to play a significant role in critical processes related to plant growth and development, such as germination, photosynthesis, flowering, circadian rhythm, and senescence (Zhang et al., 2021; Yan et al., 2020; Cao et al., 2019; Hernandez-Ruiz and Arnao, 2018; Arnao and Hernandez-Ruiz, 2015). Recent studies have shown that MT is a potent antioxidant in plants, promoting and regulating the activities of enzymes like glutathione reductase (GR), catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) in stressed plants (Manchester et al., 2000; Terron et al., 2001; Allegra et al., 2003; Rodriguez et al., 2004; Tal et al., 2011; Bahcesular et al., 2020).

In recent years, the seed priming procedure, in which seeds are soaked in various chemical solutions (such as hormones, antioxidants, and vitamins), has been increasingly applied (Savaedi et al., 2019). Previous studies have shown that seed priming not only increases germination percentages but also enhances plant growth and stress tolerance (Varier et al., 2010; Papparella et al., 2015; Liu et al., 2022; Alhammad et al., 2023). The use of MT for priming has been increasing in recent years. Several studies have demonstrated the protective effect of MT priming on plants against stress (Heshmati et al., 2019; Cao et al., 2019; Bahcesular et al., 2020; Yan et al., 2020; Khan et al., 2020).

Lentil (Lens culinaris Medic.), one of the main food crops grown in semiarid regions, is more sensitive to salinity than other legumes (Ashraf and Waheed, 1990; Katerji et al., 2001). Literature review shows that there is no study on MT priming application against salt stress in lentil. This study aims to investigate the effect of melatonin priming on seed resilience and antioxidant mechanisms under salt stress in three different lentil varieties.

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Material methods

Experimental materials and seed priming

In this study, three different winter lentil varieties (Lens culinaris Medic. cv. Çiftçi; Lens culinaris Medic. cv. Kafkas; and Lens culinaris Medic. cv. Özbek) were used as experimental materials. The seeds were obtained from the Ankara Field Crops Central Research Institute. Surface sterilization was performed by soaking the seeds in 70% alcohol for 2 min and 5% sodium hypochlorite solution for 5 min, followed by five washes with sterile distilled water. As a result of our experimental preliminary tests and literature searches, it was decided that the Melatonin (MT) concentration would be 500 µM (Rajora et al., 2022). The sterilized seeds were divided into control and experimental groups, with the control group seeds placed in sterile distilled water and the experimental group seeds in 500 µM MT solution. The seeds were primed at 25°C in the dark for 18 h.

Experimental design and salt treatment

First, the germination percentages of the Çiftçi, Kafkas, and Özbek varieties were determined. The seeds were then germinated in petri dishes containing 0, 50, 100, 150, 200, 250, and 300 mM NaCl solutions at 25°C in the dark for 7 days. At the end of this period, the germination percentages were calculated and recorded. Our preliminary studies determined that the Çiftçi variety was the most resistant to salt stress, while the Özbek variety was the most sensitive. Based on our preliminary studies, the salt concentrations to be applied to the seeds were decided (Unpublished data). The Çiftçi variety was grown in 200 mM NaCl, the Kafkas variety in 150 mM NaCl, and the Özbek variety in 100 mM NaCl MS media. 21-day-old plants were harvested, placed in liquid nitrogen, and stored at -80°C until biochemical analyses were performed.

Analysis of pigment content

Pigments were extracted by grinding the leaves in 90% ice-cold acetone with pestle and mortar and the total chlorophyll and carotenoid contents were determined spectrophotometrically (Parsons and Strickland, 1963). It was analyzed with 10 biological and 3 technical replicates.

Soluble protein content determination

The leaf samples were homogenized with ice-cold 0.1 mmol/L sodium phosphate buffer (pH 7.0). The homogenates were centrifuged at 13,000 rpm for 30 min at 4°C and the supernatants were used for the determination of total soluble protein content and enzyme activity assays. The protein content of the extracts was determined according to Bradford (1976), using bovine serum albumin as a standard. It was analyzed with 10 biological and 3 technical replicates.

