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Original Article

Influence of Methyl Jasmonate on Menthol Production and Gene Expression in Peppermint (*Mentha* x *piperita* L.)

Soheila Afkar[1], Ghasem Karimzadeh[1*], Mokhtar Jalali-Javaran[1], Mozafar Sharifi[2] and Mehrdad Behmanesh[2]

[1]*Plant Breeding and Biotechnology Department, Faculty of Agriculture, Tarbiat Modares University, Tehran, P. O. Box 14115-336, Iran*
[2]*Plant Biology Department, Faculty of Basic Sciences, Tarbiat Modares University, Tehran, Iran*

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Abstract

Peppermint has considerable commercial value and widely cultivated for essential oil production, especially menthol. The aim of this study was to determine the quantitative expression of pulegone reductase (*pr*), menthofuran synthase (*mfs*) and limonene synthase (*ls*) genes in menthol biosynthesis pathway in *Mentha* x *piperita* , using semiquantitative RT-PCR analysis and evaluating menthol production by GC/MS analysis in presence of different methyl jasmonate concentrations (MJ; 0, 0.1, 0.5 mM). RT-PCR analysis showed that *pr*, *mfs* and *ls* specifically induced by MJ treatment. The transcripts of these genes up-regulated within 4-12 h of MJ induction followed by down-regulation after 24-48 h of MJ exposure.

Key Words: *Mentha* x *piperita*, Methyl jasmonate, Gene expression, Pulegone reductase, Menthofuran synthase, Limonene synthase

Abbreviations: MJ- Methyl jasmonate, *pr*- Pulegone reductase, *mfs*- Menthofuran synthase, *ls*- Limonene synthase, JA- Jasmonic acid, SA- Saicylic acid

Introduction

The genus *Mentha* belongs to Labiatae family, including more than 25 species without numerous varieties obtained by spontaneous hybridization [1, 2]. Mint plants are vegetables of considerable commercial value and widely cultivated for their essential oil [3]. Almost all (99%) of the oil glands of peppermint are located in leaves [4]. Its mature leaves contain the highest levels of menthol: the major component of peppermint oil [5,6]. Although the menthol percentage increases steadily in peppermint through the season [4], the yield of oil increases to a peak follows by a late season decline [7,8]. Peppermint is traditionally used in the symptomatic treatment of digestive disorders; the antispastic, carminative, choleretic and cholagogic properties attributed to it are referred to the presence of the essential oil rich of menthol [9]. Menthol is derived from plants of the mint family and imparts their distinctive odor. Menthol is commonly used in food additives and has broad industrial use in oral hygiene, medicinal and other applications [10,11]. Menthol is applied to the skin elicits cooling and tingling sensations, and has anesthetic effects [12]. Limonene synthase represents the committed step of menthol biosynthesis by conducting the cyclization of the universal precursor Geranyl pyrophosphate (GPP) to the parent olefin of all subsequent p-menthane pathway intermediates. Interest in this monoterpene cyclase stems also from the fact that limonene is the precursor of carvone in the essential oil of spearmint [13]. Pulegone assumes to have a central role in monoterpene metabolism in peppermint as the precursor of menthofuran, menthone and isomenthone through the reduction of the saturated ketones, menthol, neomenthol, isomenthol and

*Corresponding author: Plant Breeding and Biotechnology Department, Faculty of Agriculture, Tarbiat Modares University, Tehran P. O. Box 14115-336, Iran
E-mail address: karimzadeh_g@modares.ac.ir

