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Effects of exogenous application of abscisic acid and melatonin on biochemical and molecular traits of Salicornia europaea under salinity stress

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Effects of exogenous application of abscisic acid and melatonin on biochemical and molecular traits of Salicornia europaea under salinity stress

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Abstract

Salinity is a major factor limiting agricultural lands that causes adverse effects on plant growth and production. The use of exogenous compounds or growth regulators has often been effective in reducing the effects of environmental stresses and/or identifying stress-coping mechanisms in plants. Among 1560 halophytes, *Salicornia europaea* is known as a salt-tolerant species. This study aimed to understand the effects of the endogenous application of abscisic acid (ABA) and melatonin hormones and their combined effects on mechanisms involved in plant resistance to salinity stress to identify these mechanisms, genes associated with ion channels, and antioxidant enzymes involved in response to stress using real-time technology. *S. europaea* metabolism compounds, namely hydrogen peroxide (H_2O_2), malondialdehyde, proline, phenol, sodium, potassium, and calcium were studied in control plants and under 400 mM salinity stress at 0, 6, 48, and 720 h (1 month) intervals. The best effects in terms of morphological, biochemical, and molecular traits were observed in spraying with both hormones at 400 mM salinity. This treatment reduced polyphenol oxidase activity, malondialdehyde content (by 35%), and H_2O_2 content, while it increased shoot (2.04 times) and root (76%) wet weights, shoot dry weight (2.45 times), K (by 19%) and Ca (by 79%) contents, and the Na/K ratio (by 30%). ABA spraying elevated catalase activity at 400 mM salinity. Spraying treatments led to the overexpression of SOD (by 21 times), SOS1 (by 1218 times), NHX1 (by 16 times), APX (by 214 times), HKT1 (by 470 times), and VHA-A (by 27 times) genes in some treatments compared to NT plants. The rapid response to stress is the main difference between stress-sensitive and tolerant plants. Spraying treatments resulted in elevated overexpression of genes involved in

stress-responding mechanisms at early hours of salinity stress compared to control treatments, thereby increasing the tolerance of *S. europaea* to salinity stress.

Keywords: Abscisic acid, Melatonin, Real-time, *Salicornia europaea*, Salinity Stress

Abbreviations: ABA: Abscisic acid, Mel: Melatonin, H_2O_2: Hydrogen peroxide, SOD: Super Oxide Dismutase, APX: Ascorbate Peroxidase, NT: Not Treatment, PPO: Poly Phenol Oxidase, CAT: Catalase, POX: Peroxidase.

1. Introduction

Environmental deterioration and human dominance of nature have increased soil salinity as abiotic stress on plants and reduced agricultural products worldwide (Zare et al. 2018; Thanabut et al. 2023). Currently, more than 20% of agricultural lands and half of the irrigation water used in farmlands are salty (Wu et al. 2010). The use of these unconventional waters in agriculture increases salty lands, which are estimated to reach over 50% by 2050 (Vinocur and Altman 2005). Because of the human need for more agricultural products in the future, many efforts have been made to increase salinity tolerance in important economic plants (Vinocur and Altman 2005; Shafiq et al. 2021). An option is to increase salt tolerance in crops through plant breeding programs and/or genetically compatible plants (Khorasaninejad et al. 2016; Mozaffari et al. 2017). However, the preliminary work results of such techniques have not been very promising. A new strategy to reuse salty lands is to utilize an economically valuable salt-tolerant (halophyte) crop (Kong and Zheng 2014).

Salicornia europaea is a hairless herbaceous annual plant with green flowers, purple fruits, and a high height (60-100 cm) from the *Caryophyllales* order and the *Amaranthaceae* family. This plant contains a basic chromosome number of X = 9, which may be diploid (2n = 2x = 18) or tetraploid (2n = 4x = 36) (Guninig, 2019). Among 1560 halophytes, *S. europaea* is known as a salt-tolerant species with a sufficient salty taste for cookery and a high nutritional value. Therefore, this plant as a popular salt alternative vegetable has been introduced to fresh crop markets in the USA and Europe, and its fresh branches are sold as marsh samphire or sea smilax (Kim and Kim 2010; Jaradat and Shahid 2012; Ventura and Sagi 2013; Izadi et al. 2020; Cardenas Perez et al., 2021). In the past, the vegetative organ of *S. europaea* was used for the treatment of asthma, bronchitis, hypertension, and hypotension (Ma et al. 2013), its seeds contain nutritive oil for human use, and its biomass can be used for fodder production. Although salt reduces growth and yield in most plants, it is used as a mineral nutrient in many halophytes such as this plant (Kotagiri and Kolluru 2017). Therefore, studies on the cellular and molecular mechanisms of halophytes against salinity, including toxic ion dilution, inhibition of salt entry into the plant, salt aggregation in less-sensitive cells, salt removal from different tissues, production of osmo-regulators, and concurrently, maintenance of growth, can provide a rich source of salinity resistance strategies in plants to transfer to crops (Kachout et al. 2012; Rezaei Moshaei et al. 2016). The use of exogenous compounds or growth regulators has often effectively reduced the effects of environmental stresses (Wei et al. 2015; Gorgini Shabankareh et al. 2021a). Growth regulators are nowadays widely used to control plant growth and improve salinity tolerance. These phyto-hormones include salicylic acid, polyamines, ABA, and melatonin. Most of these phyto-hormones are naturally found in many fruits, roots, leaves, stems, flowers, and seeds, and are thought to play a role in growth, development, immunity enhancement, photosynthetic pigment biosynthesis, callus formation, and tolerance of environmental stresses (Wang et al. 2020). Elevated gene encoding by the exogenous use of phyto-hormones, including melatonin and ABA, has been reported in many studies (Iqbal et al. 2019). Antioxidant enzymes have improved POX, SOD, and CAT activities, reduced oxidative stress activity, lipid peroxidation, and concentrations of H_2O_2 and malondialdehyde, and increased photosynthetic rates and sugar metabolism (Ma et al. 2023). Specifically, melatonin and ABA have separately been used to enhance plant tolerance to salinity stress on such crops as cucumbers, tomatoes, maize, rapeseed, etc. (Iqbal et al. 2019). In plants, melatonin and ABA are known as biostimulants that increase plant tolerance to biotic and abiotic stresses and improve plant growth and development (Tan et al. 2012). A major factor in salinity tolerance is the response of ABA, which is a phyto-hormone and plays an essential role in developing growth mechanisms under biotic and abiotic stresses in plants. ABA is actually considered the main signal of the plant's internal factory that allows plant survival in adverse environmental conditions (Massa and Melito 2019).

Genes expressed in response to salinity stress are part of a complex cascade of molecular networks and are thought to play an important role in response to environmental stresses. The identification of these practical genes using molecular biology tools is necessary to understand salinity tolerance mechanisms in plants (Chinnusamy et al. 2005). Although some halophytes can specifically counteract high salinity levels by exclusive mechanisms, little research is available on the identification of salinity tolerance genes in halophytes, and plenty of previous studies focused on salinity tolerance in model plants, such as Arabidopsis, rice, and tobacco (Kachout et al. 2012). These genes are of paramount importance due to inducing stress resistance responses in different physiological pathways.

The major transcript measurement methods are Northern blot and quantitative Real-Time PCR. The latter is an in vitro technique to replicate RNA transcribed to cDNA by primers specific to the target gene. It is the most sensitive and flexible method among quantitative RNA evaluation methods and can be used to compare mRNA levels in different samples, determine mRNA expression patterns, and differentiate between very rare mRNAs (Reischl et al. 2012).

S. europaea is a plant well adapted to water and soil salinity. Thus, this species can account for a good model plant to understand salinity tolerance mechanisms at the molecular level (Ma et al. 2013). This research investigates the effects of endogenous phyto-hormones on *S. europaea*'s resistance to salinity stress, as well as the biochemical and molecular responses of key genes and enzymes responding to salinity stress in this plant.

Materials and methods

Plant materials and stress treatment

This research was conducted at Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran, in greenhouse conditions. Plants were cultivated in pots as a factorial experiment in a completely random basic design with three replications. In this experiment, the effects of two salinities (control and 400 mM levels) were investigated on the vegetative growth of *S. europaea* and four spraying treatments (50 µM ABA, 50 µM melatonin, combined effects of both hormones, and a control without spraying) and. The hormones were sprayed on the shoots. The initial salinity of the soil used to fill seedling trays and pots was 6.6 dS/m (Table. 1). Seedlings were planted in pots with a diameter of 12 cm. The bottom of the pots was first filled with gravel for good drainage. Then, each pot was filled with soil (30%), cocopeat (20%), sand (30%), and leaf mold (Gunning 2016).

In this study, the seeds of *S. europaea* (Gorgan ecotype) were collected from the seacoast of Gomishan (Herbarium code: 2559). To prepare uninfected seedlings, seeds were disinfected using a 1% sodium hypochlorite solution for 2 min and then completely washed with distilled water. Seeds were then disinfected using 70% ethanol for 1 min, washed again with distilled water, and then fully washed with autoclaved distilled water. The seeds were sown in seedling trays in the greenhouse in the first half of March 2021. After the establishment stage, 5-6 seedlings were transferred to each pot in April 2020. Plants were kept at 28-33 °C day and night with 65-75% humidity and irrigated with unsalted water as the conventional irrigation cycle (2-3 days) for 1 month. A nutritional solution with 30 mg in 1 L of water was given twice a week, and nitrogen fertilizer (2-3 g per pot) was applied every 2 weeks. The soil was analyzed after the completion of sampling (Table. 1). Then, salinity (NaCl) treatment was applied at a level of 400 mM. Treatments were irrigated conventionally with salt water once every 2-3 days. Melatonin (50 µM), ABA (50 µM), and the combination of both hormones were sprayed once every 7 days (totally 4 times) for 1 month. Roots and stems were sampled from the control and 400 mM treatments at four intervals of 0, 6, 48, and 720 h after spraying. Samples were frozen with liquid nitrogen for the analysis of traits and molecular/biochemical assays and then kept at −80 °C.

Sample	EC ($Ds.m^{-1}$)	Ca+ Mg (Meq/L)	Na (Meq/L)	SAR
Control	6.6	15.6	60.5	21.7
400 mM	44	33.6	341.25	83.2

Table. 1 Soil chemical profiles in the experimental treatments.