MDA analysis

MDA analysis was performed according to Karabal et al. (2003). 0.2 g of leaf samples were homogenized in 1 mL of trichloroacetic acid solution (TCA, 5%) and centrifuged at 12,000 rpm for 15 min. The supernatant of the extract and thiobarbituric acid (TBA, 0.5%) solution which is dissolved in TCA solution (20%) were mixed and incubated for 25 min at 96°C in a water bath. After the incubation the tubes were cooled

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in an ice bath, and then they were centrifuged at 10,000 rpm for 5 min. The supernatant was detected spectrophotometrically at 532 and 600 nm. MDA content, the indicator of lipid peroxidation, was calculated by using extinction coefficient (155 mM^{-1} cm^{-1}).

Determination of antioxidant enzyme activities

Ascorbate peroxidase (APX; EC 1.11.1.11) activity

Cold leaf tissues were homogenized in 1 ml of 50 mM Na-P buffer (pH 7.8) containing 2 mM ascorbic acid and 1 mM EDTA-Na2.2H2O. The resulting supernatant was used for the assay (Nakano and Asada, 1981) (extinction coefficient 2.8 mM^{-1} cm^{-1}). One unit of enzyme activity corresponds to the amount of ascorbate oxidized per minute (1 µmol ml^{-1}).

Glutathione reductase (GR; EC 1.6.4.2) activity

The decrease in oxidized glutathione in the presence of NADPH was measured by the decrease in absorbance at 340 nm over 3 min (€ = 6.2 mM^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount of glutathione oxidized per minute (µmol ml^{-1}) (Foyer and Halliwell, 1976).

Peroxidase (POX; EC 1.11.1.7) activity

Activity was calculated by monitoring the increase in absorbance at 465 nm due to the oxidation of DAB (3,3’-Diaminobenzidine tetrahydrochloride). Specific enzyme activity is expressed as the amount of H2O2 consumed per minute (µmol ml^{-1}) (Kanner and Kinsella, 1983).

Catalase (CAT; EC 1.11.1.6) activity

The decrease in the amount of H2O2 was determined by the decrease in its maximum absorbance at 240 nm. CAT activity is expressed as the amount of H2O2 consumed per minute (µmol ml^{-1}) (Bergmeyer, 1970).

Statistical analysis

All data were subjected to statistical analysis using SPSS software (SPSS Inc., Chicago, IL, USA). Significant differences among the means were determined by one-way ANOVA, followed by Duncan’s multiple range test at $P < 0.05$. Data were expressed as mean ± standard error (SE).

Results

Effect of MT priming on germination rates on lentil seeds

Initially, seeds from different lentil varieties were placed in Petri dishes containing NaCl solutions ranging from 0 to 300 mM to induce germination. The results indicated that the variety “Çiftçi” exhibited the highest tolerance to salt stress, achieving a germination rate of 35% in 250 mM NaCl solution. The “Kafkas” variety showed a germination rate of 30% in 200 mM NaCl solution, while the “Özbek” variety demonstrated a germination rate of 35% in 150 mM NaCl solution. All varieties achieved a 100% germination rate in the control group (0 mM NaCl), whereas no germination was observed in the 300 mM NaCl solution.

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Following the NaCl-germination trials, one group of seeds was subjected to priming in distilled water (Control group) and another group in a 500 µM Melatonin solution for 24 h. After priming, the seeds were transferred to Petri dishes containing the selected salt concentrations for each variety, and their germination was monitored. The data showed that melatonin priming increased the germination percentage in all varieties compared to the control (*Fig. 1*).

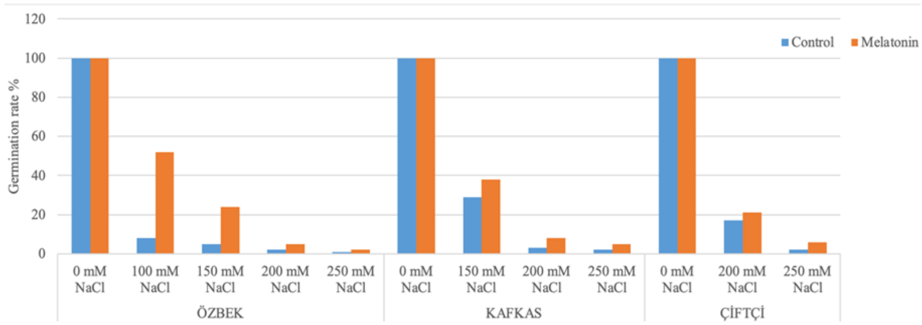

Figure 1. *The effect of melatonin priming on germination percentage in lentil varieties subjected to control and NaCl treatments*

Melatonin priming significantly improved the germination percentage in lentil varieties compared to the control, both in the absence and presence of NaCl. The enhancement in germination rates was observed across all tested varieties, indicating that Melatonin priming effectively mitigates the adverse effects of salt stress on seed germination.