neoisomenthol [14]. An important diversion from the pathway to menthol is the transformation of pulegone to menthofuran catalyzed by menthofuran synthase. Menthofuran is considered a stress metabolite whose production is favored by low light intensity, short day length, warm night temperature, nutrient and water deficiency [15-17]. Under extreme abiotic stress conditions, organic molecules such as lipids, proteins, and nucleic acids are prone to damage and/or degradation [11]. The response and adaptation of plants to such conditions are very complex and plants have developed various strategies to increase stress tolerance. Under these conditions, stress response mechanisms are activated, either to prevent the source of damage or to promote the rapid turnover of damaged molecules [18]. These strategies include changes in metabolic processes, structural changes of membranes, expression of specific genes and the production of some metabolites [19,20]. Plant defense responses are regulated through a complex network of signaling pathways that involve endogenous signaling molecules, such as SA and JA. It has become evident that the SA and JA signaling pathways are mutually antagonistic [21, 22]. Jasmonic acid and its methyl ester (MJ) have been reported to play an important role in a signal transduction process that regulates defense genes in plants [23]. However, although exogenously applied MJ is widely used in plant cell cultures to activate secondary metabolism, there are surprisingly few studies about its impact on plant growth, considering that jasmonates have a variety of biological activities, including inhibition of either seed and pollen germination [24,25] or root growth and photosynthetic apparatus [20,26-28]. Farmer et al. [29] reported that exogenous jasmonates were capable of directly inducing PIN gene expression. The role of jasmonates in controlling gene expression and secondary metabolism is, however, complicated by a number of observations, especially with regard to isoprenoid metabolism [29]. Choi et al. [30] reported that jasmonates differentially regulate HMG-CoA reductase genes in potato. On the other hand, MJ induced the accumulation of the diterpene taxol in *Taxus canadensis* cell cultures and that accumulation was correlated with the induced expression of two genes coding for downstream biosynthetic enzymes, GGPP synthase, and taxadiene synthase [31]. For example, it was shown that treatment of Norway spruce with MJ induces a complex, traumatic oleoresin response including *de novo* differentiation of traumatic resin ducts in the developing xylem, increased accumulation of monoterpenes and diterpenes, and induced enzyme activities and gene expression of monoterpene synthases and diterpene synthases [32,33]. Gene expression profiling serves one of the cornerstones of modern molecular biology and contributes to the fundamental interpretation of molecular and genetic mechanisms under certain environmental and developmental conditions [34]. In other organisms, analysis of endogenous gene expression is most commonly performed, using techniques such as northern gel blot analysis, RNase protection and primer extension. A major limitation with such techniques is that they require large amounts (tens of micrograms) of starting RNA. Alternatively, reverse transcriptase dependent polymerase chain reaction (RT-PCR) can quantify specific mRNAs in extremely small amounts of starting material (containing only a few molecules of a particular mRNA). This technique was developed initially for medical applications. In plants, although it is often used to determine the presence or the absence of a particular transcript, only in limited cases it has been used to quantify gene expression levels [35, 36]. The semi-quantitative reverse transcription-PCR (RT-PCR) offers advantages in sensitivity and specificity and has a broad range of detection.

The present work was aimed to study the expression level of *pr*, *mfs* and *ls* genes after exposure of *Mentha* x *piperita* plant to different concentrations of MJ at various time periods, using semi quantitative RT-PCR technique.

Materials and Methods

Plant Materials, MJ Treatment and Samplings

This experiment was carried out under natural light conditions in the greenhouse of College of Agriculture, Tarbiat Modares University, Tehran, Iran. The peppermint plants were supplied kindly from Iranian Institute of Medicinal Plants, Karaj, Iran. The peppermint plants initiated from 10 cm-long rhizome cuttings followed by transferring into pots. They were watered every day with the addition of fertilizer twice a month. At the early bloom stage, 0.1 and 0.5 mM MJ in 2% (v/v) ethanol was sprayed onto the peppermint plants. The untreated peppermint plants (control) were sprayed with only 2% (v/v) ethanol. Then, the MJ-treated and the control plants were zipped with a vinyl pack for 40 min. Before the vinyl pack was removed, the control plants were isolated from the treated plants, and the treated plants were left in the open air for 2 h to completely evaporate the

remaining MJ. Leaves from the untreated (control) and the MJ-treated peppermint plants were randomly sampled at 0, 4, 8, 12, 24 and 48 h after treatment. The leaves were immediately frozen in liquid nitrogen and stored at -80 °C prior to RNA extraction.

RNA Extraction, Quality Control and cDNA Synthesis

Extraction of total RNA was carried out using the Nucleo spin RNA Plant kit (Macherey Nagel, Germany). RNA was treated with DNase I (RNase-free) according to the manufacturer's instruction (Fermentase, Canada), and the purity of RNA was assessed by 1.5% (w/v) agarose gel electrophoresis, with no degradation was observed. For comparison and quantity of total RNA was examined using a NanoDrop 2000C (Thermo Scientific, NANODROP, 2000C, USA). The concentration of total RNA was determined with NanoDrop by measuring absorbance at a wavelength of the 260 nm (A260) and its purity was assessed by the ratio of absorbance values at 260 and 280 nm, where in ratio of about 2 was considered a good indication of purity. The first strand cDNA was synthesized with 200 ng of total RNA, using the RT-PCR system (Cinnagen, Iran) according to the manufacturer's protocol.

