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RESEARCH ARTICLE

Effect of exogenous melatonin application on the expression of Catalase, Superoxide dismutase and menthol biosynthesis genes in *Mentha pulegium* L.

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Abstract

Mentha pulegium L., an aromatic culinary herb, has been prevalent since antiquity for its variant pharmacological potencies. Melatonin, a versatile signalling compound, ameliorates the growth of various plants in counter to a variety of abiotic and biotic stresses via enhancing antioxidant machinery. The present survey, unprecedently, investigated the effect of exogenous melatonin application on the expression of catalase, superoxide dismutase, limonene synthase and menthol dehydrogenase. After the treatment of *Mentha pulegium* L with melatonin (0, 150 and 250 µM) for 3 weeks, the expression levels of genes were evaluated by Real-time PCR. Our results illustrated that exogenous melatonin remarkably augmented mRNA expression of CAT, 5.64 and 29.22 fold changes respectively, for 150 and 250 µM. The SOD expression was boosted by 4.11 and 19.66 fold corresponding to 150 and 250 µM of melatonin. Also, exposure to melatonin upregulated the expression of genes that cooperated with menthol biosynthesis, Limonene synthase and Menthol dehydrogenase. 150 and 250 µM of melatonin enhanced Limonene synthase expression by 3.10 and 17.89 folds and significant overexpression of menthol dehydrogenase was displayed for 150 and 250 µM of melatonin, respectively by 13.80 and 23.32 times. Our findings propose melatonin can boost the oxidative stress resistance of *Mentha pulegium* L. by upgrading both the enzymatic and nonenzymatic antioxidant protective systems. Moreover, it elevated menthol production, which is in of demand in diverse industries, from pharmaceutical and cosmetics to tobacco and food processing.

Keywords

catalase; limonene synthase; melatonin; *Mentha pulegium* L.; menthol dehydrogenase; superoxide dismutase

Introduction

Mentha pulegium L., commonly known as pennyroyal, squaw mint and pudding grass, is an aromatic and tomentose perennial herb belonging to the Lamiaceae family, naturalized in Europe, the Middle East and North Africa (1, 2). Nowadays *Mentha pulegium* L. has found uses as an insect repellent, botanical infusion, condiment plant and remedial herb (3). Its variant pharmacological potentials have been characterized, comprising hepatoprotective, anti-microbic and anti-fungal activities, anti-inflammatory, antioxidant and accordingly anti-cancer properties against cancer cell lines (4 -6).

Melatonin (N-acetyl-5-methoxytryptamine) is a multifunctional indolic compound with a broad spectrum of cellular and physiological activities in plants and animals (7). Melatonin has been identified and measured in over 140 botanical species in the past few years. It broadly disperses in a variety of herbage organs, i.e. roots, stems, leaves, flowers, fruits and seeds (8, 9). Melatonin has been correlated with stimulating the development of primary, lateral and adventitious roots, branching and growth cycles of stems and leaves, seed germination, fruit maturation, photosynthesis, the circadian rhythm; membrane integrity and Osmo control (10, 11). Furthermore, it enhances cellular antioxidant, redox network and free radical scavenging system, thereby shielding plants against abiotic damage (12).

Catalase (CAT) (EC 1.11.1.6) is a tetrameric metalloenzyme. It is observed in peroxisomes and catalyzes the conversion of hydrogen peroxide molecules into oxygen and water (13). In numerous plants, encountering different abiotic stresses leads to the upregulation of the genes associated with catalase expression (14).

The metalloenzyme superoxide Dismutase (SOD) (E.C.1.15.1.1) conducts the transformation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (13). Exposure to various environmental harshness i.e., drought, high salinity and temperature and metal toxicity, boosted SOD activity in plants (15).