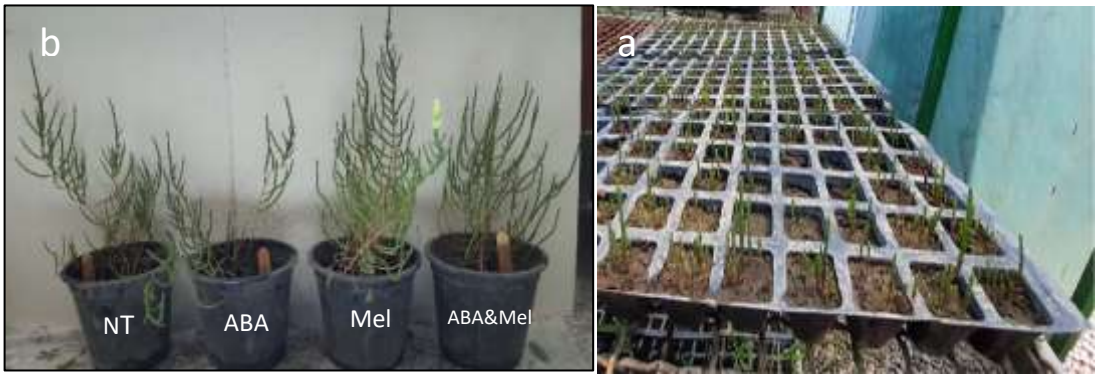


Fig. 1 a) *S. europaea* seeds sown in seedling trays; b) Salinity stress (400 mM) pots at 720 h and spraying (left to right): without hormone, ABA, Mel, and the combination of both hormones

Measurements of shoot and root fresh and dry weights within 1 month

Shoot and root fresh and dry weights (g) were measured by weighing with a balance (0.1 mg accuracy) within 1 month. Samples were dried in an oven at 70 °C for 48 h (Gorgini Shabankareh etal., 2018) and then weighed by a balance (0.1 mg accuracy) to determine total shoot DW.

Measurements of Na, K, and Ca in roots and shoots

To measure Na, K, and Ca in root and shoot samples prepared at 0 (control), 6, 48, and 720 h (after stress) in the control and 400 mM salinity levels, each sample was dried separately at 70 °C for 48 h and then weighed (g) by a balance (0.1 mg accuracy). Next, 0.5 g of powdered samples were prepared for Na, K, and Ca measurements. To extract plant samples, 0.5 g of the powdered sample was weighed and placed in a porcelain jug to convert it to ash in an electrical furnace at 550 C for 3 h. After cooling, samples were dissolved in 5 ml of 2 M HCl and then transferred to a water bath at 95 °C for 1 h. Afterward, samples were taken out from the water bath and filtered by a funnel and Whatman filter paper (Ryan et al., 2001). Na and K were measured using a flame photometer (Model PEP7, UK). Ca was measured using an atomic absorption spectrometer (Perkin-Elmer Model 3030). The ion content was expressed in mg/g DW.

Root ion leakage

Electrolyte leakage was assessed by measuring electrical conductivity (EC) as a measurement criterion for cell membrane stability. To this aim, leaf pieces (1-2 cm) were separated and washed in a Petri dish containing distilled water for several minutes. The pieces were then transferred to falcons containing 10 ml of distilled water. EC was measured in each sample 30 s after their vortex. Samples were shaken at room temperature for 24 h, followed by measuring EC1. After incubation in an autoclave for 15 min, samples were cooled at room temperature, and EC2 was measured for the third time. Electrolyte leakage (%) was then calculated with equation (1) (Zhao, 1999):

$$\text{Electrolyte leakage } (\%) = \frac{EC1-EC0}{EC2-EC0} \times 100 \qquad (1)$$

H_2O_2 and MDA measurements

H_2O_2 content in the supernatant was analyzed at 415 nm wavelength as described by Brennan and Frenkel (1977). Lipid peroxidation was measured based on the MDA biomarker measurement (Heath and Packer, 1968). In this method, the optical density of samples was read at 532 nm wavelength and subtracted from 600 nm wavelength using the extinction coefficient (ε= 155 mM^{-1} cm^{-1}). MDA content was expressed based on µM/g fresh tissue.

Proline and total phenol measurements

Based on the method proposed by Bates (1973), proline content was determined and assessed quantitatively in the samples using a standard curve and spectrophotometry at 520 nm wavelength. Total phenol content in the shoot extract was quantified by colorimetry using Folin-Ciocalteu's method, which is based on Folin's reagent reduction in an alkaline environment and a blue complex formation, showing maximum absorbance at 765 nm wavelength. A standard curve was drawn based on different Gallic acid

concentrations, and plant phenolic compounds equivalent to Gallic acid were measured as mg/g plant dry powder (Slinkard and Singleton 1977).

Evaluation of antioxidant enzyme activity

To extract the enzyme in *S. europaea*, 1 g of the frozen plant tissue from root and shoot samples was completely powdered using liquid nitrogen in a porcelain mortar. Then, it was mixed with 1 mL of ice-cold extraction buffer [100 mM of phosphate buffer (pH 7.0) with 1 mM of phenylmethylsulfonyl fluoride (PMSF) and 1% (w:v) polyvinyl-polypirrolidone (PVPP)]. Samples were then centrifuged (12,000 g at 4 °C for 20 min) and the supernatant was used for SOD, CAT, PPO, and POX enzyme activity assays. APX activity was assessed in the presence of 1 mM ascorbate (AsA). The absorbance of samples was detected by a spectrophotometer (Kontron, Milano, Italy). SOD activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin under light exposure. One unit (U) means the amount of SOD causing 50% inhibition of the NBT reduction under light exposure. CAT activity was measured by detecting the consumption of H_2O_2 at 240 nm at 25 °C for 3 min (ε_{240} = 39.4 mM^{-1} cm^{-1}). One unit of CAT activity means the amount of enzyme needed to decompose 1 µmol of H_2O_2 per min. POX activity was determined as the increase in absorbance because of the oxidation of guiacol. One unit corresponds to the amount of the enzyme producing 1 µmol min^{-1} of oxidized guaiacol. APX activity was measured by the decrease in AsA amount at 290 nm for 3 min at 25 °C (ε_{290} = 2.8 mM^{-1} cm^{-1}). One unit of APX activity means the amount of enzyme needed to oxidize 1 µmol min^{-1} of As A (Tari et al. 2015).

PPO activity was assessed according to Gonzalez et al. (1999). The first control cuvette contained 2.7 L of potassium phosphate buffer solution and 0.3 ml of the substrate, but the second cuvette contained 2.8 L of potassium phosphate buffer and 0.2 ml of enzyme extract. The absorbance of these controls was determined by measuring the absorbance increase from the sample at 420 nm wavelength. Accordingly, absorbance changes at 0.001 min per ml of the enzyme were the enzyme activity criterion.

qRT-PCR (quantitative real-time PCR)

RNA was extracted from plant tissues, and first-strand cDNA was obtained using a PrimeScript II 1st Strand cDNA Synthesis kit. Each reaction consisted of 7.5 µL of 2 × SYBR Green II mixture, 1 µL of the template, 2 µL of gene-specific primers (10 µM), and 4.5 µL of water. qRT-PCR reaction conditions were 95℃ for 30s, 95℃ for 5 s, and 60℃ for 30 s (35 cycles). The gene expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001) and SdActin (ID: DQ243702) as the internal reference gene in Arabidopsis. The qPCR primers used in this study are listed in Supplementary Table 2.

Analysis of data

Data were analyzed statistically by the analysis of variance (ANOVA) and comparisons of mean biochemical traits using SAS 9.1.3 software. Means were compared by the LSD method, and significant differences were shown at 5% and 1% probability levels. Gene expression levels were assessed and calculated using the comparative C_T ($2^{-\triangle\triangle CT}$ method. Data obtained between target genes and the reference gene from the qRT-PCR device were analyzed by REST 2000 software (Pfaffl, 2001) based on equation (2). Graphs were drawn with Excel software.

$$R = \frac{(E_{target})^{\Delta CP target\ (MEAN\ control - MEAN\ sample)}}{(E_{ref})^{\Delta CP ref\ (MEAN\ control - MEAN\ sample)}} \quad (2)$$

Table 2.

Gene name	Primer name	Annealing temp. (°C)	Primer length (bp)	Primer sequence
Se.SOS1	Forward	56.9	3292	TCGGAAGGATCTACTGCTGT
	Reverse	57.1	3419	TCTCCACCAGATTCCGAACT
Se.NHX1	Forward	57	8287	GGCTCGCCTATTGAACAGAA
	Reverse	57.7	8404	GGCTCGCCTATTGAACAGAA
Se.VHA	Forward	57.6	2065	GCTGGTTGGAGTTGTTGCT
	Reverse	56.9	2190	GCTGGTTGGAGTTGTTGCT
Se.SOD	Forward	56.5	452	TGACACCACTCCAAATCAGG
	Reverse	57.2	615	AGGTAGTATGCATGCTCCCA
Se.HKT1	Forward	58.3	1174	GATCTTTCCTCCATTGCACCAG
	Reverse	58.4	1351	GGCTGAGCTGTGAAAATAGGAC
Sb.AsPX	Forward	58.4	701	CGCCCTTTAGTTGAGCTGTATG
	Reverse	58.2	835	TACAGTGCTATCCTTGGGGC
Sd.ACT	Forward	57.5	303	TCACAACATCAGCCGAACG
	Reverse	57.8	474	GCAACGGAATCTCTCAGCTC

Table. 2 The characteristics of primers used in the qRT-PCR test (www.embnet.sk/cgi-bin/primer3_www.cgi)

Results

Root and shoot FW and shoot DW

In the interaction of salinity and hormones, root and shoot FW increased with rising salinity up to 400 mM together with spraying. The highest increase was recorded in the treatment with spraying both hormones (202 and 76% for shoot and root, respectively), which were significantly different from the control. The combined ABA and melatonin spraying led to a maximum increase in the shoot DW (245% or 2.45 times) at 400 mM salinity (Fig. 2).

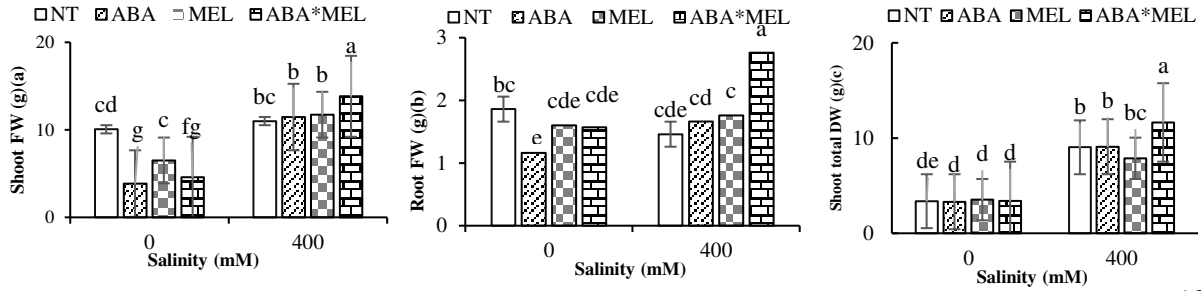


Fig. 2 The interaction effect of salinity stress and hormone on: a) plant fresh weight, b) Root fresh weight, c) plant dry weight in *S. europaea*

Ionic elements

The Na ion in the *S. europaea* root was not significantly different from NT samples in spraying with melatonin and both hormones whereas ABA spraying at 720 h elevated Na levels at both salinity levels. Shoot Na content decreased in the control salinity level at 720 h while it increased in spraying treatments. Spraying with both hormones reduced the Na level at 400 mM salinity compared to the other treatments. Unlike glycophytes, Na is a nutritive element in halophytes. In other words, the growth of halophytes decreases at low salt concentrations and cannot complete their life cycle. In a study, Na concentrations increased in shoots and roots with increasing salinity stress application (both 200 and 600 mM of NaCl at 0.01 and 1 mM concentrations) plus ABA compared to the control with 0.01 and 1 mM of ABA (Shafiq et al. 2021).