Primer design, Verification of Amplified Products and Sequencing Reactions

Four studied genes were selected from the literature for semi quantitative RT-PCR (Table 1). For normalization, an *act* (actin) fragment amplified by the primer designed from *Arabidopsis thaliana* was employed as an internal standard. The primers of *act* and the genes of interest *pr*, *mfs* and *ls* were designed with AlleleID software according to the cDNA sequences *pr* (AY300163.1), *mfs* (AF346833.1) and *ls* (EU108697.1) of *M.* x *piperita*. For each gene, the PCR conditions were modified according to the primer properties, and the PCR products were collected and quantified after various numbers of amplification cycles to determine the number of cycles that produced the largest differences among samples. PCR was performed on the samples with primers (5 pmol) and Master Mix (CinnaGen, Iran). Amplifications were performed with the following program: 94ºC for 2 min, followed by 30 cycles of 94 ºC for 45 s, (Annealing temperature for each gene) for 45 s, and 72 ºC for 45 s. The optical density of PCR products were measured by using Image Guage Software. The molecular weights of the amplification products were calculated, using 100-bp DNA ladder standards (Fermentase, Canada). The primer pairs of 4 genes (*pr*, *mfs*, *ls* and *act*) were tested with RNA extracted from the control sample (untreated plants). Of these 4 primer pairs, all of them gave specific band at different bp. Primer pairs No. 1 (*pr*) gave band with molecular size of 496 bp; primer pair No. 2 (*mfs*) gave band molecular size of 341 bp, while primer pair No. 3 (*ls*) gave band molecular size of 547 bp and primer pair No. 4 (*act*) gave band molecular size of 600 bp (Table 1). PCR products were checked on 1.5% (w/v) agarose gel, and shown to have the expected size. In order to determine the sequences of amplification products, DNA sequence was performed by Gen Fanavaran Company (Tehran, Iran). The sequences of amplification products were confirmed, using BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Compositional Analysis of Essential Oil

At the early bloom stage, the leaves were cut from the plants and total oil of leaves was estimated by steam distilling 30 g of freshly chopped leaves in a Clevenger apparatus. Subsequently, the essential oils from the 3 plants in each replicate were combined and analyzed, using a model Agilent 6890 gas chromatograph fitted with a HP-5MS column (30 m, 0.25 mm; film thickness, 0.25 µm). Flow rate of the carrier gas (Helium) was 8 ml/min. Oven temperature was programmed from 50 °C to 300 °C at 15 °C per min.

Table 1 Sequence, Tm and product size of *act*, *pr*, *mfs* and *ls* primers

Gene	Primer	Sequence 5'-3'	Tm	Product size (bp)
act	Forward	TCTGGAGATGGTGTGAGCCACAC	58	600
	Reverse	GGAAGGTACTGAGGGAGGCCAAG		
pr	Forward	ATCCTTCCTGGCTCTACTATTG	60	496
	Reverse	CATCTTCCCTCCAACATTATCG		
mfs	Forward	TCTACGCCTACATCCACCTTTC	59	341
	Reverse	CCTCCGCTCTATCTCCATTACC		
ls	Forward	CGCCTTTCTCTTTCATAACATCG	60	547
	Reverse	AGAGTGAATGAGGGTGGTGGTGATG	-	-

Table 2 Chemical composition of peppermint oil on different concentration of MJ

Essential oil constituents	Control	0.1 mM	0.5 mM
Menthol	29.99%	35.53%	34.39%
Menthone	20.23%	19.02%	20.30%
Menthofuran	12.36%	5.74%	14.10%
Pulegone	2.69%	3.72%	3.18%
Methylacetate	3.43%	1.52%	3.35%
1,8- Cineole	12.09%	13.84%	14.02%

Results and Discussion

To determine whether MJ is involved in the expression level of *pr*, *mfs* and *ls* genes the quantification of transcripts of these genes was performed by semi-quantitative RT-PCR analysis to find the changes in the transcript level of these genes under exogenous 0.1 and 0.5 mM MJ at different time points of 0, 4, 8, 12, 24 and 48 h after treatment induction. In *Mentha* x *piperita* plants, the increase of menthol levels soon after the beginning of MJ treatment at 0.1 and 0.5 mM concentration indicated a rapid response of these plants to MJ, as well as the ability of MJ to stimulate menthol biosynthesis. In our present study, the level of constituents within the essential oil varied with different MJ concentrations, leading to increases and no change in the percentage of various constituents as compared to control plants (Table 2).

