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Research article

Melatonin increases the performance of *Malus hupehensis* after UV-B exposure

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ABSTRACT

Melatonin, an evolutionarily conserved molecule, is implicated in numerous physiological processes in plants. To explore the potential roles of melatonin in response to UV-B radiation, we examined the influence of exogenous melatonin on *Malus hupehensis* Rehd. seedlings under two levels of UV-B radiation. Under UV-B stress, seedlings showed significant reduction in plant growth, biomass production, and root system development. However, 1 μM melatonin solution markedly alleviated these effects, especially at the higher dosage of UV-B radiation. The inhibitory effects of UV-B radiation on photosynthetic parameters, chlorophyll fluorescence parameters, stomatal apertures, chlorophyll levels and leaf membrane damages were also markedly alleviated with melatonin application. Melatonin treatment was also associated with higher activity and expression of genes encoding antioxidant enzymes (ascorbate peroxidase, catalase and peroxidase) and greater decline of H_2O_2 content in leaves exposed to UV-B. Moreover, exogenous melatonin treatment and UV-B stress increased the concentration of endogenous melatonin. The content of several phenolic compounds, including chlorogenic acid, phloridzin and quercetin-3-galactoside, also increased under UV-B stress, and these were further elevated significantly with melatonin addition. This study provides insight into the role(s) of endogenous melatonin in response to UV-B stress, and will facilitate application of exogenous melatonin in agriculture.

1. Introduction

Plants are sessile organisms and cannot avoid exposure to various abiotic stresses that negatively affect plant growth (Kaling et al., 2015). UV-B radiation (280–315 nm wavelength) is an important component of solar radiation and can reach the Earth's surface and affect the biosphere (Yin and Ulm, 2017). Terrestrial plants are protected from much of the solar UV-B by atmospheric ozone, and recent breakdown in the earth's ozone layer has increased the amount of UV-B radiation reaching the surface (Mckenzie et al., 2007). Climate change and chlorine- and bromine-containing pollutants in the atmosphere are important factor in ozone depletion (Turtola et al., 2006).

Different dosages of UV-B affect plants differently. As an environmental regulator, natural levels of UV-B play important roles in regulation of plant growth and development (Hideg et al., 2013; Jenkins, 2009). However, elevated UV-B dosages can induce deleterious effects, including disruption of the integrity and function of important macromolecules (including DNA, lipids and proteins), oxidative damage, reduced biomass, photosynthesis, and disruption of plant architecture (Jordan, 2002; Jenkins, 2009; Hideg et al., 2013). Extended exposures to UV-B may negatively affect growth and productivity and may produce photomorphogenic effects on plants (Ballare et al., 2011), such as smaller but thicker leaves, decreased plant height and lowered biomass (Caldwell et al., 2007; Jansen et al., 2010).

For these reasons, it is important to find ways to improve plants' ability to protect against harmful levels of UV-B. Enhanced UV-B radiation is often related to an increased accumulation of reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anion radicals and hydrogen peroxide (Strid et al., 1994). However, plants have efficient systems for scavenging ROS that involve non-enzymatic compounds, such as ascorbate and glutathione, flavonoids, tocopherol, alkaloids, and a variety of enzymes, such as catalase (CAT), ascorbate peroxidase

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(APX), peroxidase (POD), and superoxide dismutase (SOD). Flavonoids and related phenolic compounds can absorb UV-B radiation, acting as a protective shield reducing the penetration of UV-B radiation in leaves (Sullivan, 2005). Phenolic compounds can also have antioxidant activity and scavenge ROS (Wang et al., 2015). As the precursor of many kinds of polyphenols, phenylalanine is enzymatically converted to anthocyanins via the phenylpropanoid and flavonoid pathways (Jaakola, 2013). Six enzymes in this pathway are phenylalanine ammonialyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT). There are three other branches in this pathway: flavonol synthase (FLS), which synthesizes flavonols, and leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), which respectively produce trans- and cis-flavan-3-ols (Xu et al., 2014).

As a tryptophan-derived molecule, melatonin (N-acetyl-5-methoxytryptamine) has many kinds of physiological and cellular functions in most of living organisms, including both plants and animals (Arnao and Hernández-Ruiz, 2006). In mammals, melatonin not only promotes cytoprotective and immunomodulatory properties, but also signals the time of day (Posmyk and Janas, 2009). Since melatonin was first reported in higher plants (Dubbels et al., 1995; Hattori et al., 1995), successive studies have led to the increase of information about the possible physiological functions of melatonin in plants (Tan et al., 2012).

In plants, melatonin not only regulates multiple developmental processes, but also mediates defense against stresses such as UV irradiation, chemical pollutants, extreme temperatures, fungal pathogens, drought, and replant disease (Hardeland and Pandi-Perumal, 2005; Posmyk et al., 2008; Szafranska et al., 2013; Yin et al., 2013; Wang et al., 2013; Li et al., 2018). Previous studies indicated that endogenous melatonin levels were elevated in plants in natural environments exposed to high intensity of UV irradiation (Hardeland and Pandi-Perumal, 2005). UV-B exposure stimulated melatonin synthesis in the roots of *Glycyrrhiza uralensis*, suggesting that melatonin's antioxidant properties can mitigate damage caused by UV-B irradiation (Afreen et al., 2006). Expression of two genes participating in melatonin synthesis, *AANAT* and *HIOMT*, in transgenic *Nicotiana sylvestris* led to reduction in DNA damage caused by UV-B (Zhang et al., 2012).

Although a great deal of previous research has focused on the protective effect of phenolic compounds against UV-B radiation, there have been no reports of potential involvement of melatonin (Kaling et al., 2015; Ruuhola et al., 2018). In this study, a hydroponic system was employed to examine the role of melatonin in *Malus hupehensis* seedlings in response to UV-B radiation. We also tried to explore the relationship among melatonin, root system architecture, gas exchange, stomatal properties, activity of antioxidant enzymes, and accumulation of individual phenolic compounds under UV stress. We hope that our research can contribute to the understanding of the mechanisms of melatonin in protection against UV radiation, and be utilized in applied ways to increase crop productivity in environment with high UV radiation.

2. Materials and methods

2.1. Plant material and treatments

All experiments were conducted at Northwest A & F University, Yangling, Shannxi Province, China (34°20′ N, 108°24′ E). As a rootstock for apple cultivation, *Malus hupehensis* Rehd. is commonly used in China. Seeds were obtained from their native region in Pingyi, Shandong Province, China (35°07′ N, 117°25′ E). The seeds collected were kept at 4 °C for 50 days for stratification and then were planted in the open air under temperature conditions and natural light. The hydroponics system used in our experiment was based on the description of Li et al. (2012). After 40 days of growth, similarly sized plants (8–10 leaves) were transferred to black plastic containers (length 52 cm, width 37 cm, height 15 cm) filled with Hoagland's nutrient solution (20 L, half-strength). To restrict the root systems from light exposure, all tubs were wrapped with black plastic. After that, the tubs were moved to a growth chamber (16–18 °C/23–24 °C night/day). During a 14-h photoperiod, sodium lamps were used as light source with light intensity of 150 μmol m^{-2} s^{-1}. Moreover, the nutrient solution of hydroponics system was refreshed every 3 days and was aerated with the help of an air pump. The pH of hydroponics system was adjusted to about 6.5 with H_3PO_4.

To adapt to the new conditions, plants were pre-cultured for 15 days. Based on a previous trial (unpublished data), we selected 1 μM melatonin (a pretreatment level) to apply to half of the seedlings 9 days before UV-B stress period. The addition of melatonin continued throughout the entire experiment.

