The effects of foliar application of melatonin on some physiological and biochemical characteristics and expression of fatty acid desaturase gene in pistachio seedlings (Pistacia vera L.) under freezing stress

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ABSTRACT
In this study, the impacts of exogenous melatonin on freezing stress mitigation in pistachio (Pistacia vera L.) seedlings (30-days old) were investigated. Our results showed that in pistachio seedlings under freezing stress antioxidant enzyme activity, malondialdehyde (MDA) hydrogen peroxide (H2O2), electrolyte leakage, chlorophyll degradation, proline and soluble sugar and γ-aminobutyric acid (GABA) content increased. Phenolic component, phenylalanine ammonia lyase (PAL) activity and the expression of fatty acid desaturase (FAD2) gene increased whilst ascorbate and glutathione concentration significantly decreased in comparison with control. In this research, application of melatonin significantly reduced the antioxidant enzyme activity, MDA, H2O2, chlorophyll degradation, GABA concentration, soluble sugar, and proline contents under stress condition. In addition electrolyte leakage, PAL activity and transcripts of FAD2 gene markedly decreased when the leaves of pistachio seedlings sprayed with melatonin. The results of this study showed that MEL could alleviate the oxidative damages induced by freezing stress and to maintain the structure and mobility of cell membrane in pistachio seedlings under stress conditions. Considering that MEL is an inexpensive and safe bio-stimulator, it seems that MEL is a potential candidate that could be applied used to increase the resistant pistachio trees against variety of biotic and abiotic stresses in the field conditions.

Abbreviations: APX: ascorbate peroxidase; CAT: catalase; Chl: chlorophyll; GABA: γ-aminobutyric acid; GPX: guaiacol peroxidase; MEL: melatonin; MDA: malondialdehyde; RNS: reactive nitrogen species; ROS: reactive oxygen species

Introduction
Freezing temperature is an environmental condition that causes adverse effects on the growth of plants and productivity of crops (Akula and Ravishankar 2011). Cold stress has various effects on plant physiology, biochemistry and molecular biology (Chinnusamy et al. 2010). There is an agreement that a plasma membrane is the first place of freezing damage due to its fundamental function in cellular action through a freeze–thaw cycle (Griffith and Antikainen 1996). In addition, it is well established that cold stress causes the alterations of lipid composition and an increase in the fatty acid saturation index (Lado et al. 2016). Secondary responses to cold have been associated with oxidative stress and consequently, leading to increased production of reactive oxygen species such as singlet oxygen, hydrogen peroxide, superoxide anions, hydroxyl and peroxyl radicals in plant cell (Hossain et al. 2012). Thus, plant tolerance to cold stress is also closely correlated to increased capacity of scavenging and detoxifying the ROS. In addition, plants attempt to neutralize the detrimental influences of cold stress by enhancing the synthesis of osmolytes such as proline, glycine betaine, soluble protein, and soluble sugars, which can vary depending upon a variety of plant and might be interchangeable (Erdal 2012; Shao et al. 2008). It has been reported that, some of these osmolytes such as free sugars, proline, and γ-aminobutyric acid accumulate at low temperatures, share cooperative solute-like characteristics and have been proved to play a role in preserving proteins and membranes from freezing detriment (Kaplan et al. 2007).

Melatonin (N-acetyl-5-methoxytryptamine) (MEL) is an endogenously produced molecule with excellent properties as natural antioxidant (Arnao and Hernández-Ruiz 2015). MEL is a useful component for human health and plant preservation. MEL modulates circadian rhythms (Bano-Otura et al. 2020) and inflammatory processes at the intestinal level (Mannino, Caradonna et al. 2019). Additionally, MEL is a strong agent against cancer, cardiovascular, fibrosis, neuro-degenerative diseases, sepsis and osteoporosis (Li et al. 2019). Further studies by Wei et al. (2020) indicated that melatonin was an efficient and tolerable drug in the treatment sleep onset insomnia in human. Also, MEL has been reported to cause a decrease in the proliferation of tumor cells and, increase survival (Pourhanifeh et al. 2019). Bizzarri et al. (2013) concluded that MEL might act as a pro-oxidant in cancer cells. In previous study, it has been reported that melatonin can act as a direct antioxidant through the stabilization of biological membranes where it plays a relevant role in...
membrane fluidity and in counteracting lipid peroxidation under oxidative stress (García et al. 2014). Additionally, melatonin also indirectly increases the activities of reactive oxygen species scavenging enzymes (such as SOD, CAT, APX and POX) and maintaining the content of another important antioxidant such as glutathione and ascorbic acid at higher levels in comparison with control plants (Arnao and Hernández-Ruiz 2014; Wang et al. 2012). It has been showed that MEL is the first guard against the oxidative stress and could preservation of other antioxidants such as (β-carotenoids, vitamin E, vitamin C and glutathione), (Tan et al. 2012). Several reports suggest that treatment with melatonin enhances plant resistance to different types of stresses such as alkaline (Cen et al. 2020; Liu et al. 2015), cold (Kolodziejczyk et al. 2016; Turk et al. 2014), drought, heavy metal (Posmyk et al. 2008), UV irradiation (Zhang, Jia et al. 2012) and biotic stress (Lee et al. 2014; Moustafa-Farag et al. 2020) Jannatizadeh et al. (2019) also reported that chilling injury in tomato fruits was alleviated by exogenous MEL application during cold storage.