The three-way effect of salinity, hormones, and time was significant on Ca content in roots and shoots at the 1% level. It remained almost unchanged in the root no-spray treatment at 6 and 48 h after 400 mM salinity application compared to time zero while it slightly decreased in the shoots at 6 h. However, Ca content rose in both organs at 720 h, with a more noticeable increase in shoots. An increase in Ca content was observed in ABA and melatonin spraying in both control and 400 mM treatments over time, with a greater increase in shoots and melatonin spraying. Ca content remained almost unchanged in spraying with

both hormones at the first 6 h of treatment application, but Ca levels rose over time at 48 h. Root Ca content increased significantly in the control salinity level at 720 h and was uppermost (79% versus the control) among the other treatments, but it declined at 400 mM salinity. Ca levels rose continuously in shoots in the control and 400 mM salinity at 720 h.

Evidence indicates that ABA spraying during salinity stress produces various responses in element levels in *S. europaea* roots and shoots (Barragan et al. 2012). During the experiment, K reserves increased in the plant roots and shoots to counteract salinity stress, but this increase was higher in spraying treatments at both control and 400 mM salinity levels than in the treatment without spraying. The utmost rises of K were recorded in shoots and roots in spraying with both hormones, the control, and 400 mM salinity (17.64, 21.73, 19.13, and 15.72, respectively). In a previous study, the K ion concentration decreased with rising salinity stress to 600 mM (Aghaleh et al. 2010). To maintain homeostasis, plants usually try to lower the Na/K ratio (Zahedi et al. 2020). Spraying treatments positively affected the Na/K ratio. This ratio showed the highest reductions in roots and shoots in spraying with both hormones, the control, and 400 mM salinity (35, 19.23, 30.76, and 22.22, respectively) at 6 h after spraying relative to control plants without spraying. These reductions were followed by melatonin spraying in roots in the control and 400 mM salinity levels (15.78& and 15.23%, respectively) compared to control plants. The Na/K ratio reductions corresponded to the elevated K levels (Fig. 3).

Electrolyte leakage (LE)

The survival and growth of halophytes depend on high levels of ion accumulation in their tissues for turgor maintenance and osmosis regulation (Qarehkhani et al. 2016). A reduction in water potential at 100 mM and lower salinities, ionic balance disruption, and ionic toxicity compromise the growth of salinity-sensitive plants.

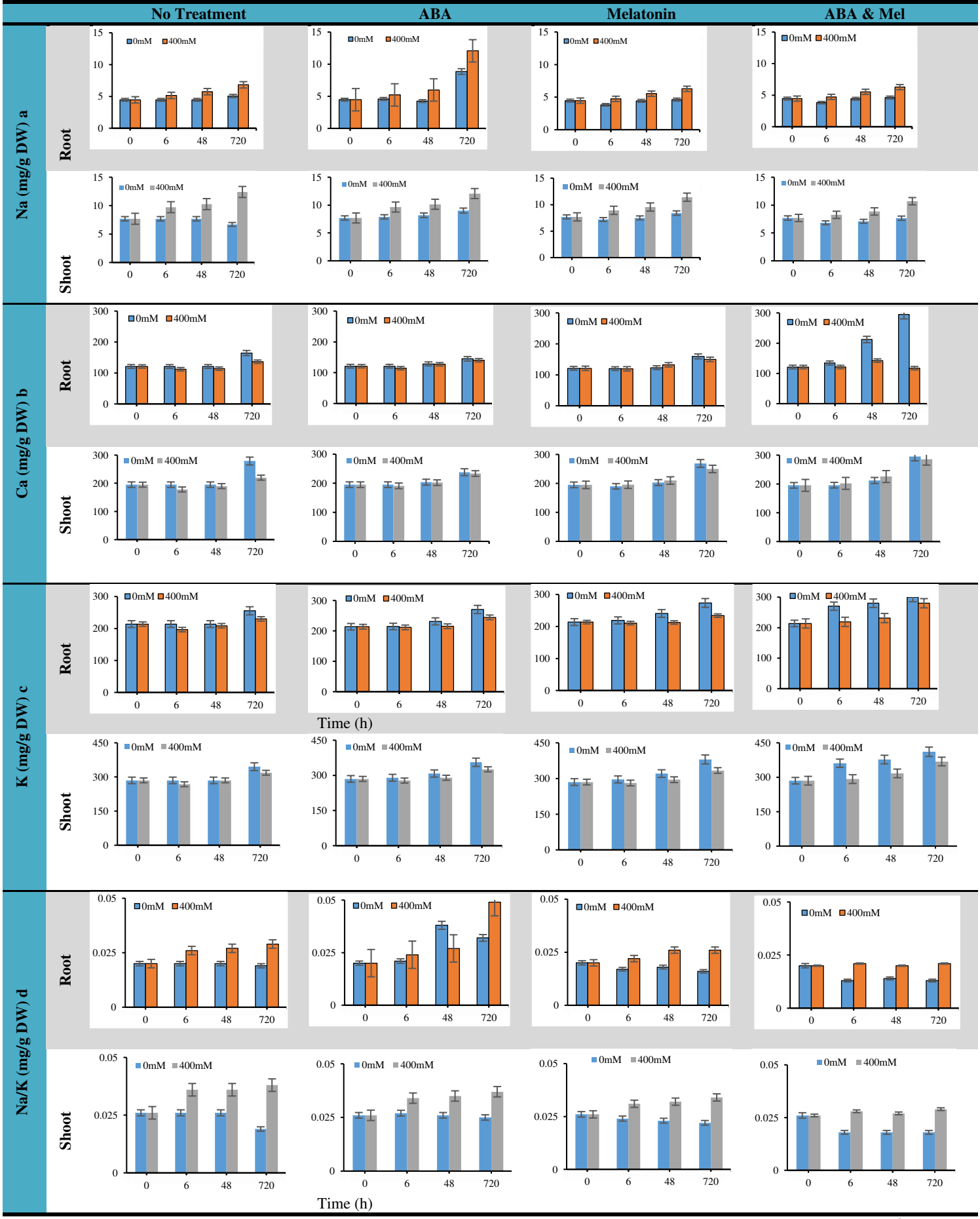


Fig. 3 Concentrations of ionic elements: a) Na, b) Ca, c) K, and d) Na/K at determined intervals and control and 400 mM salinity stress in *S. europaea* roots and shoots

However, halophytes are plants in which the water potential of their tissues becomes more negative relative to the water of the soil texture where they grow (Darvishi 2013). According to ANOVA results for the LE trait, the simple effect of the hormones and the interaction of salinity and hormones were significant at 1% and 5% levels, respectively. LE decreased in spraying treatments at the control and 400 mM salinity levels (except for ABA spraying at 400 mM salinity) in comparison to NT plants in the same group. The highest reductions were observed at the control and 400 mM salinity levels (41.32% and 48%, respectively) in exogenous melatonin application (Fig. 4).

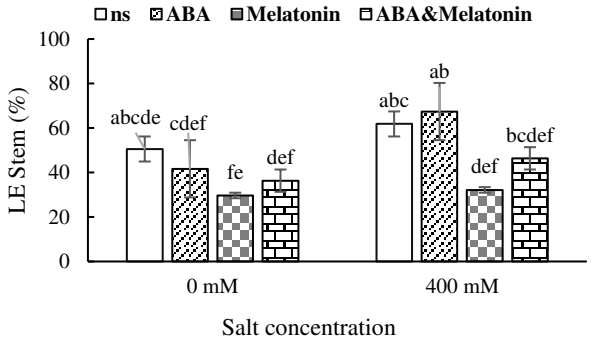


Fig. 4 Interaction effects of salinity stress and hormones on the LE stem in *S. europaea*.

PPO and total phenol in shoots

PPO is the key enzyme responsible for the oxidation of phenolic compounds. Among spraying treatments, spraying with both hormones at both salinity levels led to the lowest enzyme activity, which was not significantly different from NT plants. The reduced PPO activity can be attributed to the enzyme's rapid proteolysis and denaturation under osmosis stress (Torabi and Nicknam, 2011). The phenol content increased continuously at both control and 400 mM salinity levels in all treatments in the long term, with a greater increase at 400 mM salinity stress because is *S. europaea* a halophyte. The shoot phenol content increased by 31, 21, and 3% in treatments without spraying and ABA/melatonin spraying at the 400 mM salinity level for 6 h compared to zero time. However, lower increases were observed in spraying treatments than in unsprayed plants (Fig. 5).

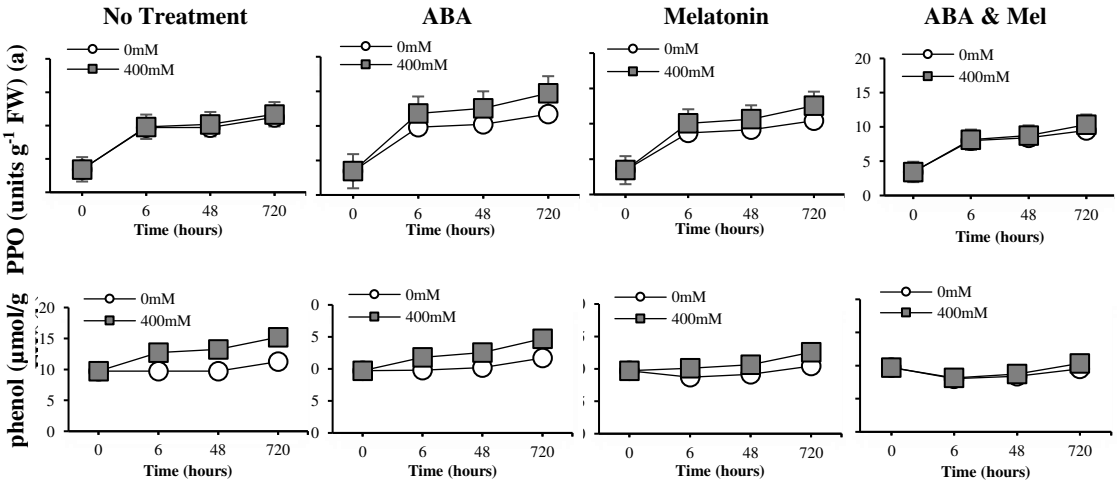


Fig. 5 The contents of a) PPO and b) phenol in control and 400 mM salinity stress levels at determined intervals in *S. europaea* shoots