As a source of UV-B radiation, UV-B lamp tubes with a spectral peak at 313 nm (40W, HUAQIANG Co., Ltd., Nanjing, Jiangsu, China) were used in our UV-B experiment. To cut off UV-C radiation below 290 nm, the lamps were covered with cellulose diacetate films (0.13 mm) and all filters were replaced weekly. Eight fluorescent lamps (40W, FSL Co., Ltd., Foshan, Guangdong, China) were used as control. Two UV-B lamp tubes and six fluorescent lamps were used as UV1, with the UV-B dosage of 0.24 W m^{-2}. Four UV-B lamp tubes and four fluorescent lamps were used as UV2, with the UV-B dosage of 0.45 W m^{-2}. The lamp fixtures were mounted in metal frames that were adjusted to a constant 0.5 m height above the plant canopies. After 15 days of precultivation, the untreated and melatonin-pretreated plants were divided into 6 groups randomly: (1) half-strength nutrient solution, eight fluorescent lamps (CK); (2) half-strength nutrient solution with 1 μM melatonin, eight fluorescent lamps (MT); (3) half-strength nutrient solution, two UV-B lamp tubes and six fluorescent lamps (CK + UV1); (4) half-strength nutrient solution with 1 μM melatonin, two UV-B lamp tubes and six fluorescent lamps (MT + UV1); (5) half-strength nutrient solution, four UV-B lamp tubes and four fluorescent lamps (CK + UV2); or (6) half-strength nutrient solution with 1 μM melatonin, four UV-B lamp tubes and four fluorescent lamps (MT + UV2). Seedlings were treated with UV-B 14 h/day for 20 days. Each treatment was performed with three replicates of 30 seedlings each. Ten subsamples for each replicate were taken to get the values for each replicate. On Day 20, the leaves were sampled from plants and quickly frozen in N_2, then stored at −80 °C throughout the remainder of the experiment.

2.2. Growth measurements and root architecture

After 20 days of UV-B treatment, shoot heights, leaf numbers, stem diameters and fresh weights were recorded from ten plants per treatment. Dry weights were then measured by drying the tissues for at least 72 h at 80 °C. Fresh weights and dry weights were recorded with 3 replicates. After the images of root system were collected with a scanner, date of root architecture was analyzed using the V4.1c WinRHIZO® image analysis system (Regent Instruments, Quebec City, QC, Canada). Root architecture data were recorded with 5 replicates.

2.3. Measurements of gas exchange parameters

The net photosynthesis rate (P_n), transpiration rate (T_r), intercellular CO_2 concentration (C_i), and stomatal conductance (G_s) were measured with a Li6400-XT photosynthesis system (LICOR, Huntington Beach, CA, USA) (Wei et al., 2018). For each treatment, data of photosynthesis were collected from 5 fully exposed mature leaves at the same position.

2.4. Observations of leaf stomata by SEM

The leaves sampled at the same position along the stem were fixed with a pH 6.8, 4% glutaraldehyde solution. After being rinsed 5 times

(5 min each time) with phosphate-buffered saline (PBS), the samples were dehydrated in a series of graded ethanol, dried in vacuum, and coated with gold. The scanning electron microscopy (SEM) observations were performed on an S-4800 microscope (Hitachi Ltd., Tokyo, Japan). Final tallies were used for recording of stomatal densities and image J software were used for recording of stomatal densities, lengths, widths, and apertures. Stomatal densities were randomly counted in 10 visual sections of leaves.

2.5. Investigation of chlorophyll fluorescence, chlorophyll, and relative electrolyte leakage

After each treatment, F_v/F_m, chlorophyll concentrations and relative electrolyte leakage (REL) were examined. F_v/F_m was measured by FluorCam FC 800-O based on the method of Pérezbueno et al. (2015) and then analyzed with the software of Fluorcam7 (PSI, Brno, Czech Republic). Eighty percent acetone was used for chlorophyll extraction and all pigment levels were measured spectrophotometrically, as described by Lichtenhaler and Wellburn (1983). REL was measured based on the method of Dionisio-Sese and Tobita (1998).

2.6. Measurements of H_2O_2 and antioxidant enzymes

H_2O_2 was measured based on the method of Patterson et al. (1984). 0.1 g leaf samples were ground with 1% (w/v) polyvinylpolypyrrolidone and then homogenized with potassium phosphate buffer (1.2 mL, 50 mM, pH 7.8) containing 0.3% Triton X-100 and 1 mM EDTA-Na_2.

CAT activity was assayed at 240 nm based on the method of Aebi (1984). APX activity was assayed at 290 nm according to the method of Nakano and Asada (1981). POD activity was determined at 470 nm based on the method of Rao et al. (1996).

2.7. Melatonin extraction and analysis

Leaves were collected 20 days after UV-B treatment. Melatonin in leaves was extracted in three biological replicates based on the method of Pothinuch and Tongchitpakdee (2011). The melatonin was detected using high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS), by a Q-TRAP tandem MS instrument (API 5500, AB SCIEX, Framingham, MA, USA) according to the method of Zhao et al. (2013).

2.8. Significant individual phenolic compounds assay by HPLC

Phenolic compounds were detected by HPLC (GL Sciences Ltd., Tokyo, Japan), based on the method described by Wang et al. (2015). The HPLC analysis was performed at 280 nm for epicatechin, procyanidin B1, procyanidin B2, catechin, syringic acid, gallic acid, and phloridzin; 320 nm for caffeic acid, chlorogenic acid, ferulic acid, and ρ-coumaric acid; 365 nm for quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin-3-galactoside, quercetin-3-arabinoside, quercetin-3-xyloside, and quercetin-3-rutinoside.

2.9. Total RNA extraction and quantitative real-time RT-PCR

Expression of *PAO*, genes encoding antioxidant enzymes, and genes involved in biosynthesis of phenolic compounds was analyzed with quantitative reverse transcription-polymerase chain reactions (qRT-PCR). The sequence of oligonucleotide primers used for qRT-PCR is shown in Table S1.

Total RNA from leaves was extracted based on the method of Yin et al. (2013). The concentration of total RNA was evaluated by the Nanodrop 2000 spectrophometer (Thermo Fisher, NY, USA). RNA integrity and quality were checked by electrophoresis using agarose gels. qRT-PCR was done using a SYBR Premix Ex Taq™ kit (Takara, Tokyo, Japan) and performed on an iQ5 Multicolor Detection System (Bio-Rad, CA, USA). In this experiment, *Malus* ELONGATION FACTOR 1 ALPHA gene (*EF-1α*) was used as reference gene. The qRT-PCR experiments were repeated three times using three separate RNA.

2.10. Statistical analysis

All data in our experiment were analyzed with SPSS software (version 20.0). Most data were expressed as means ± standard deviation (means ± SD). One-way ANOVA and Tukey's tests were used in this study. A p-value of < 0.05 was used to define a significant difference.

3. Results

3.1. Plant growth, biomass production and root system architecture

After 20 days of UV-B exposure, both low UV-B dosage (UV1) and high UV-B dosage (UV2) significantly inhibited the seedlings growth, as evident by significant decreases in values for shoot height (by 20.6% and 26.7%, respectively), leaf number (by 19.8% and 23.8%), and stem diameter (by 8.5% and 12.1%) (Fig. 1A–C). However, applying 1 μM melatonin to the roots markedly alleviated this response; those decreases were then only 10.9% and 16.1% for shoot height, and 8.7% and 9.5% for leaf number when compared with control values. After applying melatonin, we found no significant difference in stem diameter under UV-B radiation.

The protective effects of melatonin were also reflected in the data of plant weights. In the absence of UV-B, values for fresh weight, dry weight, shoot fresh weight, and shoot dry weight increased significantly after 1 μM melatonin was applied. UV-B (UV1 and UV2) exposure of 20 days resulted in decreases in values for fresh weight, dry weight, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight (Fig. 1D–I). Similarly, compared with control, the addition of melatonin markedly offset the decline in biomass production.

UV-B stress, especially high UV-B dosage (UV2) significantly inhibited root growth after 20 days of radiation. Compared with the CK, the root length, root diameter, root volume, root surface area, number of root tips, and number of root forks were reduced to 65.9%, 86.5%, 61.0%, 61.6%, 79.6%, 75.8% of the CK level after high UV-B dosage treatment. However, those values were reduced only to 88.4%, 90.9%, 78.2%, 76.2%, 102.9%, 104.8% in the melatonin-added group, respectively, when compared with CK plants (Table 1).