Pistachio (Pistacia vera L.) a member of the Anacardiaceae family has been mainly cultivated in Iran, USA, Syria, Iraq, Turkey and some areas of Europe. Pistachio consumption is useful for human health and reduces risk of heart disease. In addition pistachio has economic importance in the lives of some people (Mannino, Gentile et al. 2019). Pistachio plant often experience serious cold injury in early spring which reduces yield and product quality. The effects of cold stress are not well studied in pistachio plant. Therefore, the information on how to protect pistachio plants from cold stress is very limited. It has been reported that spring cold in the 2004 and 2005, harmed in average 60% of pistachio trees in Iran (Afshari et al. 2010). The knowledge of the physiology of stress tolerance is undoubtedly helpful to counteract this adverse effect. In some researches relationship between melatonin and GABA as two signaling molecules were reported. For example, announced that the melatonin treatment delayed postharvest spoilage in strawberry fruits by enhancing GABA shunt activity (Aghdam and Fard 2017). Turi et al. (2014) also identified and quantified melatonin and GABA in Artemisia tridentate under aseptic conditions. Nevertheless, there is no information in the literature about the relationship between melatonin and GABA in freezing-induced stress in plants. So in this research, the influence of MEL on some physiological and biochemical parameters, antioxidant enzyme activities, GABA content and the expression of fatty acid desaturase enzyme in pistachio seedlings during freezing stress were investigated.

Materials and methods

Plant materials and treatments

Ahmadaghaei pistachio cultivar is one of the most significant commercial pistachio cultivars which were considered in Kerman province. The surface of the pistachio seeds (Pistacia vera ‘Ahmadaghaai’) were sterilized with a solution of 7% (v/v) sodium hypochlorite for 5 min and rinsed with running water as well as distilled water. The sterilized seeds were imbibed in distilled water for 24 h and then germinated on moisture filter paper in the dark at 28°C. After emergence, the seedlings planted in pots of sand. Plants were grown at the constant thermal regime of 25/20°C (day/night) in a growth chamber using a 16/8-h light/dark cycle under a relative humidity of 45–50% for 30 days. Thirty-day-old seedlings were selected for foliar application of MEL (0.1, 0.25 and 0.5 µM). Deionized water was used for control with 2-day intervals extended for eight days. 0.01% v/v Tween-20 was utilized for surfactant in all treatments.

After pretreatment of seedlings with MEL, the seedlings were separated into two groups: one group was located in an incubator, in which the temperature was set 25°C. The temperature of the incubator changed to ~4°C after 30 min and seedlings were stored at ~4°C for 2 h. The other group was kept under optimal conditions. Duration and severity of cold stress were optimized in a preliminary experiment according to the severity of injury to pistachio seedlings. The stressed seedlings were transferred to the normal condition for 12 h to evaluate of biochemical and molecular changes in pistachio seedlings caused by freezing.

Malondialdehyde and other aldehydes concentration analysis

Malondaldehyde is a critical biomarker of lipid peroxidation. MDA concentration measured as illustrated by Heath and Packer (1968). The Extinction coefficient (ε) of 1.55 × 10^{-5} M^{−1} cm^{−1} was used for the determination of MDA concentration and an extinction coefficient of 0.457 × 10^{5} M^{−1} cm^{−1} was utilized for calculation of other aldehydes concentration (Meir et al. 1992).

Quantification of electrolyte leakage

Electrolyte leakage was measured according to the method of Yu et al. (2006) with some modification. Briefly, 10 leaf discs (1 cm²) were collected from pistachio seedlings leaves and washed three times with deionized water. The washed leaves were transferred into test tubes containing 15 mL distilled water and incubated at room temperature for 3 h. The initial electrical conductivity (EC_{1}) of the medium was measured using a conductivity meter. The solution was autoclaved at 121°C for 7 min. The final conductivity (EC_{2}) was measured after cooling the solution at room temperature. Electrolyte leakage was calculated as EC_{1}/EC_{2} and expressed as a percentage.

Determination of hydrogen peroxide

The exact concentration of hydrogen peroxide was determined according to the methods by Alexieva et al. (2001). Pistachio leaves were immersed in liquid nitrogen and homogenized with 0.1% (w/v) trichloroacetic acid (TCA) (1:4, w/v). Subsequently, the leaves were centrifuged at 4°C and 10,000 × g for 15 min, and the supernatants were separately collected. The reaction mixture contained 0.25 mL supernatant, 0.25 mL 100 mM potassium phosphate buffer (pH 7) and 1 mL 1 M KI. The reaction was developed for 1 h in darkness and absorbance was determined at 390 nm. The content of hydrogen peroxide was measured through a standard curve prepared with the known concentration of H_{2}O_{2}. 
**Enzyme extraction and activity determination**

To determine the activities of antioxidant enzyme, leaves (0.5 g) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) consisting of 1 mM ethylenediamine-tetraacetic acid (EDTA), 1% (w/v) polyvinylpyrrolidone (PVP), and 1 mM phenylmethylsulfonyl fluoride (PMSF). All procedures were accomplished in 4°C. The homogenate was centrifuged at 10,000 × g for 20 min and the supernatant was used for assay the activity of enzymes. Protein content was estimated using the Bradford method (1976) with bovine serum albumin (BSA) as a standard.