Based on the data obtained, after a 24-h priming treatment on the seeds of lentil varieties, the seeds were cultured in MS medium containing 200 mM NaCl for “Çiftçi,” 150 mM NaCl for “Kafkas,” and 100 mM NaCl for “Özbek” in a growth chamber.

Effect of melatonin priming on chlorophyll and carotenoid amounts

The highest total chlorophyll content was found in the “Çiftçi” variety with Melatonin priming and 0 mM NaCl application compared to all control groups. Our data indicate that Melatonin priming increased chlorophyll content in all varieties and groups (control and NaCl) ($p < 0.05$) (*Fig. 2*). Melatonin priming significantly increased carotenoid content in “Özbek,” “Kafkas,” and “Çiftçi” varieties compared to the control ($p < 0.05$) (*Fig. 3*).

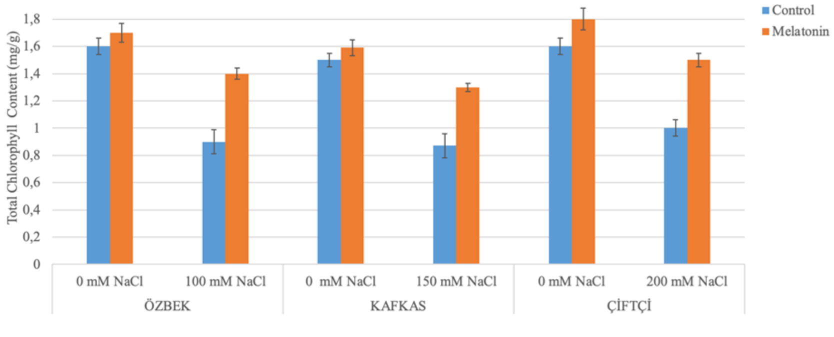

Figure 2. *The effect of melatonin priming on total chlorophyll content in lentil varieties subjected to control and NaCl treatments*

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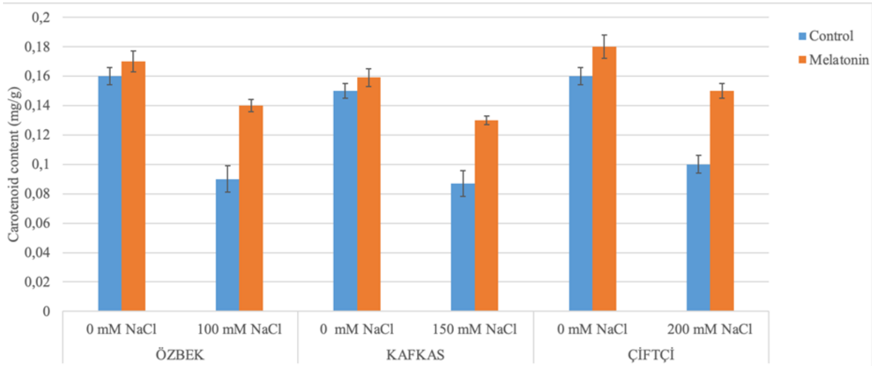

***Figure 3.** The effect of Melatonin priming on carotenoid content in lentil varieties subjected to control and NaCl treatments*

Effect of melatonin priming on MDA contents

Melatonin priming reduced MDA content compared to the control, especially in groups under salt stress ($p < 0.05$) (*Fig. 4*).