Menthol [5-methyl-2-(1-methyl ethyl) cyclohexanol; 2-isopropyl- 5-methyl cyclohexanol or p-methan-3-ol] is an antioxidant phenolic monoterpene compound, typically extracted from the aromatic Lamiaceae family, which has features conducive to fragrances (16,17). Biosynthesis of menthol in plants occurs in an 8-step pathway, initiating from the monoterpene precursor geranyl diphosphate and eventually terminating with (-)-menthol (18). Two key enzymes superoxide dismutase and catalase constitute the first line of defense antioxidants in plants (19). Menthol partakes in plant growth and development and also methanol-mediated regulation is observed during the development of plant defensive reactions in feedback to abiotic and biotic stresses (20). Moreover Menthol has been utilized in numerous domains from the pharmaceutical and cosmetic industries to tobacco and food Manufacturing, since it is applied as a flavour enhancer, preservative and for its cooling (features 16). Accordingly, any elicitor which can elevate CAT, SOD and menthol synthesis in plants is regarded as noteworthy. In previous studies the effects of exogenous melatonin elicitor on some spices of the genus *Mentha* i.e., *Mentha piperita* L., *Mentha spicata* L., *Mentha arvensis* L. were assessed (21-23). Howbeit there is no investigation evaluating the effect of melatonin on *Mentha piperita* L. Hence, in the present study, the effects of exogenous melatonin on the expression patterns of CAT, SOD, limonene synthase and menthol dehydrogenase (genes collaborating in menthol biosynthesis) in *Mentha piperita* L. were investigated.

Materials and Methods

Plant material and growth condition

This experiment was performed in the greenhouse of Shahr-e-Qods Branch, Islamic Azad University. The *M. pulegium* seeds were cultivated in 9 pots containing garden soil. After 3 weeks of germination, the plants were thinned to 4 per pot.

Preparation of melatonin and its exogenous application

The plants were randomly divided into 3 groups; melatonin (Sigma-Aldrich Chemie, Steinheim, Germany) was applied as a foliar spray in 3 doses (0, 150 and 250 µM) for 3 weeks. Melatonin was first dissolved in the minimal amount of ethanol and subsequently in Type I water. Upon completion of the trial, *Mentha pulegium* L. was harvested and the fresh weights of the plants were assessed. Eventually, they were maintained at -80 °C for gene expression analysis (Fig. 1).

RNA extraction and cDNA synthesis

The total RNA of the leaves was isolated using a column RNA isolation kit (DENA Zist Asia, Iran). The extracted RNA was qualified and quantified by agarose gel electrophoresis (1% agarose; Gibco/BRL) and Nanodrop spectrophotometer (Thermo Scientific 2000C, USA). The extracted RNA was treated with DNase I (RNase-free) based on the manufacturer's procedure (Fermentase, Canada). The complementary DNA was prepared with 500 ng of total RNA using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (#1622; Fermentas, Lithuania). 1 µL oligodT was added to 10 µL total RNA and incubated at 65 °C for 10 min. Subsequently, 2 µL RT buffer, 4 µL dNTP, 0.5 µL RiboLock RNase Inhibitor and 1.5 µL nuclease-free water were added to the mixture and maintained at 37 °C for 5 min. Accordingly, 1µL reverse transcriptase enzyme was also added and kept at 42 °C for 90 min. The mixture was incubated at 70 °C for 10 min with the aim of deactivation of the reverse transcriptase. The prepared cDNA was stored at

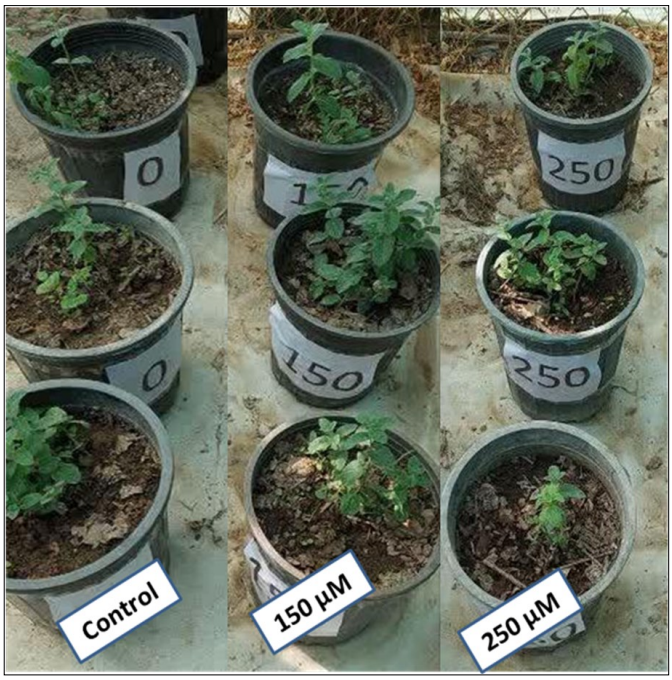

Fig. 1. The *M. pulegium* was treated with melatonin (0, 150 and 250 µM) as a foliar spray for 3 weeks.