**Catalase (CAT) activity (EC 1.11.1.6)**

Catalase activity was determined by monitoring the decomposition of H₂O₂ (extinction coefficient of 40 M⁻¹ cm⁻¹) at 240 nm following a method by Dhindsa et al. (1981).

**Guaiacol peroxidase (GPX) (EC1.11.1.7)**

Oxidation of guaiacol to tetraguaiacol calculated by the monitoring of changes in absorbance at 470 nm and using an extinction coefficient of 25.5 mM⁻¹ cm⁻¹ in order to detect the activity of peroxidase (Plewà et al. 1991).

**Ascorbate peroxidase (APX) (EC 1.11.1.11)**

Ascorbate peroxidase was established spectrophotometrically consistent with the oxidation of ascorbate. The reaction solution contained of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H₂O₂, and 150 µL enzyme extract. H₂O₂-dependent oxidation of ascorbate was followed by evaluating the decrease in absorbance within 1 min at 290 nm (extinction coefficient of 2.8 mM⁻¹ cm⁻¹) (Nakano and Asada 1981).

**PAL activity (EC 4.3.1.5)**

PAL activity was determined based on the rate of cinnamic acid production. 1 ml of 10 mM L-phenylalanine, 0.4 ml of deionized water and 0.1 ml of enzyme extract were incubated at 37°C for 1 h. The reaction was terminated by the addition of 0.5 ml of 6 M HCl and the cinnamic acid concentration was measured spectrophotometrically by the absorbance at 260 nm. One unit of PAL activity is equal to 1 µM of cinnamic acid produced per min (D’cunha et al. 1996).

**Carbohydrates and proline concentration analysis**

The proline content in fresh leaves was ascertained by using a method by Bates (1973). Total soluble sugar also was assayed using anthrone reagent and glucose as standard (Roe 1955).

**Chlorophyll and carotenoids content determination**

Chlorophyll concentration was measured in 80% acetone extracts as described by Lichtenthaler (1987). Before measuring the absorbance with a UV/visible spectrophotometer at 663.2, 646.8, and 470 nm; the extracts were centrifuged at 3000 × g. The concentration of chlorophyll was evaluated by the equations presented by Lichtenthaler (1987) given below:

\[
\text{Chl a} = (12.25 \times A_{663.2} – 2.79 \times A_{646.8}) \times \text{volume of supernatant} \times \text{dilution factor/sample mass (g)}
\]

\[
\text{Chl b} = (21.21 \times A_{646.8} – 5.1 \times A_{663.2}) \times \text{volume of supernatant} \times \text{dilution factor/sample mass (g)}
\]

\[
\text{Car} = \left[\frac{(1000 \times A_{470} – 1.8 \times \text{Chl a} - 85.02 \times \text{Chl b})}{198}\right] \times \text{volume of supernatant} \times \text{dilution factor/sample mass (g)}
\]

**Total phenolic content analysis**

Total phenolic content was determined using the Folin-Ciocalteau method by Singleton et al. (1999). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the leaf fresh weight.

**Glutathion (GSH) determination**

The reduced glutathione (GSH) content was determined by a method by Ellman (1959) with some modification. 0.2 g of frozen material was ground with 2 ml of 15% metaphosphoric acid and centrifuged at 10,000 × g for 30 min. Assay mixture was prepared by adding 2.6 ml sodium phosphate buffer (pH = 7.7), 200 µl of 6 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) and 200 µl of a sample extract. The mixture was stabilized at 25°C for 30 min. The absorbance was monitored at 412 nm.

**Ascorbate and dehydroascorbate determination**

The Ascorbate (ASC) and dehydroascorbate content were determined according to Kampfenkel et al. 1995.

This assay is based on the reduction of the ferric ion to ferrous ion by ASC. The ferrous ion then forms complexes with bipyridyl that absorbs at 525 nm. Total ascorbate (ASC + DHA) is determined through a reduction of DHA to ASC by dithiothreitol. DHA concentration was calculated by subtracting ascorbate from total ascorbate pool.

**Determination of total antioxidant capacity using DPPH method**

The DPPH scavenging activity was assayed using the method of Singh et al. (2002). Frozen leaf samples (0.05 g) were extracted with 80% methanol using a pre-chilled mortar and a pestle on an ice bath. The homogenate was filtered through one layer of muslin cloth and centrifuged for 20 min at 5000 × g at 4°C. Two mL of 100 µM DPPH methanolic solution was mixed with 20 µL of the extracted sample and allowed to stand for 30 min at 29°C in complete darkness. Radical scavenging capacity was expressed as the inhibition percentage and was calculated by the following equation:

\[
\text{Radical scavenging activity} (\%) = \left(\frac{\text{control optical density} - \text{sample optical density}}{\text{control optical density}}\right) \times 100
\]

Ascorbic acid (20, 30, 50 µM) was chosen as a standard antioxidant and antioxidant activity of the extract was expressed in µM of ascorbic acid equivalents per µL of extract.