MDA in shoots and H2O2 in roots and shoots of S. europaea

The shoot MDA content decreased in all spraying treatments (except for ABA spraying at 400 mM salinity) compared to NT plants. The highest reduction was recorded 6 h after spraying both hormones concurrently (35 and 23%, respectively) at the control salinity, which increased mildly at the next hours. The concurrent exogenous use of both hormones resulted in the utmost reduction in the MDA content; thus, the concurrent spraying of both hormones more positively affects shoot MDA content. The use of ABA (0.01 and 1 mM) plus drought treatments reduced shoot MDA content in *S. europaea*, and the treated plants

were not significantly different from the control plants. The spraying positively affected root MDA content(Fig. 6). The ABA treatment of plants under 200 and 400 mM salinity stress reduced root MDA content compared to plants under salinity stress without spraying (Darveishi 2013). All spraying treatments reduced root H_2O_2 levels in *S. europaea* compared to control plants. The greatest reductions were measured at control salinity 6 h after spraying with both hormones (52.17% decrease), followed by melatonin spraying (29.1% decrease). Although H_2O_2 levels increased in spraying treatments (except for the reduction in spraying with both hormones), their levels were significantly lower than the control plants. As with roots, shoot H_2O_2 levels decreased in all spraying treatments, and the utmost reductions were detected in the control and 400 mM salinity levels at 6 and 48 h after spraying with both hormones (29.36, 17.15, 17.18, and 27.38% reductions, respectively) and melatonin spraying (18.93, 12.13, 16.6, and 15.05% reductions, respectively). Although H_2O_2 levels decreased mildly at 720 h, this elevation was lower than that in NT plants (Fig. 7).

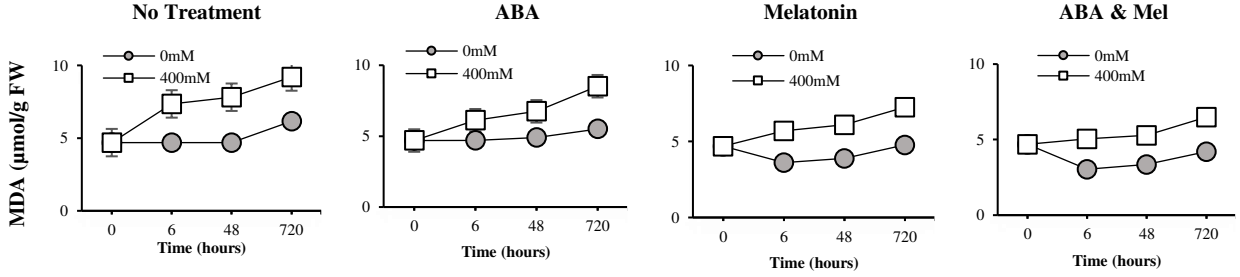


Fig. 6 MDA contents in *S. europaea* shoots in control and 400 mM salinity stress levels at determined intervals

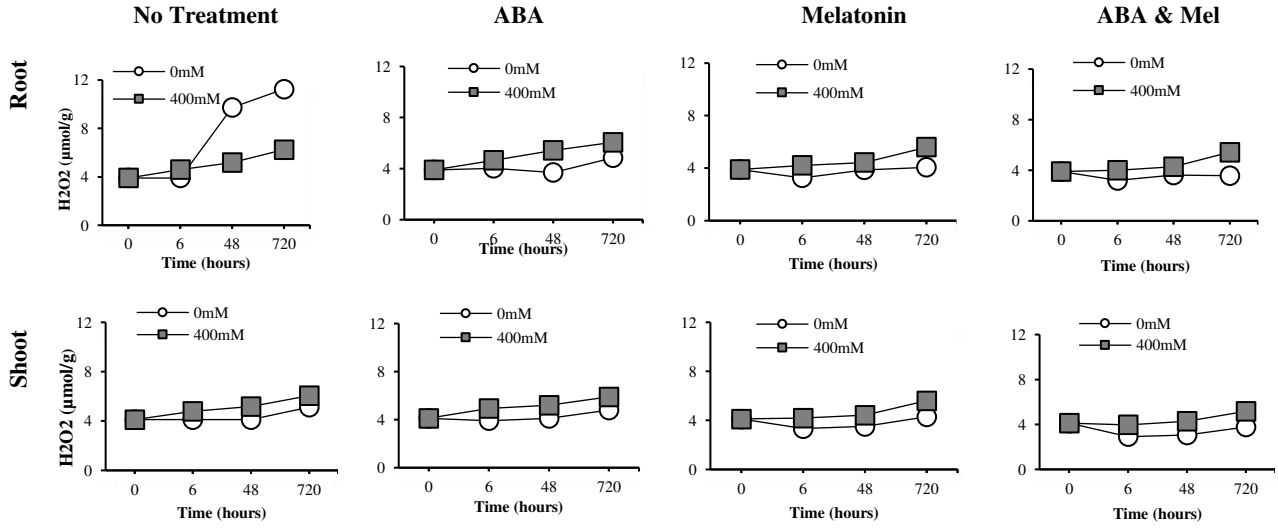


Fig. 7 H_2O_2 contents in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

Shoot proline content

Proline is involved in the protection of enzymes and cellular structures and acts as a free radical scavenger (Mosoudian et al. 2013). Proline content increased initially in ABA spraying at 400 mM salinity, but it did not change significantly at the no salinity level. Shoot proline content decreased in melatonin spraying at 400 mM salinity after 6 h, followed by a higher level than zero time. Proline content decreased heavily in spraying with both hormones at the control and 400 mM salinity levels after 6 h, and then rose at a lower level than zero time. Shoot proline content was higher in NT samples at the control and 400 mM salinity levels than in all experimental treatments, with greater content in 400 mM salinity treatment (Fig. 8). Plants treated with 50, 100, and 150 µM of ABA showed frequent changes in protein expression compared to plants in the control and 10 µM of ABA treatments. Among all concentrations, higher growth rates were observed in plants treated with 50 µM of ABA (Anbarasi et al. 2018).

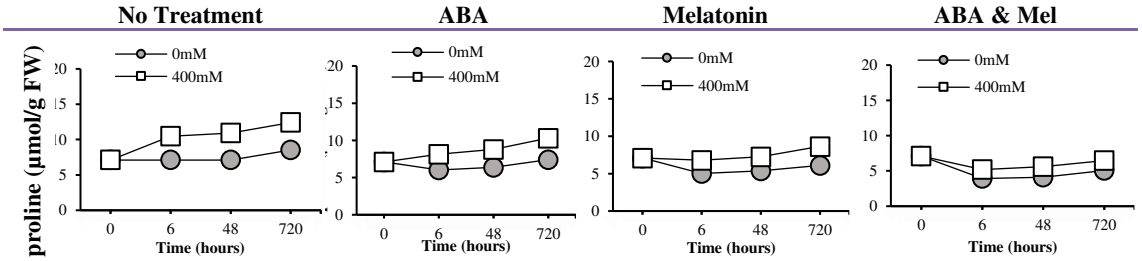


Fig. 8 Proline contents in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

Antioxidant enzymes

SOD content

SOD catalyzes superoxide to molecular oxygen and H_2O_2 and, therefore, reduces the risk of hydroxyl radical formation from superoxide (Yanase et al. 2020). The root enzyme content declined (3.43%) by ABA spraying at the control salinity after 6 h, it increased at 48 h, and then decreased at 720 h. Combined spraying with both hormones led to the highest reduction in enzyme activity (28.57% and 25.95% at control and 400 mM salinity stress levels) after 6 h. SOD activity in shoots reached a lower level in melatonin spraying than that in control plants. As with roots, SOD activity in shoots decreased by 53.35% and 35.79% after 6 h spraying with both hormones at the control and 400 mM salinity stress levels, respectively. Although the enzyme activity rose in all studied cases in the long term (720 h), this increase was lower than the enzyme activity at the control and 400 mM salinity stress levels without spraying (Fig. 9).

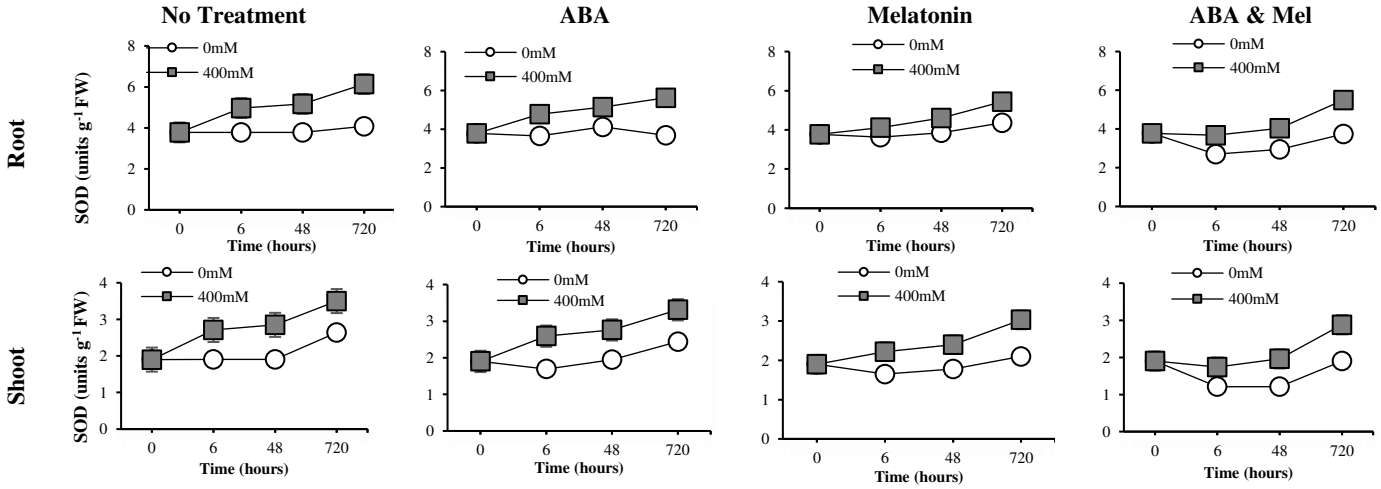


Fig. 9 SOD activity in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

POX activity

The enzyme activity in roots was almost unchanged in all treatments at early 6 and 48 h. It decreased in the long term (720 h) at 400 mM salinity, with the least reduction recorded in spraying with both hormones, followed by melatonin spraying. These two spraying treatments led to no changes in the enzyme activity at the control salinity level. The enzyme activity decreased heavily in the shoots of *S. europaea* in the 1st h after spraying treatments compared to control plants, but it rose steadily contrary to control plants without spraying. Under stress, the root uses the produced H_2O_2 as the substrate. With a longer stress duration, the substrate increases in the root, which saturates the substrate site in the enzyme and reduces enzyme activity (Fig. 10). A study on POX activity in saffron was indicated that the POX reaction rate decreased with increasing the guaiacol substrate level to 70 mM. At a constant H_2O_2 concentration, this experiment demonstrated that complete enzyme activity would be obtained when two substrate molecules bind to the enzyme or two substrate-binding sites would occur on the enzyme (Rahmani et al. 2013). Our findings are similar to those of a previous investigation (Jia et al. 2019). In tomatoes, melatonin can reportedly act as a direct free radical scavenger and an antioxidant response stimulant, both functions leading to a reduction in