-80 °C.

Primer design and real-time PCR

Primers for the genes, Catalase (CAT), Superoxide dismutase (SOD), Limonene synthase (LS), Menthol dehydrogenase (MR) and the housekeeping gene β-Actin *(ACTB)*, were designed using Geneious Ir9 and Oligoanalyzer software (Table 1). Quantitative PCR amplification was performed using a light cycler instrument (Applied Biosystems7500, USA) using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Bio DyneInc). The acquired threshold cycle (Ct) values were processed for later assessment, by the comparative C_t method. The Expression levels of the genes were normalized to the β-Actin giving the ΔC_t value. Ultimately, the fold change in the expression level of each mRNA was quantified based on the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Table 1 Name of genes, Nucleotide Sequences of the Primers Used for Real-time RT-PCR

Primer name	Sequence (5-3)	Tm	Product length
LS-F LS-R	AGTGCAAGGATAATGATGGGC GAGGTCAGTGAATTGTTCGAGT	60.2 60.1	105
MR-F , MR-R	CCCTAATTCCTCTCCTGCAAA CCTTTTGCCCATTCATTAGGC	59.5 59.2	100
CAT-F CAT-R	CACCGTCTTGGACCAAACTAT CGTCCCTATGCATGAAGTTCA	60 60	103
SOD-F SOD -F	CAGGGCATATGTTGACAACCT TGTTGAAGGGAGGAAGGAGAT	60 60	119
β-Actin-F β-Actin -R	ATGGAATTGTCAGCAACTGGG GAGGAGCCTCAGTCAAGAGAA	60 60.1	111

Statistical analysis

The experiments contained 3 groups and each group involved three plants. The gene expression assessments were repeated three times per plant. The results were illustrated as mean ± SD. Student's t-test was applied to compare the mean of the treated group with that of the control group and p-values < 0.05 were considered significant.

Results

Morphological effects of melatonin

Exposure of *Mentha pulegium* L. to 150 µM of melatonin was accompanied by an increase in fresh weight (Fig. 2) and number of leaves.

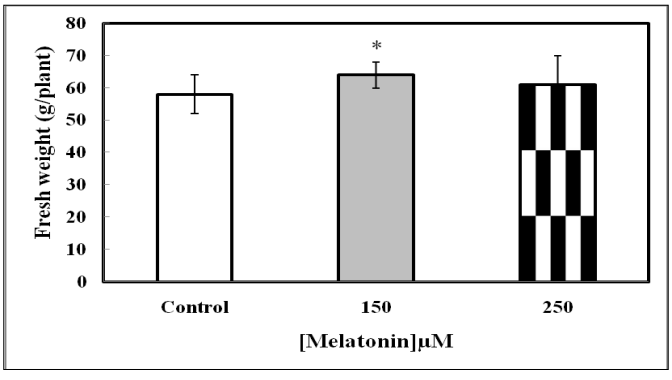

Fig. 2. The fresh weight of *M. pulegium* was treated with melatonin (0, 150 and 250 µM) as a foliar spray for 2 weeks.

Effects of melatonin on gene expression

Real-time polymerase chain reaction was implemented to evaluate the mRNA expression profile of CAT, SOD, LS and MR genes.

Catalase expression

Exposure of *Mentha pulegium* L. to 150 µM of melatonin elevated the CAT expression, i.e. 5.64 fold changes in comparison with the control. While, CAT expression was noticeably augmented, i.e. 29.22 for 250 µM (Fig. 3).

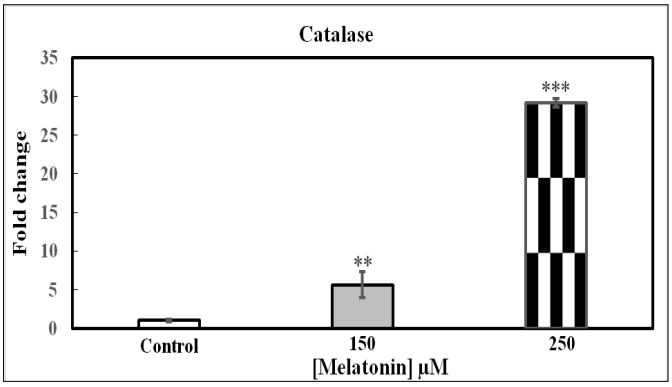

Fig. 3. The catalase gene expression in *M.pulegium* leaves was quantified using quantitative PCR and normalized to β-Actin levels. The data are presented as mean ± SD (n = 3), with asterisks denoting statistically significant differences, determined by Student's t-test with P < 0.05.