The previous studies showed that menthol and 1,8-Cineol have pharmaceutical applications. Menthol production was assayed in response to different concentrations of MJ treatments. Menthol content in the oil was increased at least 1.2- and 1.1-fold at 0.1 and 0.5 mM MJ treatments relative to the control, respectively. Menthone amount did not change after 0.5 mM MJ but after 0.1 mM MJ application reduced at least 0.9 compared to the control. We expected that menthone would be reduced because it was a precursor of menthol. Menthofuran and Pulegone amount was increased 1.1- and 1.2- fold, respectively in 0.5 mM MJ-treated plants compared to the controls. Similarly, at 0.1 mM MJ, their content was also changed by 0.5- and 1.4- fold, correspondingly compared to the untreated control plants. Increased amount of menthofuran in 0.5 mm associated with up-regulation of *mfs* gene expression. Also 1,8- Cineol production increased 1.1- and 1.2- fold at 0.1 and 0.5 mM MJ compared to control. Previous reports identified that changes in essential oil constituents were observed in a range of aromatic plants under stress, although the changes in constituents appeared to differ for different stresses and various plant materials [37,38]. In spite of the economic importance of the essential oils, relatively little is known about the regulation of the biosynthesis of terpenoids essential oil. This lack of information on metabolic controls is a serious impediment to the development of strategies for bioengineering of essential oil composition and yield [22,39,40]. It has been reported that various elicitors such as chitosan, *â*-glucan, and yeast extracts and plant hormonal chemicals such as JA and MJ can induce secondary metabolites in various plants. Those elicitors can act like biotic and abiotic stresses such as wounding, pathogen attack, UV light exposure, and temperature upon plants [41]. MJ is considered a key compound in the signal transduction pathway, involved in the induction of low-molecular weight compounds that take part in several plant responses [42].

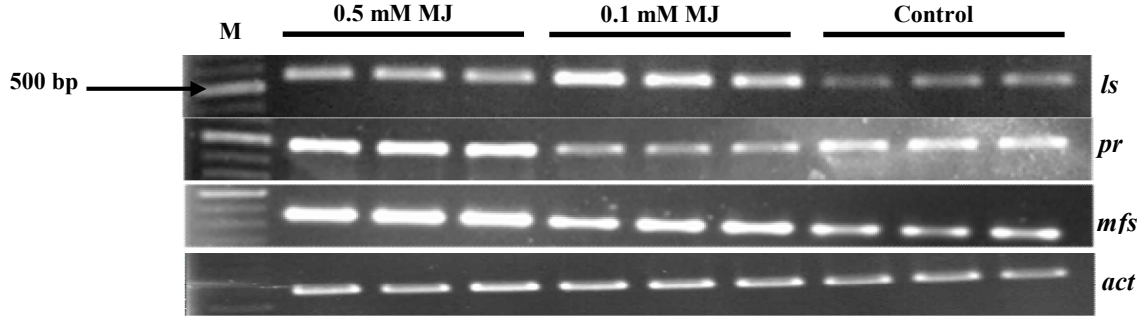

Fig. 1 RT-PCR analysis of *pr*, *mfs* and *ls* 12 h after Methyl Jasmonate treatment in *Mentha* x *piprtita*
M= molecular-weight-marker ladder (100 bp)