3.2. Gas exchange

In response to UV-B stress, P_n, an index of the assimilation efficiency of CO_2, was significantly decreased in values of 38.0% (UV1) and 64.8% (UV2) for plants pretreatment with melatonin versus a decline of 14.0% and 40.9%. Values for three other parameters of gas exchange, C_i, G_s and T_r, also decreased during the UV-B treatment period, and a similar effect of melatonin was also observed in G_s and T_r (Fig. 2).

3.3. Leaf traits and pseudo-color images

According to the results above, we concluded that the exogenous addition of 1 μM melatonin could alleviate the negative effects of UV-B, especially at high UV-B dosage (UV2) in *M. hupehensis* plants. So, we selected UV2 for further study. After 20 days of exposure to UV-B radiation, leaves from plants receiving no melatonin showed leaf surface shrinkage and a decrease in leaf area. However, the addition of 1 μM melatonin reduced this effect.

The maximum quantum efficiency of photosystem II (PSII) can also be used to test the effect of melatonin in protecting against UV-B. The pseudocolored F_v/F_m images and values revealed that the F_v/F_m value of plants treated with 1 μM melatonin was not significantly affected in

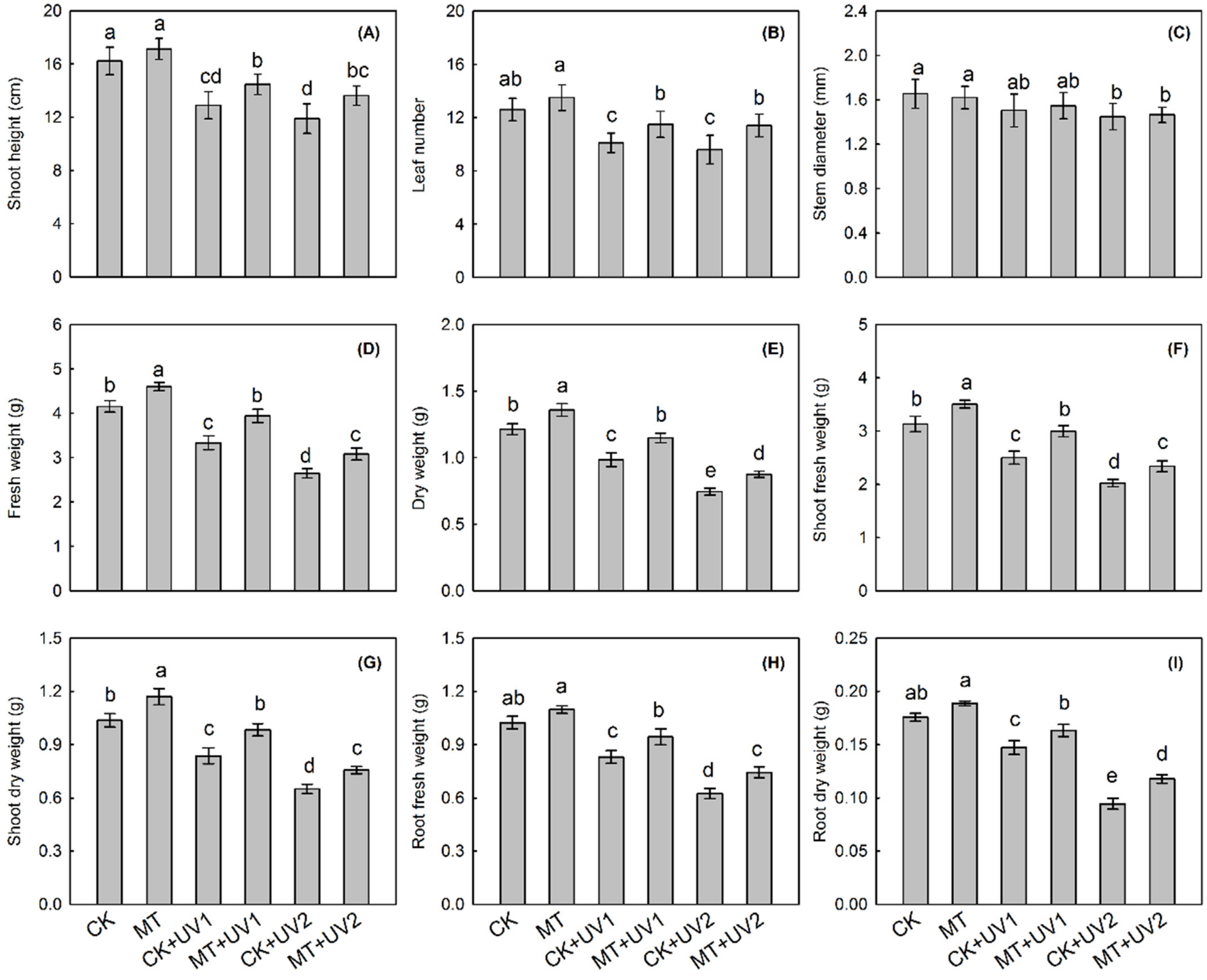

Fig. 1. Effects of 1 μM melatonin on shoot height (A), leaf number (B), and stem diameter (C) fresh weight (D), dry weight (E), and shoot fresh weight (F), shoot fresh weight (G), root fresh weight (H), root fresh weight (I) of *M. hupehensis* seedlings grown for 20 days of UV-B treatment. Values of A, B and C represent means ± SD (n = 10), values of D-I represent means ± SD (n = 3). Different letters indicate significantly different between treatments according to ANOVA and Tukey's test ($P < 0.05$).

the absence of UV-B. The F_v/F_m value was significantly reduced under UV-B stress, however. The addition of melatonin noticeably mitigated the detrimental effects of UV-B, as melatonin-enhanced leaves had higher F_v/F_m value compared with non-melatonin leaves (Fig. 3).

3.4. Stomatal properties

To assess the potential effects of UV-B and melatonin on stomatal properties, we examined leaves of UV-B-exposed and melatonin-treated seedlings using microscopy. Melatonin addition did not change stomatal properties of leaves in the absence of UV-B stress. However, values for the stomatal properties were significantly changed by UV-B radiation. Clear structural differences of leaves were also observed between treatments with or without melatonin. Stomatal density and width were higher in UV-B-exposed leaves than in the CK (by 26.5% and 10.3%, respectively), but the stomatal lengths and apertures were

Table 1
Effects of 1 μM melatonin on root length, root diameter, root volume, root surface area, number of root tips, number of root forks of *M. hupehensis* seedlings grown after 20 days of UV-B treatment. Values represent means ± SD (n = 5). Different letters indicate means are significantly different according to ANOVA and Tukey's test ($P < 0.05$).

	CK	MT	UV1	MT + UV1	UV2	MT + UV2
Length (cm)	286.49 ± 12.12 ab	327.86 ± 29.55a	239.48 ± 19.49bc	273.49 ± 36.08 ab	188.76 ± 26.42c	253.43 ± 43.18b
Diam (mm)	0.41 ± 0.03a	0.40 ± 0.01 ab	0.34 ± 0.03c	0.37 ± 0.01bc	0.35 ± 0.02c	0.37 ± 0.01abc
Volume(cm^3)	0.37 ± 0.04a	0.38 ± 0.03a	0.27 ± 0.03bc	0.28 ± 0.03bc	0.22 ± 0.02c	0.29 ± 0.03b
Surface Area (cm^2)	39.13 ± 2.94a	41.16 ± 4.65a	29.92 ± 3.02bc	31.63 ± 4.69b	24.10 ± 2.14c	29.84 ± 2.04bc
Tips	1390.20 ± 129.84a	1544.80 ± 138.08a	1426.80 ± 68.83a	1594.20 ± 127.50a	1107.80 ± 83.09b	1430.20 ± 181.82a
Forks	2498.00 ± 133.01 ab	2513.00 ± 99.68 ab	2208.60 ± 274.42bc	2655.60 ± 122.57a	1894.40 ± 102.11c	2618.20 ± 278.94a