Superoxide dismutase expression

Melatonin upregulated SOD expression in a dose-related manner (Fig. 4). The expression was boosted by 4.11 and 19.66 fold corresponding to 150 and 250 µM of melatonin compared to the untreated plants.

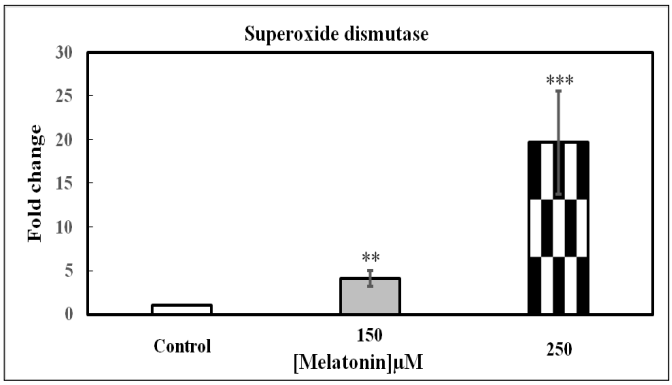

Fig. 4. The Superoxide dismutase gene expression in *M. pulegium* leaves was quantified using quantitative PCR and normalized to β-Actin levels. The data are presented as mean ± SD (n = 3), with asterisks denoting statistically significant differences, determined by Student's t-test with P < 0.05.

Limonene synthase expression

150 and 250 µM of melatonin amplified Limonene synthase expression by 3.10 and 17.89 folds in contrast with the control (Fig. 5).

Menthol dehydrogenase expression

As demonstrated in Fig. 6, remarkable overexpression of menthol dehydrogenase was observed for 150 and 250 µM of melatonin, respectively by 13.80 and 23.32 times.

Discussion

M. pulegium is a culinary and medicinal herb, presently commercialized as food and beverage flavouring (1). *M.*

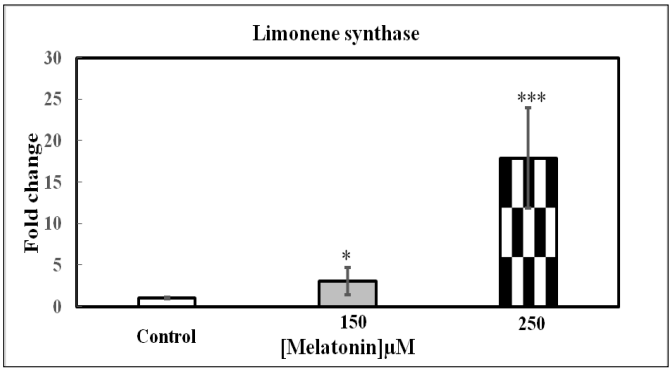

Fig. 5. The Limonene synthase gene expression in *M. pulegium* leaves was quantified using quantitative PCR and normalized to β-Actin levels. The data are presented as mean ± SD (n = 3), with asterisks denoting statistically significant differences, determined by Student's t-test with $P < 0.05$.

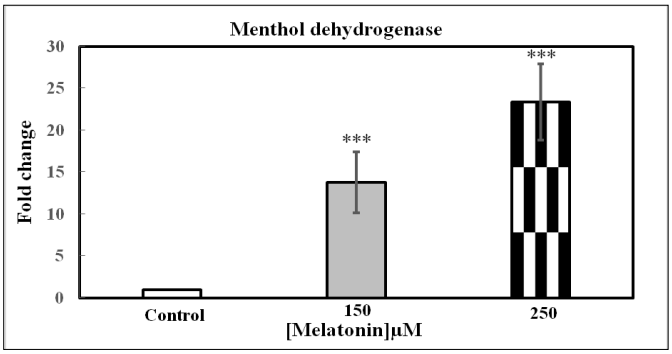

Fig. 6. The Menthol dehydrogenase gene expression in *M. pulegium* leaves was quantified using quantitative PCR and normalized to β-Actin levels. The data are presented as mean ± SD (n = 3), with asterisks denoting statistically significant differences, determined by Student's t-test with $P < 0.05$.