**Analysis of GABA by HPLC**

GABA was analyzed using HPLC on the ground of the method described by Akçay et al. (2012) with minor changes. Leaves (0.4 g) were cryopreserved in liquid nitrogen,
homogenized with 1 mL water: chloroform: methanol (3:5:12, v/v/v) solution. Following 3 min centrifugation, the supernatant was collected, 300 µL chloroform and 500 µL water was added. The resulting solution was vortexed and centrifuged for 3 min. The top phase was carefully withdrawn and dried. The dried samples were again dissolved in the mixture of 100 µL water, 150 µL Borax buffer (pH 8.0), and 250 µL of derivatization reagent 2-hydroxynaphthaldehyde (HN) (0.3%, w/v, in methanol). The solution was maintained in a water bath at 80°C for 20 min and cooled down to room temperature. The ultimate volume was set to 1 mL with methanol, and injection into the HPLC column followed by filtering the resulting mixture (Reprosil-pur C18-AQ; 200 × 4.6 mm, 5 µm). Sample injection volume was 20 µL, flow rate 0.5 mL min⁻¹; mobile phase was consisted of methanol: water (62:38, v/v), and detection (UV-detector) was performed at 330 nm. Typical retention time was 10 min for GABA, and the whole elution time was 15 min. The amount of GABA was evaluated as a ratio of the surface area under the substrate HPLC peak compared to those obtained with the untreated control.

Quantitative real-time PCR analysis

Total RNA was extracted from pistachio seedlings leaves using the CinnaPure RNA purification kit. Quantity and quality of each RNA sample were routinely determined from absorption at 260 and 280 nm. RNA integrity was detected using 2% (w/v) agarose gel electrophoresis. RNA samples were treated with DNase I (Pishgam, RNase Free) to remove genomic DNA. *FAD2* gene amplification was performed using primers designed from the plant of zucchini (Palma et al. 2015). The reference housekeeping gene (NADH dehydrogenase) designed from the plant of pistachio (Zamani Bahramabadi et al. 2011). Specific primer pairs were designed for omega-6 fatty acid desaturase (forward primers 5'-TTCTGTCTCCTGCTCATTCT-3', reverse primers 5'-GACCCATACGCCAGTAAGGA-3'), and NADH dehydrogenase (forward primers 5'-GGAGACTCAAATGGTG-3', reverse primers 5'-ACCTGCTAGTGGA GGAAAGAC-3').

cDNA was synthesized from total RNA according to the manufacturer's protocols (Thermo Scientific). Real-time PCR reactions were performed using SYBR Green real-time PCR master mix (AmpliQon, Denmark) and a Bioer fluorescent quantitative PCR detection system at 95°C for 15 min, followed by 40 cycles at 95 for 15 s, 58°C for 30 s and 72°C for 30 s. The melting curve was analyzed after the reaction was completed. Efficiency of primers was analysed by dilution method. Relative quantifications were then calculated using the correction from reference gene and transferred to a liner relationship using 2⁻ΔΔCt (Livak and Schmittgen 2001).

Statistical analysis

Experiments were established in a completely randomized pattern. Each treatment included tree replicates of 10 seedlings, and the entire procedures of the trial were repeated two times with similar results. Data were subjected to analysis of variance. Further statistical analyses were completed with the MSTATC software. LSD tests discovered considerable variations between means. P values lower than 0.05 were regarded statistically significant.

Results

**Lipid peroxidation and electrolyte leakage**

MDA and other aldehydes content and electrolyte leakage increased in those pistachios which were under freezing stress in comparison with control seedlings. Pretreatment of seedlings with exogenous MEL reduced the peroxidation of lipids and membrane leakage (50–60%) in plants which were under stress condition. In this research the 0.25 and 0.5 µM MEL was more effective than 0.1 µM (Table 1).

**H₂O₂ production rate**

The H₂O₂ contents increased in pistachio seedling during freezing stress, and was significantly alleviated by MEL application (Table 1). Hydrogen peroxide contents decreased about 50% in stressed-plants which were pretreatment with MEL.

**Antioxidative enzymes activity**

All of the antioxidant enzyme activity (GPX, CAT, and APX) increased in freezing stressed seedlings compared to the non-treated group. Melatonin pretreatment decreased these enzymes activity in seedlings during freezing stress (APX, GPX and CAT decreased about 50%, 75% and 50–75%, respectively), as compared to non-pretreated plants (Table 2).

**Glutathione (GSH), ascorbate and dehydroascorbate content**

The glutathione content significantly decreased in freezing stressed seedlings as compared with the control. MEL pretreatment markedly suppressed the reduction of glutathione content when plants were subjected to freezing stress (Table 3). Data showed that in plants which were pretreated with MEL, GSH content increased 3–4 more than in non-pretreated plants.

Similarly, low ASC content induced by freezing stress was markedly increased by MEL pretreatment (about 5 folds). Also the ratio of ASC/DHA was calculated under freezing stress (Table 3). Data showed that in plants which were pretreated with MEL, GSH content increased 3–4 more than in non-pretreated plants.

**Total antioxidant capacity**

In normal condition, MEL pretreatment with different concentrations had positive effects on radical scavenging activity. It also was shown that freezing stress increased antioxidant capacity and melatonin pretreatment resulted in further increases in antioxidant capacity as compared to freezing-stressed ones alone (Table 3).