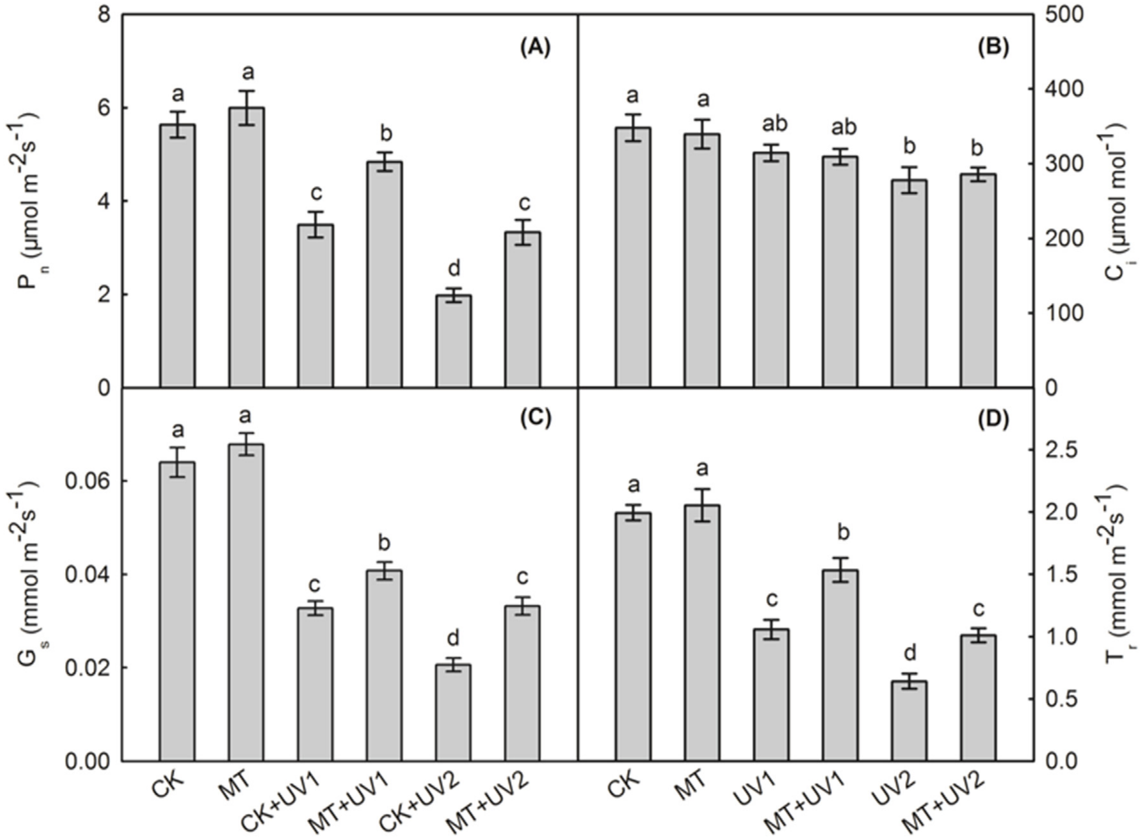

Fig. 2. Effects of 1 μM melatonin on (A) net photosynthesis rate (P_n), (B) intercellular CO_2 concentration (C_i), (C) stomatal conductance (G_s), and (D) transpiration rate (T_r) of *M. hupehensis* seedlings after 20 days of UV-B treatment. Values represent means ± SD (n = 5). Different letters indicate significantly different between treatments according to ANOVA and Tukey's test ($P < 0.05$).

significantly decreased (by 37.2% and 12.2%) (Fig. 4). Exogenous melatonin led to the increase in stomatal density and reduced the decrease in stomatal apertures, but no significant difference was found in stomatal lengths and widths after 20 days of exposure to UV-B radiation.

3.5. REL, chlorophyll and H_2O_2 contents and the activities of antioxidant enzymes

REL is often used as an index to assess the extent of leaf membranes injury. After 20 days of UV-B radiation, REL increased by 24.8% when compared with the CK. But that rise was subdued by the addition of melatonin, which tempered this increase by 11.9% (Fig. 5A). After 20 days of UV-B exposure, total chlorophyll concentrations were noticeably lower than in non-UV-B controls. Moreover, total chlorophyll was reduced by 9.0% and 3.1% in non-melatonin and melatonin-treated plants, respectively. Chlorophyll *b* concentrations showed the same change trend with total chlorophyll concentrations, while no significant change was found in chlorophyll *a* concentrations under UV-B stress (Fig. 5B–D).

Compared with the CK, UV-B stress noticeably increased leaf H_2O_2 content by 36.9% after 20 days of radiation treatment. Under UV-B stress, the addition of melatonin significantly suppressed the production of H_2O_2, with an increase of 17.6% as compared to the CK (Fig. 5E). The activities of enzymes involved in H_2O_2 scavenging changed noticeably after 20 days of the UV-B treatment. When comparing non-UV-B and UV-B stressed plants, the activity of APX was not significantly changed, while activities of CAT and POD increased by 79.8% and 24.6%, respectively. Pretreatment with melatonin led to much higher activities of APX, CAT, and POD. When compared with the control, these values increased by 35.6%, 143.6% and 64.3% (Fig. 5F–H), respectively.

3.6. The concentrations of melatonin in leaves

In order to understand the role of melatonin under non-UV-B and UV-B stress conditions we measured the concentrations of melatonin at 20 days after the onset of UV-B treatment. Without exogenous melatonin application, the level of melatonin under no UV-B stress conditions was 0.35 ng g^{-1} FW. After UV-B stress, melatonin concentration in stressed plants, reached 0.80 ng g^{-1} FW. Additionally, application of melatonin led to significant increase in endogenous melatonin concentrations (10.0 ng g^{-1} in UV-B stress and 15.0 ng g^{-1} FW in no stress plants; Fig. 6).

3.7. Levels of individual phenolic compounds

Phenolic compounds are produced as secondary metabolites and can enhance stress resistance as natural antioxidants in plants. The major groups of phenolics in plants are anthocyanins, benzoic and hydroxycinnamic acids, dihydrochalcones, flavanols and flavonols. Two anthocyanins, cyanidin-3-glucoside and cyanidin-3-galatoside, were quantified in this study, but were not detected in leaves in all treatments due to their low levels (data not shown). Without UV-B stress, there were no significant changes in the levels of any individual phenolic compound that was detected after melatonin pretreatment for 20 days (Table 2).

Four types of hydroxycinnamic acids and two types of benzoic acidswere identified in our study. After UV-B stress, gallic acid, chlorogenic acid, ρ-coumaric acid, ferulic acid increased by 76.5%, 148.4%, 25% and 500.0%, respectively, when compared with the CK. The content of chlorogenic acid was noticeably higher after exogenous melatonin compared with no melatonin pretreated leaves under UV-B radiation. Syringic acid and caffeic acid were significantly decreased by