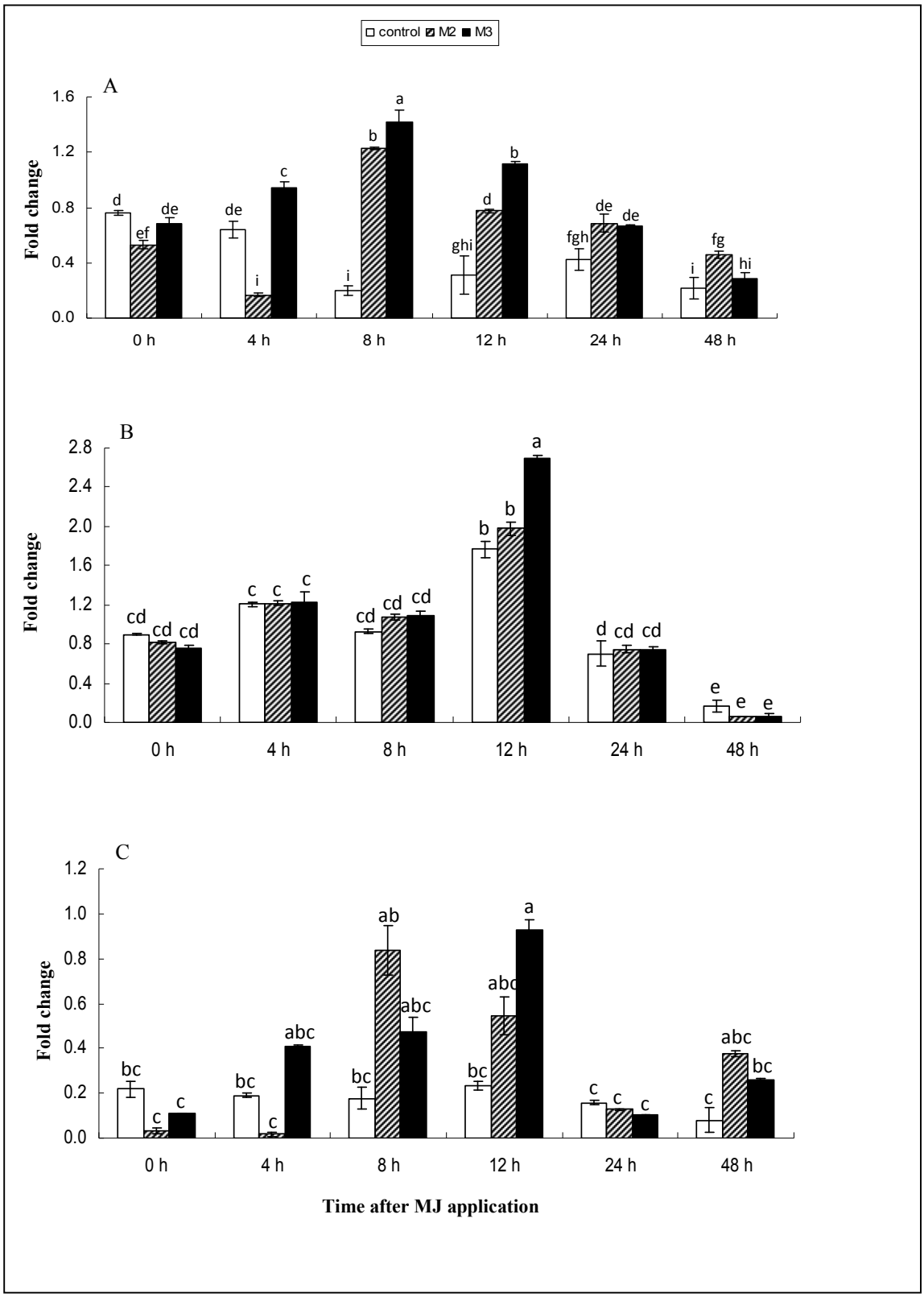

Fig. 2 Effect of MJ on A) *pr*, B) *mfs* and C) *ls* genes expression in peppermint (*Mentha* x *piperita* L.) Control = 0 mM, M2 = 0.1 mM, M3 = 0.5 mM

The effect of either MJ or any elicitor (biotic or abiotic) is dependent on a number of factors which may interact. These include the elicitor's specificity and concentration, the duration of treatment and the growth stage of the culture [43]. Our results showed that application of MJ elicit menthol and 1,8-cineol as a secondary metabolite, also previous studies showed that application of exogenous MJ treatment was investigated to increase the contents of secondary metabolites in various plants such as *Ocimum basilicum*, *Nicotiana attenuate* and *Rubus carolinianus* [44].