**Chlorophyll and carotenoids contents**

Chlorophyll content was significantly decreased in pistachio seedlings which were exposed to freezing stress. The application of MEL improved chlorophyll content in seedlings
which were under stress condition. Freezing stress and MEL pretreatment had no significant effects on carotenoids content (Table 4).

**Soluble carbohydrates and proline**

Soluble sugar and proline content increased in freezing stressed seedlings. Pretreatment of seedlings with MEL decreased the content of soluble sugar and proline (about 50-70%) in plants which were under stress conditions (Table 5).

**Total phenolic content and PAL activity**

PAL activity and phenolic compounds increased in freezing stressed seedlings. Melatonin pretreated plants showed significantly lower levels of phenolic compounds in comparison with non-treated plants (70% decline in phenolic compounds and 50% decrease of PAL activity). There was a positive correlation between phenolic contents and PAL activity (Table 5).

**GABA concentration**

The GABA content increased in freezing stressed seedlings as compared with the control plants. MEL pretreatment decreased the GABA content in freezing stressed plants.

**Effect of freezing stress and melatonin pretreatment on expression of FAD2gene**

Freezing stress increased the expression of FAD gene in comparison with the control samples. Pretreatment with MEL significantly decreased the transcripts of this gene under stress condition. The most remarkable decreases were observed in the seedlings treated with 0.5 µM melatonin (Figure 1).

**Discussion**

Subjecting plants to low temperature is believed to initiate biochemical and physiological modifications in plants, leading to loss of vigor. Low temperature causes some alterations in cell structure, cell membranes and cell wall compound. The systems of Cell membrane are the initial sites of cold injuries (Kratsch and Wise 2000).

Lipid peroxidation assessed by MDA content is a widely used indicator of membrane injury owing to the reaction of free radicals with phospholipids and fatty acids, which induces peroxidation of lipids, and increases the amount of MDA (Wan et al. 2015). Electrolyte leakage is also a good indicator of membrane permeability or the loss of membrane integrity under stress condition (Li et al. 2012). The results of this research showed that the concentration of MDA, other aldehydes, and membrane leakage in stressed plants pretreatment with melatonin were remarkably decreased compared with those seedlings which were just under stress. This is accompanied with depletion in superoxide anion and H2O2 content in melatonin pretreated plants. It seems that melatonin could attenuate oxidative stress caused by freezing stress.

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**Table 1** Effects of exogenous application of melatonin on malondialdehyde (MDA), other aldehydes, membrane leakage and hydrogen peroxide (H2O2) content of pistachio seedlings exposed to freezing stress.

| Freezing stress | Melatonin concentration (µM) | MDA content (nmol g⁻¹ FW) | Other aldehydes content (nmol g⁻¹ FW) | Membrane leakage (%) | H2O2 content (µmol g⁻¹ FW) |
|-----------------|-----------------------------|--------------------------|--------------------------------------|----------------------|---------------------------|
| −               | 0                           | 3.44                     | 98.18                                | 30.75                | 1.40                      |
| −               | 0.1                         | 4.00                     | 98.18                                | 26.88                | 2.22                      |
| −               | 0.25                        | 6.32                     | 109.41                               | 26.17                | 2.41                      |
| −               | 0.5                         | 9.08                     | 127.79                               | 30.61                | 2.93                      |
| +               | 0                           | 23.61                    | 458.21                               | 77.25                | 5.128                     |
| +               | 0.1                         | 14.93                    | 173.01                               | 42.00                | 2.62                      |
| +               | 0.25                        | 9.29                     | 120.79                               | 36.21                | 1.60                      |
| +               | 0.5                         | 10.54                    | 97.88                                | 34.87                | 2.13                      |

Different letters in the same column indicate significant differences according to LSD tests (p < 0.05), (−): not freezing treated seedlings, (+): freezing treated seedlings.

**Table 2** Effects of exogenous application of melatonin on activities of guaiacol peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) in pistachio seedlings exposed to freezing stress.

| Freezing stress | Melatonin concentration (µM) | GPX activity (U mg⁻¹ protein) | CAT activity (U mg⁻¹ protein) | APX activity (U mg⁻¹ protein) |
|-----------------|-----------------------------|-------------------------------|-------------------------------|-------------------------------|
| −               | 0                           | 0.16                         | 0.59                         | 0.14                         |
| −               | 0.1                         | 0.11                         | 0.59                         | 0.14                         |
| −               | 0.25                        | 0.22                         | 0.72                         | 0.99                         |
| −               | 0.5                         | 0.19                         | 0.19                         | 0.16                         |
| +               | 0                           | 0.35                         | 4.67                         | 1.63                         |
| +               | 0.1                         | 0.27                         | 4.67                         | 1.63                         |
| +               | 0.25                        | 0.27                         | 4.67                         | 1.63                         |
| +               | 0.5                         | 0.14                         | 4.67                         | 1.63                         |

Different letters in the same column indicate significant differences according to LSD tests (p < 0.05), (−): not freezing treated seedlings, (+): freezing treated seedlings.