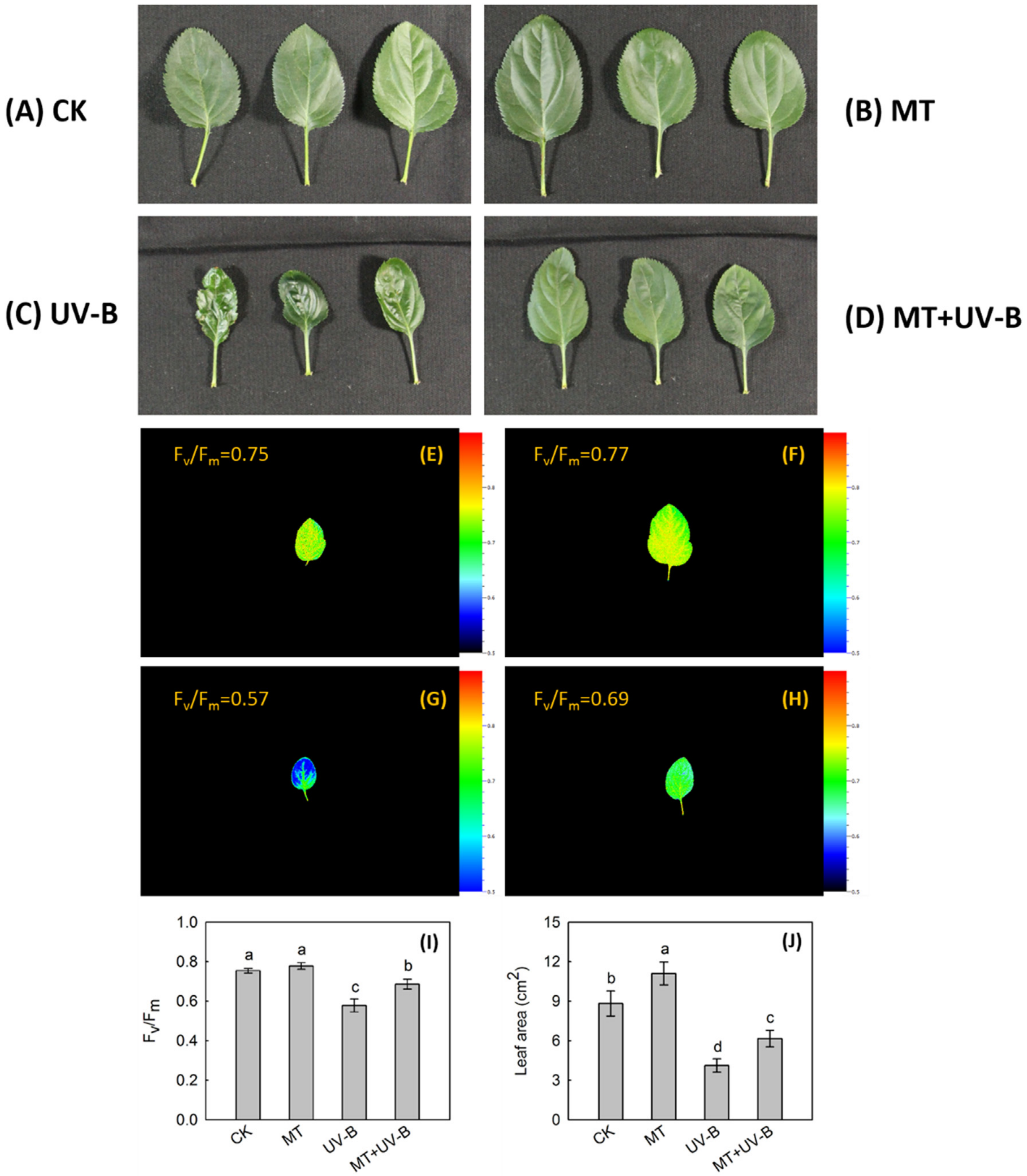

Fig. 3. Effects of 1 μM melatonin on the leaf traits (A, B, C and D), chlorophyll fluorescence images (E, F, G and H), F_v/F_m (I), and leaf area (J) of *M. hupehensis* seedlings after 20 days of UV-B treatment. (A and E) Control, without UV-B or melatonin treatments; (B and F) 1 μM melatonin treatments; (C and G) UV-B treatments; (D and H) with UV-B and 1 μM melatonin treatments. Values represent means ± SD (n = 5). Different letters indicate significantly different between treatments according to ANOVA and Tukey's test ($P < 0.05$).

34.8% and 47.6%.

Phloridzin is the dominant dihydrochalcone in *Malus,* and our experimental results showed that phloridzin constitutes 74.5% of total phenolic compounds in control leaves. After 20 days of UV-B radiation, the content of phloridzin was markedly increased by 11.3% and melatonin pretreatment significantly increased this value to 21.6% compared with the CK. Four types of flavanols, procyanidin B1, procyanidin B2, catechin and epicatechin, were identified and quantified in this study. After 20 days of UV-B radiation, no significant changes were found in the level of these flavanols, however, significantly higher levels of epicatechin were detected with the addition of melatonin compared with the CK.

In this study, quercetin glycosides (quercetin-3-galactoside, -glucoside, -xyloside, -arabinoside, -rhamnoside) were the only flavonols found in plant leaves. In UV-B-treated seedlings, the levels of these glycosides increased by 193.6%, 123.4%, 142.9%, 101.7% and 131.6%, respectively, when compared with the CK. After UV-B stress, quercetin-3-galactoside was the only quercetin glycoside we detected, with significantly higher levels in leaves that had undergone melatonin pretreatment than leaves that had not. The content of total phenolics in CK were 2210.4 mg/kg FW, and this increased to 2517.3 mg/kg FW by UV-B radiation, and further increased to 2742.5 mg/kg FW in UV-B stress conditions after melatonin pretreatment. Moreover, the level of total dihydrochalcones and total flavonols showed the same change trend in

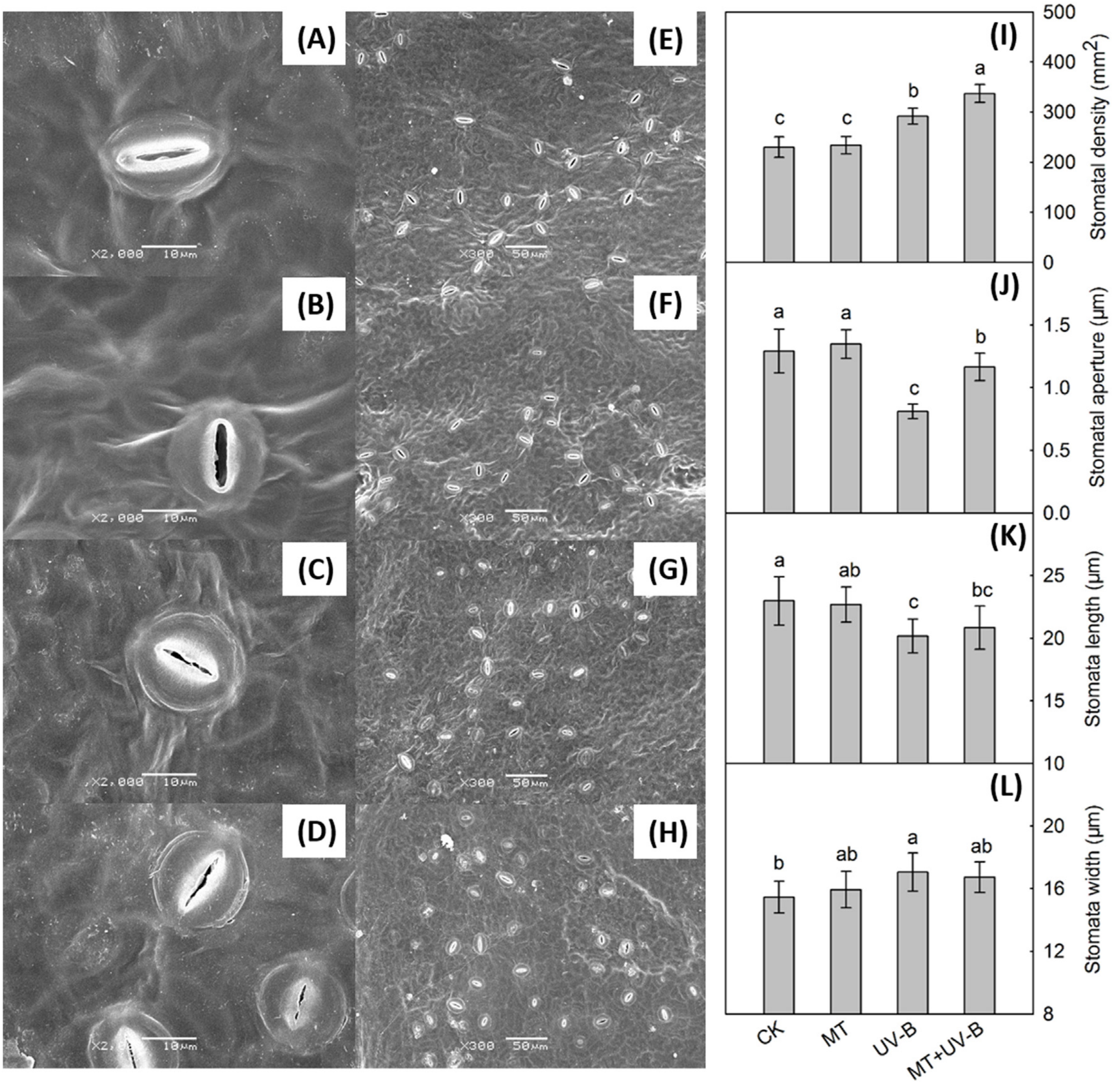

Fig. 4. SEM images of stomata and stomatal properties from *M. hupehensis* seedlings with exposed for 20 days of UV-B treatment. (A and E) Control, without UV-B or melatonin treatments; (B and F) 1 μM melatonin treatments; (C and G) UV-B treatments; (D and H) with UV-B and 1 μM melatonin treatments. (I) Stomatal density, (J) Stomatal length, (K) Stomatal width, (L) Stomatal aperture. (A, B, C and D) magnification 2000 × , scale bars = 10 μm; (E, F, G and H) magnification 300 × , scale bars = 50 μm. Values represent means ± SD (n = 10). Different letters indicate significantly different between treatments according to ANOVA and Tukey's test ($P < 0.05$).

our experiments.