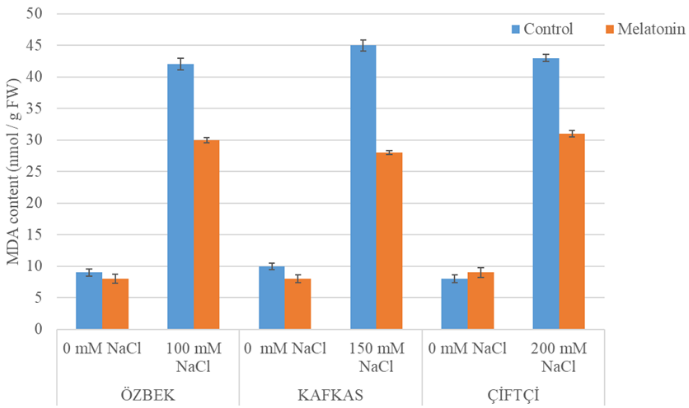

***Figure 4.** The effect of melatonin priming on MDA content in lentil varieties subjected to control and NaCl treatments.*

Effect of MT priming on antioxidant enzyme activities

APX

As shown in *Figure 5*, Melatonin priming increased APX activity compared to the control ($p < 0.05$). This increase was relatively higher in groups under salt stress (*Fig. 5*).

GR

Melatonin priming significantly increased GR activity, especially in groups subjected to NaCl stress compared to the control ($p < 0.05$) (*Fig. 6*).

POX

As shown in *Figure 7*, Melatonin priming caused an increase in POX activity in all groups compared to the control ($p < 0.05$) (*Fig. 7*).

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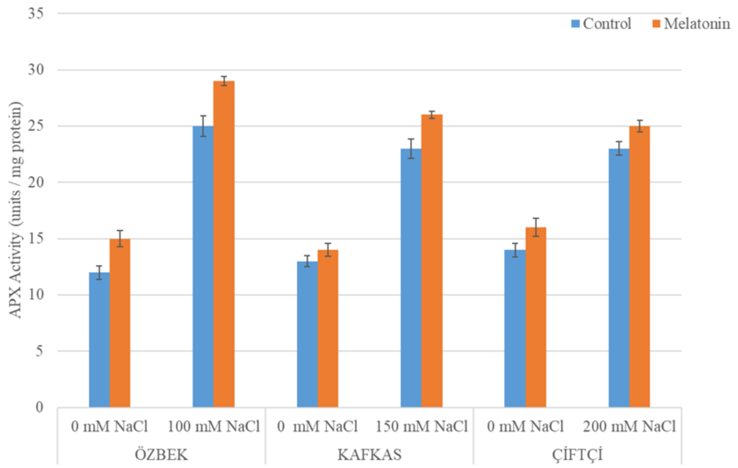

Figure 5. *The effect of Melatonin priming on APX activity in lentil varieties subjected to control and NaCl treatments*

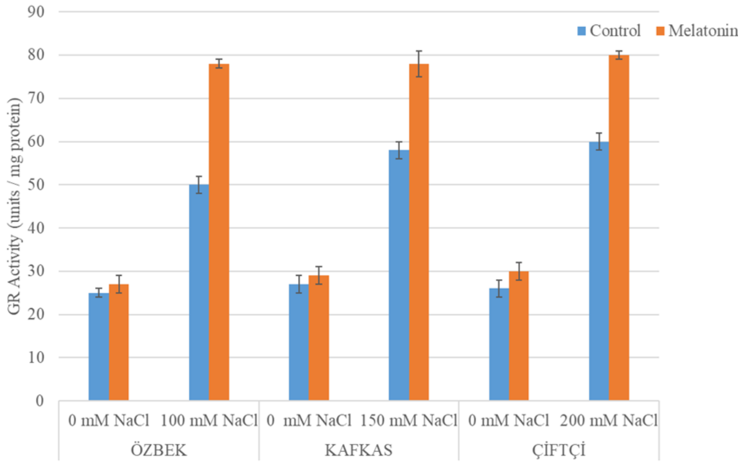

Figure 6. *The effect of melatonin priming on GR activity in lentil varieties subjected to control and NaCl treatments*

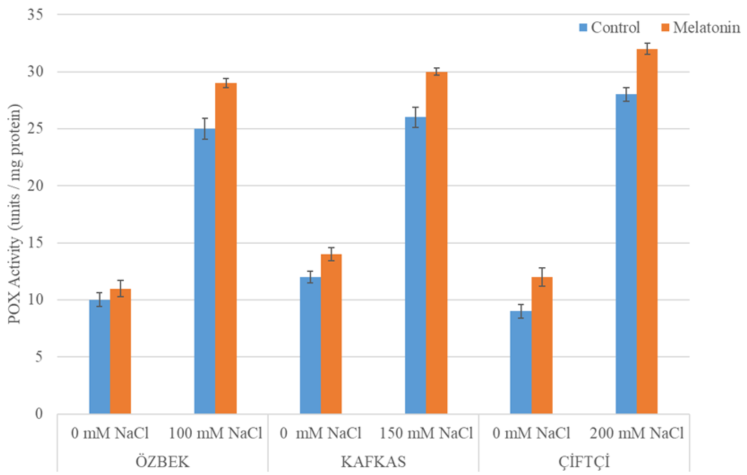

Figure 7. *The effect of melatonin priming on POX activity in lentil varieties subjected to control and NaCl treatments*