**Table 3** Effects of exogenous application of melatonin on concentrations of glutathione, ascorbate, dehydroascorbate, ratio of ascorbate to dehydroascorbate and radical scavenging capacity in pistachio seedlings exposed to freezing stress.

| Freezing stress | Melatonin concentration (µM) | GSH content (µmol/g FW) | Ascorbate content (mg/g FW) | Dehydroascorbate content (mg/g FW) | Ascorbate/dehydroascorbate ratio | Inhibition (%) |
|-----------------|-----------------------------|-------------------------|-----------------------------|------------------------------------|----------------------------------|----------------|
| −               | 0                           | 0.38                     | 0.39                       | 1.61                               | 0.24                             | 29.78          |
| −               | 0.1                         | 0.42                     | 0.55                       | 1.33                               | 0.42                             | 34.18          |
| −               | 0.25                        | 0.41                     | 0.72                       | 1.37                               | 0.53                             | 34.64          |
| −               | 0.5                         | 0.42                     | 0.69                       | 1.42                               | 0.43                             | 39.51          |
| +               | 0                           | 0.12                     | 0.20                       | 2.72                               | 0.07                             | 33.37          |
| +               | 0.1                         | 0.34                     | 0.75                       | 2.03                               | 0.39                             | 38.00          |
| +               | 0.25                        | 0.33                     | 0.85                       | 1.68                               | 0.51                             | 39.28          |
| +               | 0.5                         | 0.40                     | 1.14                       | 1.61                               | 0.71                             | 40.55          |

Different letters in the same column indicate significant differences according to LSD tests (p < 0.05), (−): not freezing treated seedlings, (+): freezing treated seedlings.
in pistachio seedlings. It has been reported that in the cell membranes, melatonin can be placed between the polar heads of polyunsaturated fatty acids and therefore, can reduce the lipid peroxidation and maintain the optimal fluidity of the membrane (Cerallo et al. 1999; Janas and Posmyk 2013). Positive impact of MEL on the membrane integrity, lipid peroxidation and maintain the optimal fluidity of the membranes, melatonin can be placed between the polar heads of polyunsaturated fatty acids and therefore, can reduce the lipid peroxidation and maintain the optimal fluidity of the membrane (Cerallo et al. 1999; Janas and Posmyk 2013). Overproduction of ROS is a mechanism, which resists the adverse impact of reactive oxygen species (Lee and Lee 2000). Oxidative stress leads to changes in the certain antioxidant activity of an enzyme. These changes are illustrated as a function of melatonin in the decreasing of stress and scavenging of ROS directly or indirectly through raising the levels of antioxidants and the activities of related enzymes to scavenge ROS (Arnao and Hernández-Ruiz 2015; Li et al. 2012). These actions provide a way to assist in enhancing abiotic stress resistance in diverse plants (Zhang et al. 2014). Among the defense systems CAT, GPX, and APX are essential enzymes in breaking down H₂O₂ to H₂O in plant cells (Mittler et al. 2011). In the present experiment, freezing stress provoked a significant increase in CAT, APX, and GPX activity in plants, whereas the exogenous application of MEL decreased the activity of these antioxidant enzymes in plants which were under stress. Considering the reduction of MDA, H₂O₂ and superoxide anion as the stress index, and weak activities of CAT, APX, as well as GPX in MEL- pretreated plants, it seems that melatonin did not induce the antioxidant activity and probably scavenged the ROS directly and alleviated oxidative damages caused by freezing stress. The direct interacts of melatonin with hydroxyl radical, hydrogen peroxide, singlet oxygen and superoxide anion was reported previously (Kostopoulou et al. 2016). In the present work, DPPH antioxidant capacity was higher in melatonin treated plants compared to control plants which is recommended the directly role of melatonin as excellent free radical scavengers and broad-spectrum antioxidant.

Cold stress leads to changes in the certain antioxidant activity of an enzyme. These changes are illustrated as a mechanism, which resists the adverse impact of reactive oxygen species (Lee and Lee 2000). Overproduction of ROS damages the lipids, plant proteins, carbohydrates, and DNA, which eventually causes cell death (Wang, Miao et al. 2013). A great deal of preceeding research concentrated on the function of melatonin in the decreasing of stress and scavenging of ROS directly or indirectly through raising the levels of antioxidants and the activities of related enzymes to scavenge ROS (Arnao and Hernández-Ruiz 2015; Li et al. 2012). These actions provide a way to assist in enhancing abiotic stress resistance in diverse plants (Zhang et al. 2014). Among the defense systems CAT, GPX, and APX are essential enzymes in breaking down H₂O₂ to H₂O in plant cells (Mittler et al. 2011). In the present experiment, freezing stress provoked a significant increase in CAT, APX, and GPX activity in plants, whereas the exogenous application of MEL decreased the activity of these antioxidant enzymes in plants which were under stress. Considering the reduction of MDA, H₂O₂ and superoxide anion as the stress index, and weak activities of CAT, APX, as well as GPX in MEL- pretreated plants, it seems that melatonin did not induce the antioxidant activity and probably scavenged the ROS directly and alleviated oxidative damages caused by freezing stress. The direct interacts of melatonin with hydroxyl radical, hydrogen peroxide, singlet oxygen and superoxide anion was reported previously (Kostopoulou et al. 2016). In the present work, DPPH antioxidant capacity was higher in melatonin treated plants compared to control plants which is recommended the directly role of melatonin as excellent free radical scavengers and broad-spectrum antioxidant.