3.8. Transcriptional regulation of related genes

To evaluate potential effects of UV-B and melatonin on gene expression, we analyzed one gene related to chlorophyll degradation, three genes related to antioxidant enzymes, and eight genes related to phenolic compound metabolism. As a nuclear-encoded enzyme, pheide a oxygenase (PAO) is induced by a gene related to chlorophyll degradation. Under UV-B stress, the *PAO* gene was upregulated 1.9-fold compared to CK, while application of melatonin noticeably inhibited the expression of *PAO* after 20 days of exposure to UV-B. In *M. hupehensis* leaves, *APX, CAT,* and *POD* were significantly up-regulated by 1.5-fold, 2.4-fold, and 1.9-fold compared to CK after 20 days of UV-B treatment. *APX* and *CAT* were significantly up-regulated by 2.7-fold, 4.0-fold with melatonin addition. To further study melatonin modulation of phenolic compounds' metabolism after UV-B stress, the mRNA levels of related genes were investigated via qRT-PCR. Relative to levels on Day 20, transcripts of *PAL, CHS, F3H, ANS, UFGT, LAR, FLS* were markedly increased by 4.3-, 4.1-, 4.4-, 8.9-, 8.5-, 2.9-, and 8.9-fold compared to CK in the leaves, respectively. However, the expression levels of *CHS, F3H, ANS, UFGT, LAR,* and *FLS* were noticeably higher in melatonin pretreated leaves than in the non-melatonin treated group under UV-B stress (Fig. S1).

4. Discussion

Melatonin is an evolutionarily conserved molecule that is implicated in numerous biological processes and stress responses in plants. In the last few years, there has been an increasing interest in the mechanistic roles of melatonin in regulating plant growth and development (Kanwar et al., 2018). We carried out a comprehensive investigation to evaluate the effects of melatonin pretreatment on plant response to UV-B, and to explore the mechanistic roles of melatonin in this process.

UV-B is a shorter wavelength of solar UV with high energy and can induce a large number of deleterious effects in plants as a stressor (Hideg et al., 2013). Long-term exposure to UV-B can have many detrimental effects on plant growth and productivity (Ballare et al., 2011).

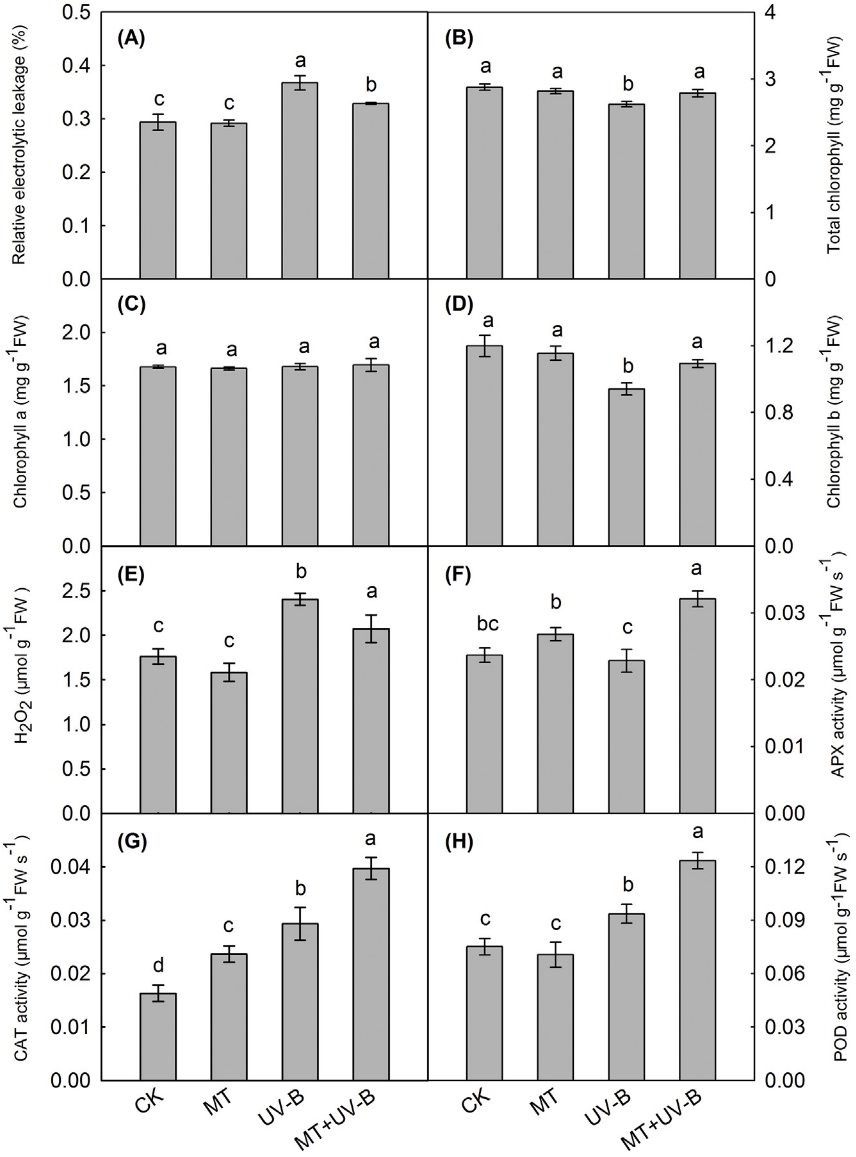

Fig. 5. Effects of 1 μM melatonin on (A) relative electrolyte leakage, (B) total chlorophyll, (C) chlorophyll *a*, (D) chlorophyll *b*, (E) H_2O_2 and main antioxidative enzymes: APX (F) CAT (G) and POD (H) of *M. hupehensis* seedlings after 20 days of UV-B treatment. Values represent means ± SD (n = 3). Different letters indicate significantly different between treatments according to ANOVA and Tukey's test ($P < 0.05$).

Our study showed that almost all of the parameters related to growth and biomass production measured here were significantly reduced in seedlings grown under UV-B. In addition, we found that those inhibitory effects were significantly alleviated with the application of exogenous melatonin. These results support previous suggestions that melatonin can promote plant growth under various stresses such as salt, cold and drought (Antoniou et al., 2017; Shi et al., 2015).

As the first plant organ to sense changes of water and minerals in the soil, the root plays a significant role in plant survival and performance (Liang et al., 2018a). Root growth and architecture are crucial for the plant's ability to explore the soil for their supplies of water and minerals efficiently (Gruber et al., 2013). We found here that values of root length, root diameter, root volume, root surface area, and numbers of root tips and forks were reduced for seedlings exposed to UV-B. However, exogenous melatonin application significantly alleviated these inhibitory effects, as evident from measurements of root fresh and dry weight. In sweet cherry rootstocks grown *in vitro*, melatonin promotes the growth of root at a low concentration, but inhibits the growth of root at high concentrations, indicating the action of melatonin is similar to IAA (Sarropoulou et al., 2012).