The variation in the percentage of some constituents within the essential oil showed trend as a result of the MJ exposure treatment. GC analysis of the essential oils indicated some variation in chemical constituents of peppermint oil. *pr* and *mfs* genes are linked in the monoterpene biosynthesis pathway because pulegone is converted to either menthofuran or menthone then to menthol [40,45]. In peppermint, along with the menthol increase the expression of the *pr* and *ls* genes were also significantly ($P < 0.001$) enhanced in response to MJ induction. These genes encode the enzymes that have key role in the committed and final step of menthol biosynthesis. *pr* and *mfs* genes production clearly resembled these genes expression profile, thus indicating that the stimulatory effect of MJ on pulegone and menthofuran production could be due to up-regulation of the these genes. On the basis of these results, these genes, being regulated at the transcriptional level by MJ, could be a suitable candidate for metabolic engineering strategies to improve menthol. The expression of *pr* and *ls* genes was increased in *Mentha x piperita* leaves during 4-12 h following MJ application which thereafter decreased. The *pr* mRNA level increased strongly within 4 h of MJ elicitor treatment, reaching a maximum at 8 h and then decreased rapidly from 24 to 48 h (2- to 5-fold lower than the maximum). The transcript levels of *ls* gene were approximately 2.1- and 4-fold higher at 4 and 12 h exposure to 0.5 mM MJ, respectively than those in the controls. Moreover, at 8 h MJ treatment, the transcript levels of *ls* was almost higher 4.7-fold at 0.1 mM MJ than that in the control (Figs. 1 and 2). In menthol pathway *pr* and *ls* genes respond to MJ more quickly than *mfs* gene, meaning that the expression of *mfs* gene was increased/started increasing after *pr* and *ls* genes. It was found that not only transcript levels of these genes changed during the induction time but also the rate of change varied from gene to gene. For example, after 0.5 MJ treatment at 4 h time induction, the expression of *pr* and *ls* genes increased 1.5- and 2.1-fold, respectively compared to the controls whereas the *mfs* gene expression did not change. Several reports have shown that exogenous application of MJ can induce the expression of plant genes for various biosynthetic pathways [46,47], confirming our results. Plants react to environmental stresses by modulating the expression of genes involved in both primary and secondary metabolism. Industrial plants producing essential oils, such as peppermint, respond to changes in environmental conditions through the alteration of quality and quantity of the essential oil components [48,49]. It is important to determine the molecular mechanisms of abiotic stress signal transduction and to identify methods to improve the tolerance of plants [50]. In the present work, such results indicate that *pr* and *ls* genes are important genes in the menthol biosynthesis pathway in response to MJ induction. This is consistent with research on terpenoid biosynthesis [51,52], demonstrating that MJ induction of gene expression is involved in biosynthesis of secondary metabolites. In our study, in the case of gene expression at 12 h of MJ induction, the expression of the *mfs* gene in *Mentha* x *piperita* was significantly ($P<0.001$) different from that of control and this level of transcripts was decreased from 24 to 48 h of treatment exposure. For *mfs* gene expression, our results confirm that there are no significant different between the control and MJ treatment during 4-8 h application of MJ treatment exposed to peppermint plants. Previous report showed that wounding and MJ both enhanced and suppressed the expression of several genes for terpenoid biosynthesis. The most highly up-regulated genes from this class were the terpene synthase genes, limonene cyclase homolog and S-linalool synthase. Also in this study, it was found that exogenous MJ application induced expression of some genes in menthol pathway [53]. Fig. 2 shows that all of these genes were up-regulated after MJ induction. However, the highest transcription levels for several genes were not observed at the optimal MJ concentrations necessary for maximum menthol production. Our study showed that *pr*, *mfs* and *ls* genes expression increased at 0.5 mM MJ while the highest level of menthol obtained at 0.1 mM MJ treatment. The lack of correlation between gene expression and essential oil production might depend on the different rates of terpenoid emissions from the secretory structures. In control healthy plants, the monoterpene volatilization from glandular trichomes occurs at a very low rate [54,55] and also the gene expression can be regulated at transcriptional, post- transcriptional, post-translational and metabolic levels. An opposite trend was found for *pr* with *mfs* gene expression, with a down-regulation in 12 h and up-regulation after treatment, respectively (Figs. 1 and 2). This result is consistent with findings reported by Mahmoud and Croteau [56]. They suggested that the expression of *mfs* (and the production of relatively high levels of menthofuran) results in the suppression of *pr* expression, or the accelerated turnover of the *pr* message [56]. In general, we

conclude that MJ can potentially induce the biosynthesis of menthol through the transcriptional induction of genes in menthol pathway and also our results suggested that MJ increases the pharmacological quality of *Mentha* x *piperita.*

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