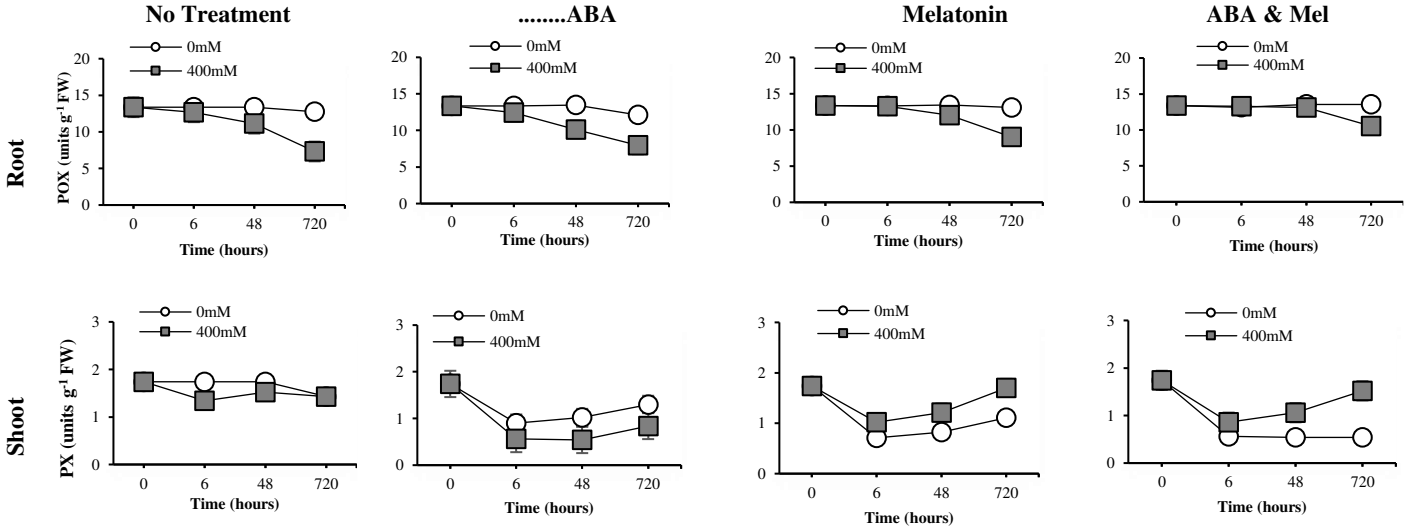


Fig. 10 POX activity in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

ROS levels. This role of melatonin may be the reason for reductions in some antioxidant enzymes (Xu et al. 2016).

CAT activity

CAT activity was only studied in shoots. In spraying control samples, the enzyme activity decreased in salinity-free control treatment at 6 and 48 h and then increased at 720 h. CAT activity increased significantly at 400 mM salinity at early 6 h, and this increase continued over the long period. In all spraying treatments (ABA, melatonin, and both hormones), CAT activity did not change markedly in the salinity-free sample at 6 and 48, then rose in the 1-month period, and increased at 400 mM salinity from the beginning (Fig 11). CAT and POX act the same as two sides of a scale (Rahmani et al. 2013). Although POX activity seems to be reduced during the experiment to scavenge H_2O_2, this reduction is compensated for by an increase in CAT activity. In *S. persica*, CAT activity decreased at 100 mM of NaCl salinity and then increased with rising salinity stress (Aghaleh et al. 2010).

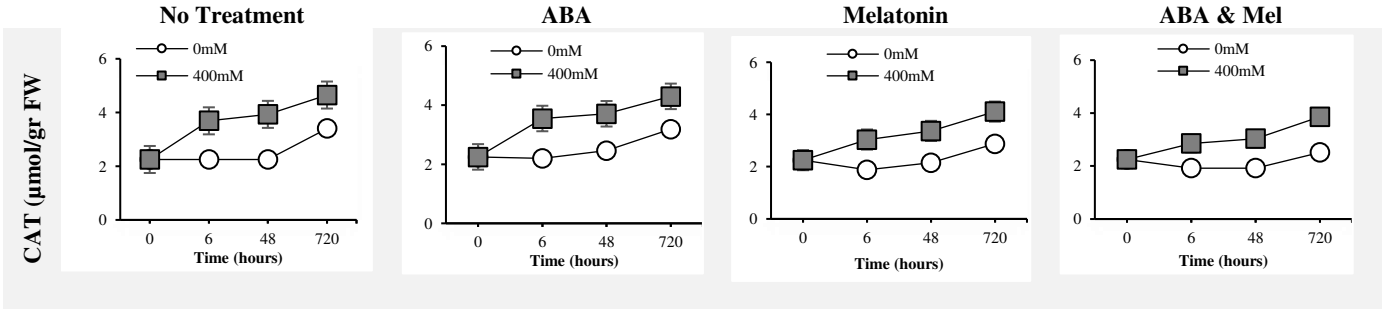


Fig. 11 CAT activity in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

Molecular results

SOD gene expression levels

The root is the first organ facing salinity stress. As mentioned above, the difference between halophytes and glycophytes in resistance to stress is their rapid responses to stressful conditions (Mou et al. 2020). *SOD* gene overexpression was observed in *S. europaea* roots by ABA spraying at control salinity and by spraying with both hormones and melatonin at 400 mM salinity after 6 h spraying treatments. The highest increase belonged to the concurrent exogenous use of both hormones. In *S. europaea* shoots, however, *SOD* gene overexpression only occurred in melatonin spraying after 6 h spraying. The *SOD* gene was also overexpressed in long-term (720 h) ABA spraying at 400 mM salinity and the exogenous use of both hormones at the control salinity level (Fig. 12). The expression of *SOD* and *CAT* genes increased in *Camellia sinensis* by 100 µM of melatonin spraying after 12 h compared to untreated plants (Li et al. 2019).

No Treatment **ABA** **Melatonin** **ABA & Mel**

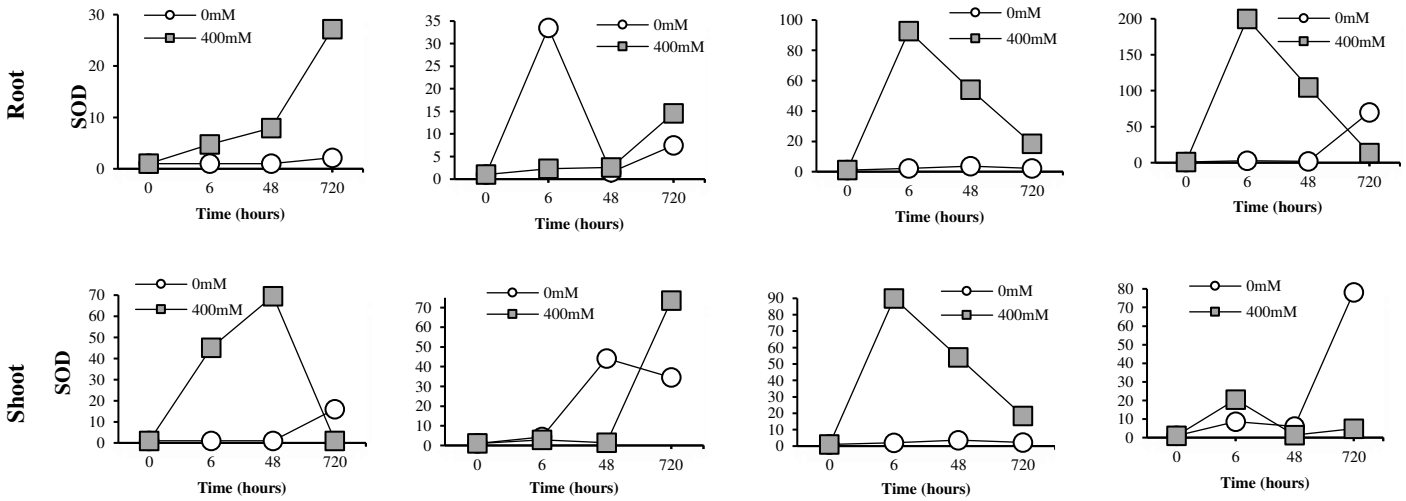


Fig. 12 Effects of spraying and salinity stress on *SOD* gene expression levels in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

SOS1 gene expression

In *S. europaea* roots, *SOS1* gene expression was maximized at 400 mM salinity in NT samples. The gene expression increased in ABA spraying at 48 h, in melatonin spraying at 6 and 720 h at 400 mM salinity, and in spraying with both hormones at control salinity after 6 h. *SOS1* gene expression increased mildly after spraying with both hormones at 400 mM salinity stress. Unlike roots, *SOS1* gene expression remained almost unchanged in *S. europaea* shoots at 400 mM salinity stress while the control salinity spraying caused the gene overexpression at some hours. The gene overexpression was observed in ABA and melatonin spraying without salinity stress after 6 h, which was conspicuously higher in melatonin spraying (Fig. 13).