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CAT

Similar to other antioxidant enzyme activities, Melatonin priming increased CAT activity compared to the control ($p < 0.05$) (*Fig. 8*).

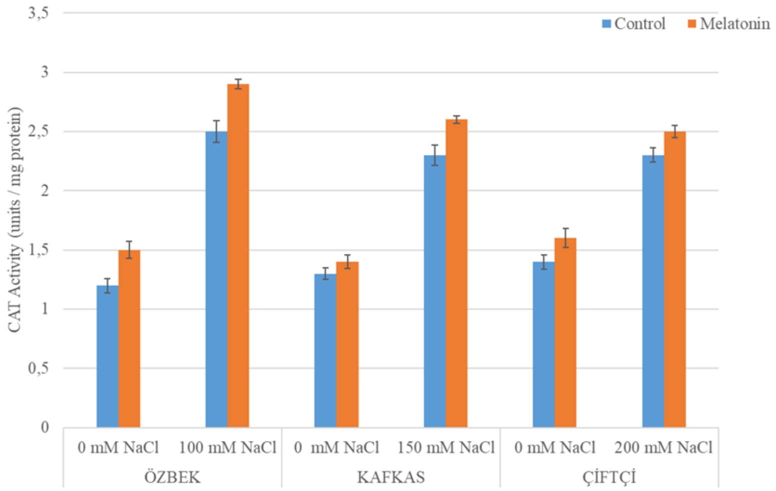

Figure 8. *The effect of melatonin priming on CAT activity in lentil varieties subjected to control and NaCl treatments*

Discussion

Global warming has exacerbated abiotic stresses such as salinity, drought, and temperature fluctuations, which adversely affect both plant growth and development, as well as crop yields. Climate change, encompassing multifaceted and dynamic alterations in environmental conditions, threatens the biosphere by inducing both biotic and abiotic stressors. By constraining plant development through changes in temperature, precipitation, and atmospheric conditions, climate change leads to yield losses and the emergence of new weeds and pathogens (Abbass et al., 2022; Chaudhry and Sidhu, 2022). Given the global and unstoppable nature of climate change, enhancing plants' tolerance to abiotic stresses is crucial (Dutta et al., 2020; Chaudhry and Sidhu, 2022). Drought and salinity are among the primary factors negatively impacting plant growth, particularly wheat production (Hossain et al., 2021). In arid and semi-arid regions, soil salinity intensifies due to the evaporation of soil moisture, resulting in physiological drought as plants fail to absorb sufficient water from the soil (Angon et al., 2022). While both drought and salinity inhibit root and shoot growth, salinity has a more severe impact on root development compared to drought (Angon et al., 2022). Reactive oxygen species (ROS) are byproducts of numerous processes in plants, primarily photosynthesis, and play a role in signal transduction under optimal conditions. ROS have multiple detrimental effects on plants, including protein denaturation, DNA damage, lipid peroxidation, carbohydrate oxidation, pigment degradation, and inhibition of enzyme activity (Angon et al., 2022). The effects of ROS are regulated by an antioxidant system comprising enzymatic components such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST), and glutathione peroxidase (GPX), as well as non-enzymatic components like glutathione (GSH), ascorbate (AsA), tocopherol, carotenoids, alkaloids, and phenolic compounds (Hossain et al., 2021).