Under stress, non-enzymatic antioxidant systems such as ascorbate (ASC), reduced glutathione (GSH) and carotenoids are directly or indirectly responsible for ROS removing from cells (Balabusta et al. 2016). The changes in the ratio of ASC to DHA are pivotal for the cell to sense oxidative stress and respond accordingly (Potters et al. 2002). In this study, under freezing stress, the ratio of ASC to DHA was decreased significantly when compared with controls. However, exogenously applied melatonin significantly increased the ratio of ASC/DHA under freezing or normal conditions. So, melatonin may leads to proper function of plant against

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**Table 4. Effects of exogenous application of melatonin on chlorophyll a, chlorophyll b, total chlorophyll and carotenoids content of pistachio seedlings exposed to freezing stress.**

| Freezing stress | Melatonin concentration (µM) | Chlorophyll a content (µg g⁻¹ FW) | Chlorophyll b content (µg g⁻¹ FW) | Total chlorophyll content (µg g⁻¹ FW) | Carotenoids content (µg g⁻¹ FW) |
|-----------------|-------------------------------|-----------------------------------|-----------------------------------|----------------------------------------|-------------------------------|
| −               | 0                             | 4.70±0.00c                       | 1.03±0.00d                       | 5.74±0.00cd                            | 1.29±0.00ab                  |
| −               | 0.1                           | 4.06±0.00c                       | 1.23±0.00d                       | 5.28±0.00bc                            | 1.04±0.00bc                  |
| −               | 0.25                          | 4.39±0.00c                       | 1.09±0.00d                       | 5.67±0.00cd                            | 1.30±0.00ab                  |
| −               | 0.5                           | 4.59±0.00c                       | 1.40±0.00c                       | 6.37±0.00cd                            | 1.27±0.00bc                  |
| +               | 0                             | 4.12±0.00c                       | 0.68±0.00c                       | 4.80±0.00c                             | 1.08±0.00bc                  |
| +               | 0.1                           | 4.89±0.00c                       | 1.98±0.00c                       | 6.86±0.00bc                            | 1.09±0.00bc                  |
| +               | 0.25                          | 5.15±0.00c                       | 1.84±0.00c                       | 6.98±0.00bc                            | 1.20±0.00bc                  |
| +               | 0.5                           | 4.40±0.00c                       | 1.68±0.00c                       | 6.08±0.00bc                            | 0.82±0.00bc                  |

Different letters in the same column indicate significant differences according to LSD tests (p < 0.05), (−): not freezing treated seedlings, (+): freezing treated seedlings.

**Table 5. Effects of exogenous application of melatonin on concentrations of proline, soluble sugars, phenolic component, GABA (γ-aminobutyric acid) and activity of phenylalanine ammonia lyase (PAL) in pistachio seedlings exposed to freezing stress.**

| Freezing stress | Melatonin concentration (µM) | Proline content (µmol g⁻¹ FW) | Soluble sugars content (µmol g⁻¹ FW) | Total phenol content (mg/g FW) | PAL activity (U/mg protein) | GABA content (µmol g⁻¹ FW) |
|-----------------|-------------------------------|-------------------------------|-------------------------------------|-------------------------------|----------------------------|-----------------------------|
| −               | 0                             | 5.06±0.00bc                   | 64.91±0.00c                        | 5.48±0.00bc                   | 12.68±0.00bc                 | 39.08±0.00bc                |
| −               | 0.1                           | 1.28±0.00c                    | 44.91±0.00c                        | 5.00±0.00bc                   | 12.69±0.00bc                 | 41.81±0.00bc                |
| −               | 0.25                          | 2.52±0.00c                    | 38.41±0.00c                        | 5.50±0.00bc                   | 10.71±0.00bc                 | 45.23±0.00bc                |
| −               | 0.5                           | 2.59±0.00c                    | 40.69±0.00c                        | 2.81±0.00d                    | 7.90±0.00d                   | 47.63±0.00bc                |
| +               | 0                             | 10.16±0.00c                   | 90.66±0.00c                        | 13.03±0.00c                   | 21.08±0.00bc                 | 51.33±0.00bc                |
| +               | 0.1                           | 3.68±0.00c                    | 47.26±0.00c                        | 4.50±0.00d                    | 14.15±0.00bc                 | 49.13±0.00bc                |
| +               | 0.25                          | 3.57±0.00c                    | 46.10±0.00c                        | 5.28±0.00bc                   | 13.63±0.00bc                 | 42.88±0.00bc                |
| +               | 0.5                           | 3.99±0.00c                    | 36.35±0.00c                        | 2.98±0.00d                    | 9.94±0.00d                   | 38.51±0.00bc                |

Different letters in the same column indicate significant differences according to LSD tests (p < 0.05), (−): not freezing treated seedlings, (+): freezing treated seedlings.