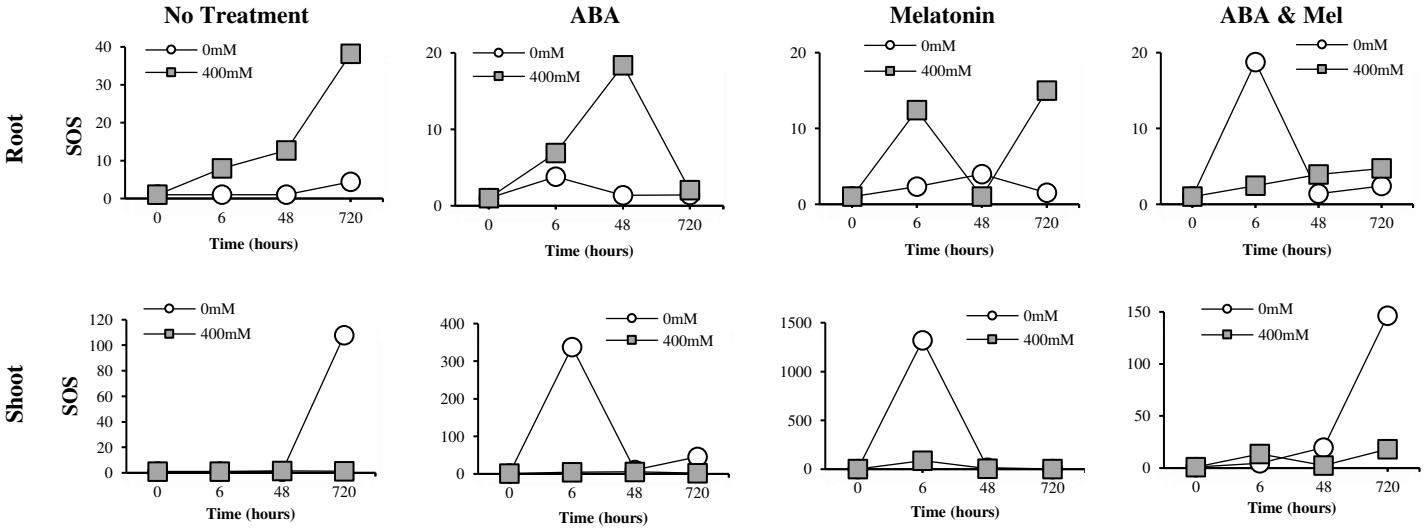


Fig. 13 Effects of spraying and salinity stress on *SOS1* gene expression levels in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

NXH1 gene expression

In *S. europaea* roots, *NXH1* gene expression in NT samples was almost unaffected in all experimental treatments, but it was overexpressed at 400 mM salinity stress in the early hours. The gene expression was uppermost in spraying with both hormones 48 h after the treatment application. In *S. europaea* shoots, the maximum *NXH1* gene expression was recorded at 720 h after spraying with both hormones at the control salinity level. Melatonin spraying at the 400 mM salinity level caused gene overexpression at all studied hours (Fig. 14).

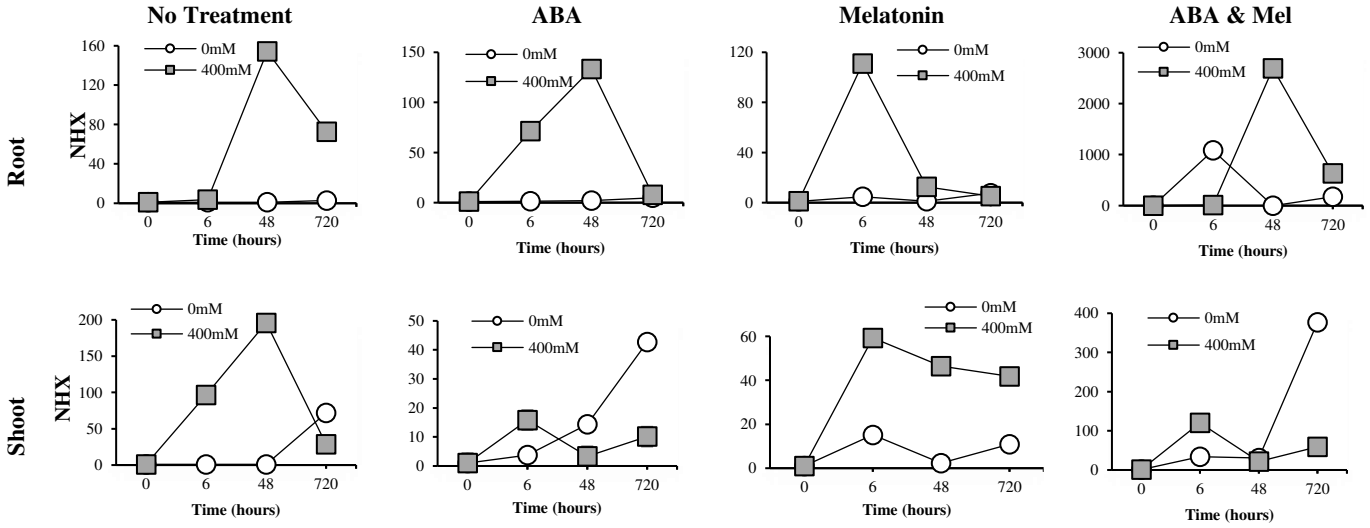


Fig. 14 Effects of spraying and salinity stress on *NXH1* gene expression levels in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

APX gene expression

Unlike NT plants, *APX* gene expression increased in *S. europaea* roots by spraying at the control salinity level. The highest gene expression (45.36 times) was observed 6 h after spraying with both hormones at 400 mM salinity. Although the gene expression was upward in NT samples, the highest gene expression (2.72 times) was recorded 6 h after ABA spraying. A high gene expression in shoots was noticed in NT samples at the control salinity level after 720 h, while it was almost zero in the other treatments. The gene expression rose by spraying treatments at both salinity levels. The gene was expressed 6 h after ABA and melatonin spraying and 720 h after spraying with both hormones (119, 214.26, and 35.66 times, respectively) at the control salinity. Melatonin spraying at 48 h led to the highest gene expression level at 400 mM salinity stress (Fig. 15).

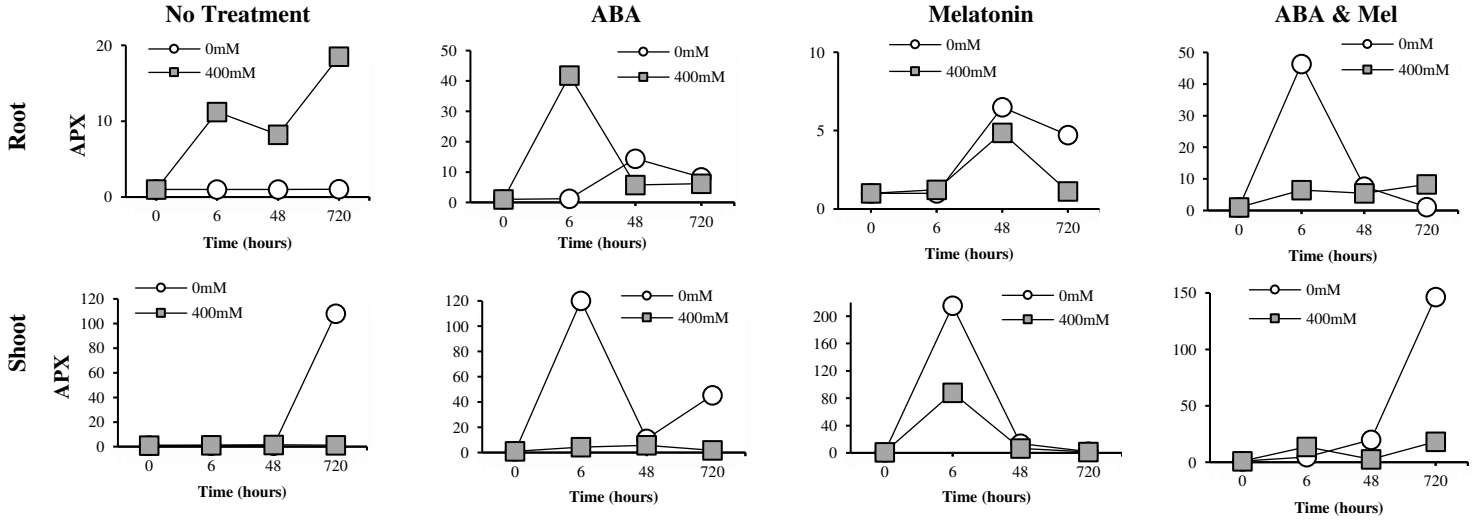


Fig. 15 Effects of spraying and salinity stress on APX gene expression levels in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

HKT1 gene expression

Compared to NT plants, *HKT1* gene expression increased in *S. europaea* roots by all spraying treatments, particularly at 400 mM salinity stress. At this level, the highest gene overexpression (23.59% versus the control) was observed 6 h after spraying with melatonin. As with roots, the gene expression rose in *S. europaea* shoots in all spraying treatments as opposed to control treatments. A high overexpression was

noticed 6 h after melatonin spraying at the 400 mM salinity level, 6 h after ABA spraying, and spraying with both hormones at the control salinity level (Fig. 16).

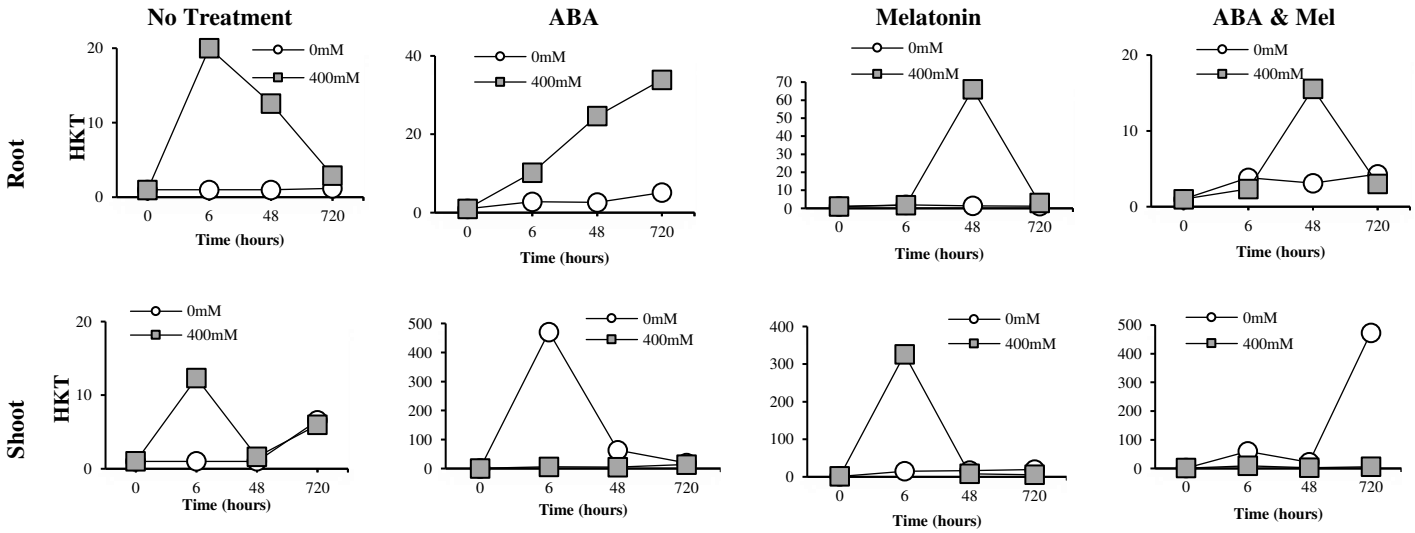


Fig. 16 Effects of spraying and salinity stress on HKT1 gene expression levels in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

VHA-A gene expression

Compared to control plants, *VHA-A* gene expression increased in *S. europaea* roots by all spraying treatments. The highest gene overexpression was observed at 400 mM salinity stress 48 h after spraying with both hormones. The gene expression rose in *S. europaea* shoots in all spraying treatments. At the control salinity level, the gene expression was maximized 6 h after ABA spraying and 6 h after melatonin spraying (27.52 times) at 400 mM salinity stress (Fig. 17).