Abiotic stress reduces photosynthesis, leading to decrease in biomass production (Lidon and Ramalho, 2011). We found that UV-B exposure caused a rapid decrease in photosynthetic rates and decrease in almost all photosynthesis-related parameters. However, in our hydroponics trial, the negative impact of UV-B was significantly reduced when plants were pretreated with melatonin, consistent with previous observations (Li et al., 2012; Liang et al., 2018a). Some interrelated parameters as P_n, T_r, and G_s showed the same pattern of response, indicating that the process of photosynthesis was somewhat dependent on stomatal activity. Under UV-B stress, the reduction in P_n was accompanied by reduced G_s and T_r, implying that stomatal limitations might be the reason for P_n reduction. The protective role of melatonin in the process of photosynthesis may be accomplished through enhanced stomatal activity.

In this study, two levels of UV-B were used to evaluate the protective effect of melatonin under different environmental conditions. Within a certain range UV-B, plant would be influenced by more serious stresses in plant growth, development and yield (Jansen et al., 2010). Melatonin pretreatment reduced the negative impact of both levels of UV-B, but had more remarkable protective effects at the higher dose.

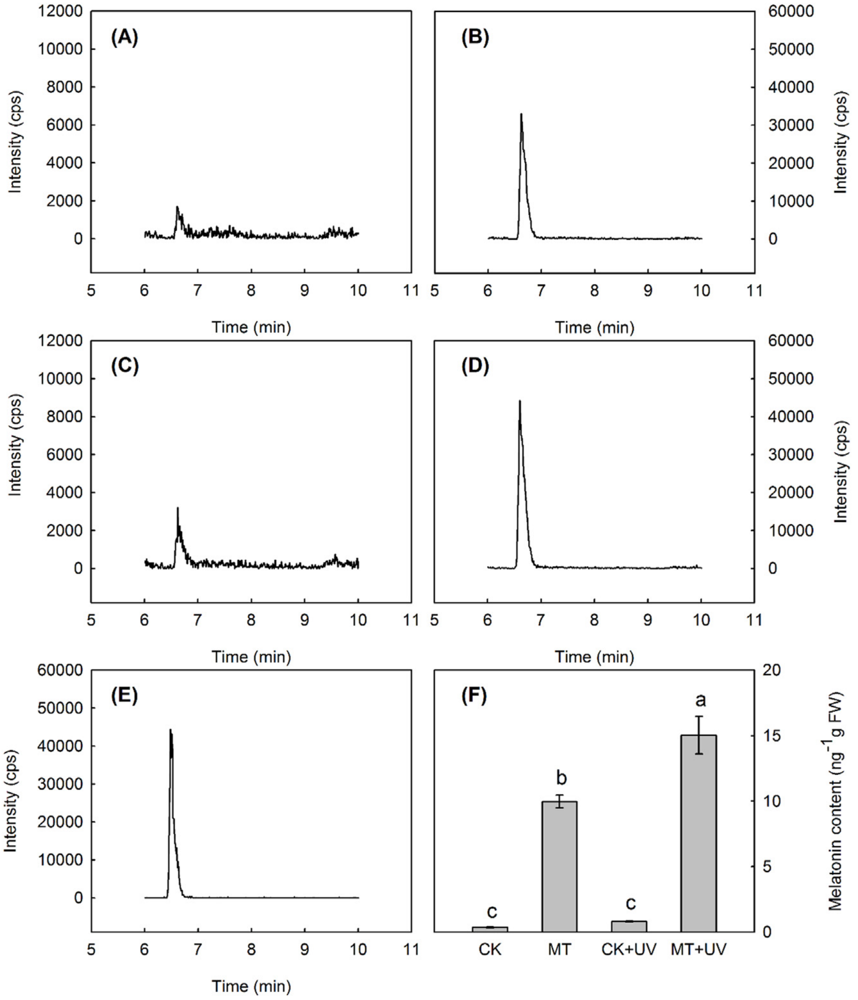

Fig. 6. HPLC-MS/MS spectra (A–E) and endogenous level of melatonin (F) in leaf samples of *M. hupehensis* seedlings with exposed after 20 days of UV-B treatment with 1 μM melatonin addition. HPLC-MS/MS spectra of CK (A), MT (B), UV (C), and CK + UV (D), melatonin standard sample (E), both retention times are 6.64. Effects of 1 μM melatonin on (A) relative electrolyte leak, (B) total chlorophyll, (C) chlorophyll *a* and (D) chlorophyll *b* of *M. hupehensis* seedlings after 20 days of UV-B treatment. Values represent means ± SD (n = 3). Different letters indicate significantly different between treatments according to ANOVA and Tukey's test ($P < 0.05$).

Table 2
Effects of 1 μM melatonin on concentrations of phenolic compounds in *M. hupehensis* seedlings after 20 days of UV-B treatment. Values represent means ± SD (n = 3). Different letters indicate means are significantly different according to ANOVA and Tukey's test ($p < 0.05$).

	Phenolic concent(mg/kg FW)			
	CK	MT	UV-B	MT + UV-B
Gallic acid	1.7 ± 0.1b	1.9 ± 0.3b	3.0 ± 0.2a	3.3 ± 0.3a
Syringic acid	32.8 ± 3.1a	37.4 ± 1.6a	21.4 ± 1.4b	22.9 ± 1.6b
Chlorogenic acid	6.2 ± 0.3c	6.9 ± 0.6c	15.4 ± 0.8b	18.4 ± 0.2a
Caffeic acid	2.1 ± 0.3a	2.1 ± 0.2a	1.1 ± 0.1b	2.1 ± 0.3a
ρ-Coumaric acid	0.4 ± 0.04b	0.5 ± 0.05 ab	0.5 ± 0.03a	0.6 ± 0.06a
Ferulic acid	0.1 ± 0.02c	0.2 ± 0.04bc	0.6 ± 0.04a	0.2 ± 0.03b
Sum of benzoic acids and hydroxycinnamic acids	43.4 ± 3.5 ab	49.0 ± 1.9a	42.1 ± 2.0b	47.6 ± 1.9 ab
Phloridzin	1647.7 ± 29.2c	1674.0 ± 32.8c	1834.3 ± 59.6b	2004.0 ± 67.6a
Sum of dihydrochalcones	1647.7 ± 29.2c	1674.0 ± 32.8c	1834.3 ± 59.6b	2004.0 ± 67.6a
Procyanidin B1	409.5 ± 10.2a	414.4 ± 12.4a	441.4 ± 20.3a	449.4 ± 25.8a
Catechin	9.6 ± 1.3a	12.3 ± 2.4a	13.1 ± 2.0a	11.5 ± 1.8a
Procyanidin B2	24.4 ± 3.8a	20.9 ± 4.9a	26.3 ± 5.2a	28.2 ± 2.1a
Epicatechin	16.7 ± 2.3bc	14.4 ± 1.1c	20.1 ± 0.9b	37.9 ± 2.2a
Sum of flavanols	460.3 ± 11.7b	462.1 ± 6.1b	500.9 ± 27.6 ab	527.1 ± 29.5a
Quercetin-3-galactoside	10.9 ± 0.6c	11.3 ± 1.0c	32.0 ± 1.4b	46.0 ± 4.4a
Quercetin-3-glucoside	21.4 ± 1.6b	22.1 ± 0.2b	47.8 ± 4.2a	52.0 ± 3.9a
Quercetin-3-xyloside	3.5 ± 0.2b	3.8 ± 0.3b	8.5 ± 0.9a	8.3 ± 1.1a
Quercetin-3-arabinoside	6.0 ± 0.5b	6.2 ± 0.8b	12.1 ± 0.9a	13.4 ± 2.6a
Quercetin-3-rhamnoside	17.1 ± 0.4b	17.0 ± 0.5b	39.6 ± 4.9a	44.0 ± 4.0a
Sum of flavonols	59.0 ± 1.5c	60.4 ± 1.6c	140.1 ± 9.7b	163.8 ± 14.9a
Sum of individual flavonoids	519.3 ± 10.3b	522.6 ± 5.6b	641.0 ± 37.1a	690.9 ± 44.4a
Sum of individual phenolics	2210.4 ± 33.1c	2245.5 ± 32.3c	2517.3 ± 94.0b	2742.5 ± 27.1a

Therefore, we selected the high level of UV-B for further study.