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In recent years, the seed priming procedure, in which seeds are soaked in various chemical solutions (such as hormones, antioxidants, and vitamins), has been increasingly applied (Savaedi et al., 2019). Seed priming agents can include water, solutions, hormones, minerals, stress conditions like gamma irradiation, or heat shock (Siyar et al., 2020; Paul et al., 2022). The critical role of germination in plant life and its impact on yield highlight the importance of seed priming in enhancing abiotic stress tolerance. Various priming agents have yielded positive results in many plant species, but the type and duration of application vary depending on the plant species and expected outcomes (Paul et al., 2022). Common priming agents include proline, tryptophan, glutathione, mannitol, inositol, ascorbic acid, glycine betaine, alpha-tocopherol, and melatonin (Godoy et al., 2021), as well as auxin, ethylene, ABA, salicylic acid, jasmonic acid (JA), and brassinosteroids (Adak et al., 2020). It has been reported that priming, a physiological technique involving the hydration and drying of seeds to enhance pre-germination metabolic processes, increases germination rate, seedling growth, and yield under biotic and abiotic stress conditions and induces plant tolerance (Rahman et al., 2021; Bruce et al., 2007).

The germination percentages of lentil varieties in different NaCl concentrations and the effect of melatonin priming on germination are shown in *Figure 1*. The control group without NaCl treatment had the highest germination percentages for all varieties. However, increasing NaCl concentrations significantly reduced germination percentages. The Çiftçi variety exhibited the highest tolerance to salt stress, while the Özbek variety showed the least tolerance. Melatonin pretreatment significantly improved germination percentages under salt stress conditions, suggesting that melatonin priming alleviates the negative effects of salinity on germination.

The chlorophyll content of lentil seedlings grown under different salt concentrations and melatonin priming is presented in *Figure 2*. Chlorophyll content decreased with increasing NaCl concentration in all varieties. However, melatonin pretreatment significantly increased chlorophyll content under salt stress. The highest chlorophyll content was observed in the Çiftçi variety pretreated with melatonin and grown under 200 mM NaCl.

The soluble protein content of lentil seedlings under salt stress with and without melatonin priming is shown in *Figure 3*. Salt stress reduced soluble protein content in all varieties, with the Özbek variety showing the most significant reduction. Melatonin-priming significantly increased soluble protein content compared to untreated control under salt stress conditions.

The MDA content, indicative of lipid peroxidation, is presented in *Figure 4*. MDA levels increased with higher NaCl concentrations, indicating enhanced oxidative stress. Melatonin-priming significantly reduced MDA content under salt stress in all varieties, suggesting its protective role against oxidative damage.

The activities of antioxidant enzymes APX, CAT, and POX in lentil seedlings under salt stress with and without melatonin priming are shown in *Figures 5*, *7*, and *8*, respectively. Salt stress increased the activities of all three enzymes, with the highest increase observed in the Çiftçi variety. Melatonin pretreatment further enhanced the activities of these enzymes, indicating an improved antioxidant defense system in melatonin-primed seeds.

When MT priming is applied to different seeds under various stress conditions, it has been observed that MT plays a significant role in reducing and counteracting the effects of ROS (Cao et al., 2019). It has been reported that MT application inhibits H_2O_2

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accumulation in tomato and wheat leaves under chilling stress (Liu et al., 2015; Tan et al., 2000). The effect of MT priming on the antioxidant enzyme system demonstrates the defense and protection mechanisms that MT induces in plants against salt stress. Previous studies also support these findings (Khan et al., 2019; Cao et al., 2019). Chlorophyll loss is one of the most important symptoms observed in plants under stress conditions. In our study, MT priming increased chlorophyll content under both stress and control conditions. The protective effect of melatonin on chlorophyll has also been demonstrated under drought stress (Ye et al., 2016). The high concentrations of APX, POX, CAT, and GR, along with the low concentrations of MDA observed as a result of melatonin priming application, indicate that MT priming enhances the redox defense system in lentil seedlings under salt stress.

Conclusion

This study demonstrates that melatonin priming improves salt stress tolerance in lentil seeds by enhancing germination, chlorophyll content, soluble protein content, and antioxidant enzyme activities, while reducing lipid peroxidation. The findings suggest that melatonin priming could be a promising strategy to mitigate the adverse effects of salt stress on lentil cultivation, potentially contributing to sustainable agricultural practices in salt-affected areas.

Author contributions. All authors conceived research. Dr. Nihal Gören-Sağlam and Dr. Orkun Yaycılı designed the experimental framework. All authors participated the experimental analysis. Dr. Nihal Gören-Sağlam drafted the manuscript. All authors have read and approved the manuscript.

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