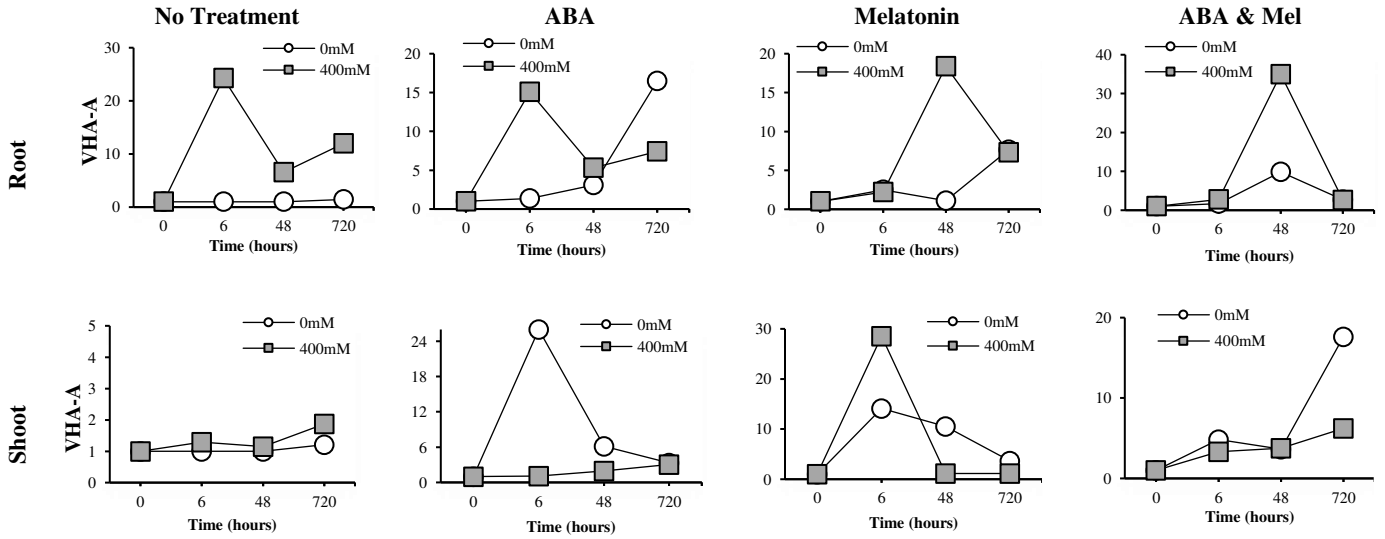


Fig. 17 Effects of spraying and salinity stress on VHA-A gene expression levels in *S. europaea* roots and shoots in control and 400 mM salinity stress levels at determined intervals

Discussion

In salinity stress conditions, plant growth decreases for two reasons: salt accumulation in shoots and the onset of processes counteracting salinity. These two phenomena are closely associated with plant response time: immediate response (from a few minutes to a few days) and long-term response (from a few days to a few weeks) (Mou et al. 2020). Accordingly, the biochemical and molecular traits of *S. europaea* were investigated at short times (0, 6, and 48 h) and long-term (720 h or 1 month) response phases of the plant to salinity stress, as well as the effects of spraying with hormones (ABA, melatonin, and both hormones concurrently) affecting plant tolerance to biotic and abiotic stresses.

In the primary plant's response, the osmotic effect of salt, which reduces primary growth, is observed in both resistant and sensitive species. Thus, the difference between halophytes and glycophytes is related to the second phase and, consequently, to the plant's ability to prevent salt accumulation in tissues and its toxic effects (Liu et al. 2020). In less-tolerant plants, slow responses to regulate water potential lead to water loss and less-efficient tolerance mechanisms (Hrynkiewicz et al. 2019). Thus, such methods and exogenous applications, which can produce rapid plant response (in the early hours of stress incidence), will effectively increase the plant's tolerance to environmental stresses.

ABA is the first hormone produced when the plant suffers from stress. A plethora of studies indicate that the melatonin level increases heavily as stress clues when the plant is exposed to rapid environmental changes. These stresses may include heat, cold, salinity, drought, excessive irrigation, UV light, and chemical/metal contaminants (Franzoni et al. 2019). Melatonin also exerts a regulatory effect on the expression of genes responsible for ABA and gibberellic acid production (Zhang et al. 2014a). Therefore, the exogenous use of melatonin and ABA and the effect of both hormones were investigated in this study.

The interaction effect of salinity, hormones, and time of root Na content was significant at the 1% probability level. The interaction effect of salinity and hormones was significant on *S. europaea* shoots at the 1% probability level. Thus, simultaneous spraying with both hormones reduced the Na content in *S. europaea* shoots and roots (Fig. 3a). In halophytes, Na acts as a nutrient and effective osmo-regulator to maintain cell permeability, which increases growth and photosynthesis capacity, probably suggesting the presence of an Na-specific catalyst enzyme in halophytes (Lv et al. 2020). Na abundance in the soil disturbs K uptake by the plant (Shafiq et al. 2021). Salinity stress in the plant rapidly increases the Na^+ level in the cytoplasm through Nonselective Cation Channels (NSCCs) and then via the K^+ transporter, HKT1. Elevated cell Na levels disturb cytosolic Ca and increase the Na released in the cell (Frukh et al. 2019). Ca^+ is reportedly involved in both early and long-term responses to salinity stress (Tuteja 2007). The utmost Ca contents were observed by the concurrent exogenous application of melatonin and ABA in roots and shoots, as well as by exogenous melatonin application in shoots, after 720 h of the treatments (except for the 400 mM root treatment) (Fig. 3b). A feature of stress-tolerant plants is their maintenance of a low Na/K ratio, which is important to produce ionic homeostasis and proper function of plant enzymes in exposure to salinity stress (Zahedi et al. 2020). Among spraying treatments, the combined spraying with both hormones increased K content in roots and shoots after 6 h of spraying compared to NT treatments (Fig. 3c), and consequently, the minimum Na/K ratio was obtained in these treatments (Fig. 3d). In a study on rice seedlings, exogenous melatonin maintained Na/K homeostasis and boosted rice tolerance to salt stress (Yan et al. 2020). Na/K homeostasis seems to be maintained by melatonin through increasing the activity of H^+ pumps creating the gradient required for more activity of SOS, NHX, and HKT antiporters (Yan et al. 2020). In an investigation on the strawberry plant, salinity effects were not apparently reduced with melatonin treatments completely, and a significant increase in Na levels and reduced concentrations of Mg, P, K, and Ca were observed compared to non-salty controls treated with the same melatonin dose (Zahedi et al. 2020).

Exogenous melatonin application reduced cell K content (Noor shah et al. 2021) and resulted in the highest level of LE reduction in our study (Fig. 4). At determined intervals, shoot phenol content increased in the long term, but this increase was lower in spraying treatments than in NT ones (Fig. 5b). Phenolic compounds are a large group of natural plant substances, including flavonoids, tannins, and anthocyanin. Flavonoids and other phenolic compounds are widely distributed in plants, and the biological activities of these compounds include antioxidant, antimicrobial, and anti-inflammatory properties. They also activate antioxidant enzymes and reduce free radicals and α-tocopherol, prevent oxidases, and increase uric acid levels (Di Domenico et al. 2012; Beheshti 2021). In a study on the effect of spraying with 20 mg/l of ABA on the cannabis plant, the maximum and minimum leaf phenol contents were respectively observed at 100 and 75 mM of NaCl stress. The production of phenolic compounds depends on leaf carbon content; polyphenol accumulation begins when carbon production is more than the metabolic need for growth (Behshti 2021).

Phenolic compounds are considered the substrate of PPO (Chong et al. 2011). As mentioned in the results, PPO activity increased or decreased (Fig. 5a) along with increasing or decreasing shoot phenol content (Fig. 5b). PPOs are enzymes that can oxidize phenolic compounds to orthoquinones, resulting in the browning of plant product tissues, inappropriate appearance, and reduced quality of fruits and vegetables (Kadioglu and Yavru 1998).

Exogenous hormone treatment led to a lower proline content accumulated in *S. europaea* plants than that in NT plants under the control and 400 mM salinity stress levels (Fig. 8). To increase osmotic potential, a strategy can be increasing active metabolites, such as proline, glycine, betaine, etc. Plants produce more proline levels under salt stress, whereas our hormone-treated plants produced lower proline contents, which

can be explained by ABA levels in plants. ABA controls proline biosynthesis and, on the other hand, melatonin exerts a regulatory effect on ABA biosynthesis genes (Abdelaziz et al. 2019). Melatonin suppresses ABA production to postpone leaf senescence (Noor shah et al. 2021), which also influences the production of compounds associated with ABA synthesis. Treatment of cherry seedlings with melatonin (29 mg L^{-1}) indicated an increase in plant melatonin content in the initial days of heat stress and then decreased in subsequent periods; proline content in cherry leaves was contrary to melatonin content (Jia et al. 2019). In salinity stress conditions, a high ABA increase in treated plants reduces salinity-induced toxic effects. Therefore, the exogenous use of these hormones reduces toxic effects, thereby lowering the need for secondary osmolytes, including proline and phenol (Abdelaziz et al. 2019).

The exogenous use of these hormones reduced MDA content in all treatments. Among spraying treatments, the greatest reduction (60.54%) belonged to the combined exogenous use of both hormones a 400 mM salinity stress (Fig. 6). Although MDA content rose in the long term, this increase was significantly lower than in NT plants. The exogenous use of melatonin (29 mg L^{-1}) reduced MDA accumulation in cherry seedling leaves. MDA is a key indicator to measure the peroxidation rate of membrane lipids and their reduction. Adding a suitable concentration of melatonin can eliminate ROS to resist high-temperature stress (Jia et al. 2019).