pulegium is an auspicious source of phytochemicals, which could be worthwhile for controlling oxidative stress-related diseases (24). Recent evidence indicated that the antioxidant activity of *Mentha pulegium* L. is associated with is flavonoids and phenolic constituents (25). Melatonin, a multipotent signalling molecule pervasively distributed in varied parts of plants, stimulated numerous physio-chemical responses to unfavourable environmental conditions. There is mounting evidence confirming that exogenously applied melatonin can enhance the stress tolerance of plants by adjusting both the enzymatic and non-enzymatic antioxidant protection systems (26). Extensive studies have surveyed the physiological and pharmaceutical potential of *Mentha pulegium* L. Although previous research has studied the effect of exogenous melatonin on some spices of the genus *Mentha* i.e., *Mentha piperita* L., *Mentha spicata* L., *Mentha arvensis* L. (21-23), however, the effects of melatonin on its physiological properties have not elucidated. Therefore, in the present survey, the effects of melatonin exposure on the expression levels of CAT, SOD and the collaborative genes in menthol biosynthesis, in *Mentha pulegium* L. were investigated.

The SOD catalyses superoxide radicals into hydrogen peroxide and molecular oxygen. Subsequently, the catalase serves, as the eliminator of H_2O_2, converting hydrogen peroxide into water and molecular oxygen. Accordingly, 2 harmful oxygen species, the superoxide radical and hydrogen peroxide are eliminated and converted to water (27). Our results unprecedentedly elucidated that in the absence of stress conditions, melatonin exposure of *Mentha pulegium* L. significantly elevated mRNA expression of CAT and SOD, in a dose-dependent manner. Previously, a growing body of published literature assessed the effect of exogenous melatonin under adverse conditions on varied crops. It was illustrated that the activity of the expression of enzymatic antioxidants, CAT and SOD was coµMonly promoted under harsh physicochemical conditions (26). It was revealed that heat stress remarkably regresses menthol biosynthesis in *Mentha arvensis* L., while melatonin spray enhanced the activity of CAT and SOD in 2 spices of menthe genus, *Mentha* × *piperita* L. and *Mentha arvensis* L. (21). It is worth mentioning that exogenous melatonin increases the levels of endogenous melatonin. Subsequently, endogenous melatonin prompted the enzymatic activity and mRNA levels of superoxide dismutase and catalase (28).

It has been displayed that exogenous melatonin noticeably augmented the biosynthesis of menthol via the upregulation of limonene synthase and menthol dehydrogenase expressions. Our results are consistent with reports that menthol biosynthesis and concentrations were elevated in *Mentha* × *piperita* L. under temperature ranges 14-32 °C and a spray dose of 30 M (21). Earlier investigations on cell suspension culture of *Mentha pulegium* L., showed that salicylic acid and yeast extract elicit or increase the amount of menthol. It was demonstrated that the maximum amount of pulegone (intermediate in menthol biosynthesis pathway), as the product of pulegone reductase and menthol, as the product of menthol dehydrogenase, produced was in the cell culture treated with salicylic acid 6 mg/L (which was more than in the natural plant). It was also shown that the amount of menthol increased with increasing doses of yeast extract elicitor (29).

Conclusion

The results of the present survey displayed that enzymatic antioxidants, catalase and superoxide dismutase, were augmented under melatonin exposure in *Mentha pulegium* L. Furthermore, it is demonstrated that melatonin is capable of elevating menthol biosynthesis in the *Mentha pulegium* L. Since, there is a positive correlation between melatonin and limonene synthase and menthol dehydrogenase gene expressions. Exogenous melatonin can reinforce the oxidative stress tolerance of *Mentha pulegium* L. by enhancing both the enzymatic and nonenzymatic antioxidant protective systems.

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Authors' contributions

FGH contributed to all assays and collecting results. SKH designed the study and had the responsibility of supervising and conducting plant culture and treatment. LA Contributed as an advisor of research, conducted gene expression studies and wrote the manuscript. All authors have reviewed the final version of the manuscript and approved it for publication.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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