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**Figure 1.** Effects of exogenous application of melatonin on expression of fatty acid desaturase (FAO) gene in pistachio seedlings exposed to freezing stress. Different letters indicate significant differences according to LSD tests (p < 0.05).
environmental stresses, such as salt stress (Liu et al. 2015), on alleviation of oxidative damage by stimulating antioxidant free radicals. Also, Wang, Sun, Chang et al. (2013) have found that significant decrease of proline in the root of the cherry rootstock PHL-C (Prunus avium × Prunus cerasus) followed by melatonin treatment. It seems that melatonin could protect pistachio seedlings against oxidative stress through detoxifying ROS directly and indirectly with maintaining the ascorbate and reduced glutathione content than the control plants.

Soluble carbohydrates piled up in plants function as a cytoprotective combination, which is able to avoid or slow ice crystal formation (Karimi and Ershadi 2015). Apart from carbohydrates, trees accumulate proline during exposure to cold, which provokes osmotic regulation, maintains turgor pressure in cells during drought stress, and enables plants to endure dehydrative stresses (Zhang, Wu et al. 2012). There are contradictory reports regarding the function of proline in chilling tolerance. In a low-temperature-stressed plant, the high content level of proline had been reported as a factor responsible for conferring chilling tolerance (Chen et al. 1999). In contrary, accumulation of proline has also been regarded as an indication of stress-induced injury to plant tissue (Upadhyaya et al. 1989). In this research, we observed the high content of soluble sugars and proline in freezing-stressed pistachio. When the plants were pretreated with MEL, the levels of soluble sugars and proline were decreased. Similar effects of melatonin treatment have been reported by Sarropoulou et al. (2012) that showed melatonin treatments significantly drop the levels of proline in the root of the cherry rootstock PHL-C (Prunus avium × Prunus cerasus) compared to those of the control. Also, Wang, Sun, Chang et al. (2013) have found that significant inhibition of hexose (fructose and glucose) accumulation and delaying leaf senescence of Malus hupehensis followed by exogenous application of melatonin. In contrast, it has been reported that melatonin elevates levels of proline and carbohydrates (glucose, fructose, malrose, sucrose), which brings about resistance to stresses in plant (Fan et al. 2015; Qian et al. 2015; Shi et al. 2015). It might be concluded that melatonin has a regulatory function in carbohydrate metabolism of plants. However, additional studies will be required to ascertain the association between melatonin, proline, and carbohydrates.

Chlorophyll is vulnerable and can be simply injured by ROS which is produced during the environmental changes such as cold or hot temperatures and UV or visible light irradiation (Triantaphylides and Havaux 2009). Our data for chlorophyll a, chlorophyll b, and total chlorophyll amount demonstrated that chlorophyll degraded in freezing-stressed leaves and MEL application increased the chlorophyll content (Table 4). It seems that MEL can be protected chlorophylls as an antioxidant. The chlorophyll-protective efficacy of melatonin was also reported in seedling of cucumber exposed to heat stress (Xu et al. 2010) and wheat seedlings subjected to cold stress (Turk et al. 2014).

GABA is a metabolite that has public cryoprotective characteristics and have been proposed to have a function in responses to stress signaling (Kaplan et al. 2007). In this experiment, GABA content increased significantly during freezing stress however it decreased in MEL pretreatment plants compared to the non-pretreated plants. We also found that the effect of MEL on the relation of GABA content was dose-dependent. It has been reported that melatonin elevates levels of proline which increased under stress condition decreased in MEL-pretreated plants because of MEL alleviation effects.

Different degrees of cold tolerance can be found among plant genotypes possessing same levels of unsaturated fatty acids which confirm that other components of plant metabolism are also necessary for whole cold stress resistance in plants (Ariizumi et al. 2002).

The effect of melatonin in model membranes (lipid vesicles) has been demonstrated that melatonin increases fluidity and lipid dynamics (Severcan et al. 2005). Our results showed that fatty acid desaturase (FAD2) expression increased remarkably in plants under freezing stress. By contrast, in melatonin pretreated plants, the FAD2 expression decreased when compared with non-pretreated plants. It should be noted that the lower transcription levels were recorded in plant treated with higher concentration of melatonin (0.5 μM). It has been proposed that the positioning of melatonin in the bilayers might be responsible for the reduction of lipid peroxidation and increasing of membrane fluidity (Garlic et al. 2014).

The findings of the current study verified that melatonin and its related catabolites might act as excellent free radical scavengers directly and by maintaining the fluidity of cell membrane, plays protective roles in pistachio seedlings under freezing stress. It seems that MEL concentration in plant tissues might influence antioxidant system homeostasis and potential resistance to chilling stress. Considering that MEL is economical and safe for animals and humans, its utilization as a bio stimulator could be an invaluable and affordable method in agriculture. It seems that by scavenging ROS through the cell, melatonin.

Conclusion

The results of this study showed that MEL could alleviate the oxidative damages induced by freezing and to maintain the structure and mobility of cell membrane in pistachio seedlings under freezing stress. These results were also confirmed by analysis of FAD gene expression. Considering that MEL is an inexpensive and, safe bio-stimulator, it could be applied in the food and agriculture industry. According to recent reports on the effect of abiotic stresses such as salinity, freezing and, drought on growth parameters and bud abscission in pistachio, it seems that MEL is a potential candidate that could be used to increase the resistant pistachio trees against variety of biotic and abiotic stresses in the field conditions.

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No potential conflict of interest was reported by the author(s).

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