Plants respond to UV-B stress by adjusting their growth and morphology (Neugart and Schreiner, 2018; Robson et al., 2015). After 20 days of UV-B radiation, we observed reduction of the leaf area and decrease in the value of F_v/F_m. As an important chlorophyll fluorescence parameter, the F_v/F_m is often used to monitor photosynthetic performance, and decreases are frequently observed when plants are exposed to environmental stresses. Pretreatment with external melatonin or overexpression of *ASMT* substantially increased the value of F_v/F_m, highlighting a potential role for melatonin as a regulator of photosynthesis (Xu et al., 2016). In order to further study the relationship between a reduction in photosynthesis and stomatal limitations, we examined the stomatal properties using SEM. Under UV-B stress, stomatal density increased and addition of melatonin led to a further increase. Previous research has reported various effects of UV-B radiation on stomatal density. In rice, stomatal density decreased in all cultivars tested after 4 weeks of UV-B exposure, and a similar effect was also observed in soybean grown under UV-B (Gitz et al., 2005; Dai et al., 1995). However, adaxial stomatal density increased with UV-B treatment according to an experimental test of whether different levels of UV-B affect stomatal development. Potentially, the change in stomatal density is the result of UV damage to developing stomatal initials rather than the result of a true photomorphogenic process.

In this study, pretreatment with melatonin was associated with increased stomatal aperture and stomatal density in plants under UV-B stress. Under high light or heat stress, the supply of CO_2 to Rubisco is impaired, leading the photosynthetic apparatus to greater energy dissipation and decline of photosynthesis (Chaves et al., 2008). Higher stomatal density and larger stomatal aperture might explain why melatonin pretreated plants were able to maintain higher photosynthetic activity under UV-B stress.

Under UV-B radiation, the enhanced REL and the decline of total chlorophyll content were alleviated by melatonin, similar to our previous research in detached apple leaves (Wei et al., 2018). Similar results were found with drought-stressed apple plants, in which the addition of melatonin allowed the leaves to maintain stable levels of chlorophyll, less electrolyte leakage, and higher photosynthetic performance (Wang et al., 2013). A key gene related to chlorophyll degradation, *PAO,* was induced by UV-B radiation and was suppressed by exogenous melatonin. Our data for *PAO* expression and total chlorophyll contents indicated that the application of melatonin slowed chlorophyll degradation in UV-B-treated leaves.

We found that UV-B stress led to increase of H_2O_2 in the leaves, while the application of melatonin suppressed this production. Excessive accumulation of H_2O_2 causes lipid peroxidation, which leads to electrolyte leakage and membrane damage (Sairam and Srivastava, 2002). APX, CAT, and POD are antioxidant enzymes involved in scavenging H_2O_2; activities of CAT and POD increased significantly during the UV-B radiation. However, the application of melatonin was linked to higher APX, CAT, and POD activities compared with plants receiving no UV-B stress. In plants, melatonin not only plays a role as a generous scavenger of ROS and RNS, but also acts as a regulator of many antioxidant enzymes to protect plants against oxidative damage (Arnao and Hernández-Ruiz, 2015; Posmyk and Janas, 2009; Reiter et al., 2016). Moreover, expression of *APX, CAT, POD* were up-regulated after pretreatment with melatonin. This response may help seedlings reduce damage caused by UV-B radiation, similar results were also found in drought stress (Wang et al., 2013).

In the absence of exogenous melatonin, melatonin content in the CK was relatively low, while the content was increased by 128.6% with UV-B radiation. In previous studies, a higher level of melatonin was detected in plants from alpine and Mediterranean environments with high intensity of UV irradiation (Hardeland and Pandi-Perumal, 2005). In the study of *Glycyrrhyza uralensis*, the endogenous melatonin content was approximately 7-fold over control plants after UV-B radiation treatment (Afreen et al., 2006). The application of exogenous melatonin led to high levels of endogenous melatonin concentrations, especially under UV-B stress. As an effective free radical scavenger, the elevated melatonin levels may help plants to resist UV-B stress. Moreover, transgenic technology has been successfully used in apple and it may be helpful to our research work in the future (Meng et al., 2018a, 2018b).

As secondary metabolites and antioxidants in plants, phenolic compounds may scavenge excessive ROS (Fini et al., 2012). The accumulation of these compounds on the leaf surface is well known as a protective mechanism against UV-B radiation (Neugart and Schreiner, 2018). Although this has been widely studied, there is still no report on the effect of melatonin on individual phenolic compounds under UV-B stress. In the present study, we measured the content of individual phenolic compounds and the expression of related genes. Under UV-B radiation, no significant difference was found in the content of total phenolics, or almost all individual phenolics, after melatonin pretreatment compared with no melatonin control. However, increased content of total phenolics by UV-B radiation was also observed, which illustrates the role of phenolic compounds in defense to UV-B stress, especially at higher UV-B radiation level (Jansen et al., 2008). In plant leaves, phenolic compounds may act as antioxidants and shielding components at high UV-B levels (Agati and Tattini, 2010).

In the present study, exogenous melatonin was associated with the increased levels of phenolic compounds in UV-B stressed plants. In tomato, exogenous melatonin increased total anthocyanin content during fruit ripening (Sun et al., 2016). In kiwifruit, melatonin delayed leaf senescence and enhanced accumulation of flavonoids (Liang et al., 2018b). We found that three types of phenolic compounds (chlorogenic acid, phloridzin, quercetin-3-galactoside) were markedly increased with melatonin in plants under UV-B radiation, which is in accordance with the change of the level of total phenolic compounds. Phloridzin is the most abundant among these phenolics. In apple leaves, dihydrochalcone constitutes more than 90 percent the soluble phenolics (Gosch et al., 2009). The mitigating effects of melatonin under UV-B stress may be related to the increase in phloridzin, as phloretin is an excellent antioxidant (Liaudanskas et al., 2014). In addition, the increase in expression of the *CHS* gene which encodes a key enzyme in phloridzin biosynthesis, is correlated with the synthesis of phloridzin. Transcripts of most other genes related to phenolic metabolism were markedly enhanced under UV-B stress, and these genes were up-regulated by melatonin under stress condition, suggesting a mechanism for the observed increase in phenolic compounds (Liang et al., 2018b). Furthermore, *ANS* and *UFGT* are important genes for the synthesis of anthocyanins, and these genes were also up-regulated by melatonin under UV-B stress. However, anthocyanins were not detected in the leaves, potentially due to the absence of substrate, spontaneous chemical degradation, or some other reasons (Tsao et al., 2005).

5. Conclusions

In this study, a protective role for exogenous melatonin was confirmed in *M. hupehensis* after UV-B exposure. The application of melatonin showed mitigating effects in plant growth, biomass production, root system development and alleviated the inhibitory effects of UV-B radiation on photosynthetic systems, chlorophyll levels, stomatal apertures and leaf membranes damages. The increased activity of antioxidative enzymes, increased content of melatonin and phenolic compounds and up-regulation of related-genes expression were also observed in this study. Although this study complements several previous studies of the effects of melatonin on UV-B response, further work should be done to elucidate the potential mechanisms. To better understand the role of melatonin in UV-B stress, melatonin transgenic plants may be useful, and crosstalk between melatonin and phenolic compounds mediating stress signaling is worth further research.

Conflicts of interest

The authors declare that they have no conflict of interest.

Contribution

Z. Wei and F. Ma designed the experiments; Z. Wei performed the experiments with assistance from C. Li, T. Gao, Z. Zhang, B. Liang, and Z. Lv; Z. Wei and C. Li performed the data analyses and wrote the manuscript; and F. Ma and Y. Zou critically revised the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.026.

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Abbreviations

APX: ascorbate peroxidase
CAT: catalase
C_i: intercellular CO_2 concentration
G_s: stomatal conductance
HPLC: high performance liquid chromatography
HPLC-MS/MS: high performance liquid chromatography-tandem mass spectrometry
MT: melatonin
P_n: net photosynthesis rate
POD: peroxidase
qRT-PCR: quantitative reverse transcription-polymerase chain reactions
REL: relative electrolyte leakage;
SEM: scanning electron microscope
SOD: superoxide dismutase
T_r: transpiration rate