The H_2O_2 content declined in *S. europaea* roots and shoots in spraying treatments compared to the control NT plants. The H_2O_2 content in *S. europaea* roots and shoots (Fig. 7) corresponded to SOD activity (Fig. 9) at 6, 0, 48, and 720 h after spraying in the control and 400 mM salinity stress levels. In ABA spraying, H_2O_2 content was downward and increased to a level lower than the control sample within 6 months. In *S. persica*, H_2O_2 content decreased up to 300 mM salinity, but it rose by increasing salinity up to 600 mM (Aghaleh et al. 2010). In kiwi seedling treatments, H_2O_2 content at 100 mM salinity stress was significantly more than the control and very higher than the melatonin and salinity treatment group. Melatonin affects the functions of SOD, POX, H_2O_2, and CAT in eliminating reactive oxygen radicals and preventing their excessive accumulation, leading to salinity stress reduction to a particular extent (Liang et al. 2018). Heavy metal stress reduction by exogenously applied melatonin indicates the involvement of melatonin adaptation to environmental stress (Tal et al. 2011). Although SOD is the only enzyme producing H_2O_2 in plant tissues, the balance between the activity of this enzyme and H_2O_2-inhibiting enzymes in cells is of paramount importance in determining superoxide and H_2O_2 levels. The balance between the activity of H_2O_2-producing enzymes and their inhibition level plays an important role in creating the plant's defense mechanism against oxidative stress. The effect of melatonin on growth and metabolism in different plants is highly dependent on its concentration. SOD cannot be considered the only enzyme responsible for membrane protection against peroxidation because this enzyme converts O^{2-} into H_2O_2, which is a ROS and has to be scavenged by CAT and POX (Torabi and Nicknam 2011). In kiwi seedling treatments, SOD activity was significantly lower in a hydroponic environment with 0.1 µM of melatonin and 100 mM salinity than in the control and a melatonin-free salinity group. Melatonin actually caused seedlings to partially adapt to the hydroponic environment (Liang et al. 2018). Agarwal et al. (2005) presented evidence that SOD activity increased significantly at 0.5 mM of ABA on days 30 and 45. Although SOD activity increased at 1 mM of ABA in treated plants compared to control plants, this enzyme elevation was lower than 0.5 ABA treatment, clearly indicating that ABA boosts plant tolerance to oxidative stress and, consequently, increases the activity of oxidative enzymes. SOD is the first defense line against ROS toxic effects (Reddy and Srivastava, 2003).

In the early hours after spraying treatments, the gene expression level in roots was maximized 6 h after spraying with both hormones and melatonin (41.37 and 18.63 times, respectively) at 400 mM salinity. The maximum gene expression level (119.03%) in shoots was observed 6 h after spraying with melatonin at 400 mM salinity stress (Fig. 12). The incongruity of the enzyme activity changes with changes in the gene expression pattern can be attributed to the presence of three types of SOD and their influence on the measurements (Anbarasi et al. 2015).

Ca^{2+} channels are distributed at the cytoplasmic membrane surface and different membranes of organelles (vacuoles, chloroplasts, and mitochondria). Changes in Ca levels regulate downstream signals, one of which is the V-ATPase antiporter that is responsible for Na accumulation in vacuoles. V-ATPase has different catalytic and non-catalytic subunits (Seidel et al. 2005). The activity of ATPase pumps, undertaken by the *VHA-A*
gene encoding the A unit, a V-ATPase pump starter, creates the gradient necessary to activate such antiporters as NHX1 in the vacuole membrane. V-ATPase is the predominant pump in most plant cells. Under different stress conditions (e.g., salinity and drought), cell protection is heavily dependent on the regulation of this gene activity (Seidel et al. 2005).

NHX1 is an H^+/Na^+ antiporter that imports Na ions into vacuoles from the cytoplasm using the electromagnetic gradient produced by H^+ membrane pumps, such as V-H-ATPase (Katschnig et al. 2015).

In most of the studied cases, the *VHA-A* gene expression level corresponded to the NHX1 antiporter, and the *NHX1* gene overexpression was observed with the elevated *VHA-A* gene expression by spraying treatments at the determined intervals. In these periods, *VHA-A* and *NHX1* gene expression levels were uppermost in roots at 400 mM salinity stress and spraying with both hormones (431.91% and 1644%, respectively) after 48 h compared to control plants (Figs. 14 and 17). The increased expression of the genes in the early sampling hours indicates the rapid preliminary response of *S. europaea* to stress, which is of paramount importance in plant tolerance to environmental stresses (Massa and Melito 2019). Our findings are in line with those of Rezaei Moshaei et al. (2014). The exogenous melatonin improved H^+ pump activity in plasma and vacuole membranes and increased ATP content in rice roots (Yan et al. 2020).

The role of Ca^{2+} as a signal molecule for salinity tolerance is best clarified in the study of the Salt Overly Sensitive (*SOS*) signaling pathway. Salinity stress tolerance and maintaining a good ionic ratio in the plant's cytoplasm are associated with the *SOS1-SOS3* gene pathway. The study of the *SOS* function pathway highlights the role of Ca^{2+} in maintaining ionic balance (Abdelaziz et al. 2019). SOS3 is a strategic protein binding to Ca^{2+} ions for salt tolerance and plays a critical role as a Ca^{2+} ion sensor to produce a salt stress signal. To re-establish ion homeostasis in plants, the SOS2/SOS3 complex stimulates the activity of the Na^+/H^+ antiporter, called SOS1 (Zhang et al. 2020a). In our study, the *SOS1* gene expression level increased by spraying with the control and 400 mM salinity levels at the determined intervals in the early hours (6 and 48 h after spraying) compared to control plants (Fig. 13). As described earlier, rapid response to environmental stresses is an important factor in increasing plant tolerance to stresses; thus, spraying treatments could effectively increase *S. europaea* tolerance to salinity stress. Melatonin spraying and the control salinity resulted in the *SOS1* gene overexpression in *S. europaea* shoots 6 h after spraying (Fig. 15). In the cucumber plant, the use of exogenous melatonin led to the overexpression of *SOS1, SOS2*, and *SOS3* genes and photosynthesis protection (Zhang et al. 2020a).

By suppressing ABA production, exogenous melatonin postpones leaf senescence and activates *SOS* and *NHX1* genes (Noor Shah et al. 2021). In addition to a regulatory effect on the *SOS1* gene, the SOS2/SOS3 complex activates the AtNHX1 vacuole protein (Abdelaziz et al. 2019). After the SOS pathway, AtNHX1 plays an important role in salt tolerance. The *HKT* gene family includes Na/K antiporters (VanBezouw et al. 2019), which are of high interest owing to their prominent role in plant salinity tolerance (Chen et al. 2023). The SOS2/SOS3 complex also has a regulatory effect on the *HKT1* gene. In control and 400 mM salinity levels at the determined intervals, the highest *HKT1* gene expression levels in shoots were obtained in spraying treatments with ABA in the control salinity at 6 h (470.9 times), combined hormone application in the control salinity at 720 h (71.73 times), and melatonin application at 400 mM salinity at 6 h (25.43 times) (Fig. 16). The increased *HKT1* gene expression in the early hours after shoot spraying suggests the effect of spraying on increasing gene expression, transferring accumulated Na in shoots to older tissues, and its transport from the phloem to the xylem to increase plant salinity tolerance (Guduru et al. 2019).

Stomata are closed and CO_2 entry for photosynthesis decreases in salt-stressed plants. Besides, electron decreases excessively in cell organelles, and electron transfer to the photosynthesis cycle generally declines, resulting in the overproduction of free radicals and serious damage to the lipid membrane (Gorgini shabankareh et al. 2021b). Plants have evolved key biochemical strategies to control ROS overproduction and counteract the adverse effects of abiotic stresses (Fan et al. 2015). The melatonin molecule structure allows ROS identification. A melatonin molecule can eliminate eight or more ROS through the cascade of free radical scavenging (Garcia et al. 2014). ROS produced by NADPH oxidase activates a cascade of ABA signals in the plasma membrane; for example, it increases the activities of antioxidant enzymes, such as SOD, CAT, and POD (Kopyra and Gwozdz, 2003). Melatonin spraying increases osmolyte accumulation (50%), antioxidants (25%), and biomass (45%) (Noor shah et al., 2021).

APXs are the major antioxidant enzymes in eliminating intracellular H_2O_2. A variety of APXs have a high affinity to H_2O_2 and use ascorbic acid as a substrate to convert it into water (Rabiei et al., 2020). Actually, APX can help the plant in toxicity removal through its high attachment strength to H_2O_2. Although CAT and POX antioxidant enzymes can elevate the speed of this reaction, they have low attachment ability to H_2O_2 and cannot inhibit it properly. The same as a molecular signal, APXs can regulate and track H_2O_2. APXs exist in several isoforms found in different parts of plant cells, enabling them to regulate ROS levels in different cellular compartments for signaling or defense goals (Zhang et al. 2014c). APX can enhance ascorbic acid production in addition to improving stress resistance in crops (Khan et al. 2011). The activity of CAT and POX in ROS toxicity removal is associated with each other. A reduction in the activity of one enzyme, particularly POX, which decreases with exacerbated salinity stress and stress duration due to the saturation of substrate sites, ROS increases in the cell and thereby elevates the activity of the other enzyme (Figs. 10 and 11). Our findings on the accordance of CAT and POX activity agree with those of other studies (Darvishi 2013; Ramani et al. 2013; Zhang et al. 2014c).

Rapeseed treatment with 30 µM of exogenous melatonin under salinity stress treatment increased CAT, POX, and APX activities and reduced H_2O_2 levels, but increasing exogenous melatonin concentrations reduced the activity of these enzymes and even led to e negative effect (Liu et al. 2018). The impact of exogenous melatonin on plants is highly dependent on the plant type and melatonin concentrations in spraying and, therefore, it differently affects plant mechanisms at stress times (Liu et al. 2018). Oxidative stress caused by cherry seedling treatment with high-temperature stress significantly reduced CAT, POX, and APX activities on day 27 and APX activity on day 9 after stress application. However, exogenous melatonin application at 29 mg L^{-1} increased the activity of these enzymes (26, 45.5, 76.5, and 6.6%, respectively) at a high temperature at the same time (Jia et al. 2019). Exogenous melatonin is used in studies to reduce oxidative stress caused by abiotic stresses to maintain ROS homeostasis (Yadu et al. 2018; Xu et al. 2016). Exogenous melatonin application also elevates endogenous melatonin levels and reduces oxidative stress in plants (Ahmmed et al. 2019). Increasing active metabolites can be a strategy to increase osmotic potential. Increases in osmolyte levels, such as sugars, proline, and glycine, induce the metabolic pathways of increasing plant salinity tolerance (Hill et al. 2013; Gorginishabankareh et al. 2021b). In agreement with biochemical and molecular results, the highest morphological traits of root FW and shoot FW and DW (76, 202, and 245%, respectively) were obtained in spraying treatment with both hormones (Figs. 2).

Conclusion

Our results agree with those of other studies reporting that endogenous melatonin application could preliminarily purify stress toxicity effects in products, thereby lowering free radicals and increasing antioxidant activity. Based on the levels of elements and enzymes, direct melatonin spraying on shoots produced a more positive effect on plant tolerance to salinity stress in the short time whereas ABA spraying has a better effect on plant salinity tolerance in the long term. ABA spraying results in plant adaptation to stressful conditions by controlling physiological activities (by affecting such elements as K), such as stomatal opening and closing, through an effect on the activity of guard cells and melatonin by a regulatory effect on key genes. Both hormones increased the activity of antioxidant enzymes to counteract ROS, leading to more desirable results in the exogenous application of both hormones, which was also observed in the results of morphological traits.

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