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Original Article

Expression of 4 Genes in *Ocimum basilicum* and their Relationship with Phenylpropanoids Content

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Abstract

Recent data showed that phenylpropanoid compound, methylchavicol is essential component of Iranian cultivars of basil. Studying their occurrence during development of plant may help to elucidate the role of phenylpropanoids in plant cell physiology. We followed the phenylpropanoids concentration and the expression of genes related to their biosynthesis during growth and development of two cultivars of Iranian basil. Cinnamate 4- hydroxylase (C4H), 4-Coumarate CoA ligase (4CL), Eugenol O- methyltransferase (EOMT) and Chavicol O-methyl transferase (CVOMT) are known as key enzymes regulating phenylpropanoids production. The yield of essential oils and concentration of phenylpropanoid, methylchavicol, increased during growth of the plant to reach a peak before pre-flowering stage. Gene expression analyses showed that the expression of the genes encoding C4H, 4CL, EOMT and CVOMT are increased during the plant development in parallel to the methylchavicol reaching a maximum before pre-flowering. These correlations showed that the biosynthesis of phenylpropanoid may regulated at transcriptional level.

Key words*: Ocimum basilicum* L., Phenylpropanoid, 4CL (4Coumarate CoA ligase), C4H (Cinnamate 4-hydroxylase), EOMT (Eugenol O- methyltransferase), CVOMT (Chavicol O-methyltransferase).

Introduction

Plant essential oils, that have in general terpenoids and phenylpropanoids as major components, are important sources of aromatic and flavoring chemicals in food, industrial, and pharmaceutical products [10].

Phenylpropanoids are a group of small phenolic molecules, which are key elements in many important herbs and spices, including peppercorns, cloves, nutmeg, cinnamon, allspice, pimenta, basil (*Ocimum*) and tarragon [19].

Ocimum (2n=48), a member of the Lamiaceae family, comprises annual and perennial herbs native to the tropical and subtropical regions of Asia, Africa and Central South America [23]. The plant, traditionally used as medicinal herb in the treatment of headaches, diarrhea, coughs, warts, worms and kidney malfunctions [4, 21, 32, 43].

Basil is widely cultivated for the production of essential oils and containing high proportions of phenylpropanoids derivatives, such as eugenol, methyleugenol, chavicol, methylchavicol, myristicin, methylcinnamat and elemicin, often combined with various proportions of linalool, a monoterpens, and sesquiterpenes [3]. These compounds synthesized and stored in peltate glands found on the surface of leaves, stems, and flowers [19]. Biosynthesis of phenylpropanoid compounds is passed from shikimate pathway and it regulated by several groups of enzymatic reactions through metabolic channels in which these enzymes are loose or joint on cell membranes [12]. All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the deamination action of phenyl alanine amonia-lyase (PAL) which is one of the most important enzymes and has a key role in regulation of phenylpropanoid production in plants [1, 19]. Cinnamate 4-hydroxylase (C4H) catalyzes the hydroxylation of *trans*-cinnamic acid to 4-hydroxycinnamate (*p*-coumaric acid) and is the second key enzyme of phenylpropanoid biosynthetic pathway [11]. Core enzymes of phenylpropanoid metabolism are believed to form an enzyme complex, and C4H plays a pivotal role at the interface between

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cytosolic phenylpropanoid pathway and membrane-localized electron-transfer reactions [22].

Down-regulation of C4H in transgenic plants and C4H mutation induce pleiotropic effects on phenolic patterns including reduction in chlorogenic acid, flavonoids and lignins [33]. Some plant genera are assumed to contain only one gene for C4H, such as *Pisum* [15], *Arabidopsis* and *Petroselinum* [22], while in maize [5] and alfalfa, they encoded through a small gene family. Also in *Brassica napus* two isoforms was reported [11].

4-Coumarate: CoA ligase (4CL) family catalyzed the activation of 4-coumarate and a few related substrates to the respective CoA esters and thus channels the common, phenylalanine-derived building block into the other wise widely distinct branches of general phenylpropanoid metabolism [18]. 4CL is encoded by multigene families in all plant species investigated [7]. It has been reported that the mRNA activities encoding the PAL, C4H and 4CL are regulated in a coordinated manner [31, 40]. Eugenol O-methyltransferase (EOMT) and chavicol O-methyltransferase (CVOMT), catalyze the final step to convert eugenol and chavicol, respectively, to methyleugenol and methylchavicol using *S*-adenosylmethionine (SAM) as the methyl donor [16, 26, 43]. Methyeugenol is an important insect pollinator attractant in many flowers [38].

Little is known about regulation of phenylpropanoids, such as chavicol and its derivatives. Gang *et al.* (2002) observed higher CVOMT transcript accumulation in young basil whole leaves compared to older leaves. It is known that phenylpropanoid content and composition changes with age concentration of growth factors, herbivory, tissue wounding, pathogenic attack, UV irradiation and low temperature [7, 28, 34, 35, 42]. On the other hand, there are chemotypes of basil rich in specific aromatic terpenes and phenylpropanoids [42].

Because of the economic importance of essential oil production, basil is considered as a target for bioengineering [39]. In this research to gain new insight about the biosynthesis of the essential oil in basil, the developmental regulation of genes expression of C4H, 4CL, EOMT and CVOMT and phenylpropanoid composition were evaluated in leaves of two cultivars (green basil and purple basil) at different stages of growth and development.

Materials and Methods

Plant materials

Two *Ocimum basilicum* cultivars (*Ocimum basilicum* L. cv green (Sweet basil) and *Ocimum basilicum* L. cv purple) seeds were obtained from Tehran University Experimental Research Station, and grown in plastic pots containing a mixture of soil: sand (1:1), under natural light condition in a greenhouse. The plants were watered every other day and maintained at day/night temperature of 27-30 and 18-20 ºC, respectively [44]. The leaves were harvested at different developmental stages [10 leaves plants (10L), 50 leaves plants (50L), pre-flowering (PF) and flowering (F)]. The samples from different stages were used for essential oil analysis. A part of the samples were immediately frozen in liquid nitrogen and preserved at -80 °C for molecular analysis.

Essential oil extraction and Identification of essential oil components and methyl chavicol measurment

The dried plant material (250 g) was used for essential oil extraction by hydrodistillation in a Clevenger type apparatus. Essential oil components were analyzed using a thermoquest-finnigan instrument, Gas Chromatograph (GC), equipped with a DB-5 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 µm). Oven temperature was raised from 60 ºC to 250 ºC at a rate of 5 ºC/min. Injector and detector (FID) temperatures were kept at 250 ºC and 280 ºC, respectively. Nitrogen was used as the carrier gas at the constant flow of 1.1 ml/min. The split ratio was 1/50. Quantitative data were obtained electronically from FID area percent data. Gas chromatography/mass spectrometry (GC/MS) analysis was carried out on a thermoquest- finnigan trace GC/MS instrument equipped with the same column and temperature programming as mentioned for GC. Transfer line temperature was 250 ºC. Helium was used as the carrier gas at a flow rate of 1.1 ml/min with a split ratio equal to 1/50. Identification of individual compounds was made and comparing of their mass spectra with those of the internal reference in the mass spectra library (Wiley 7.0) or with those of authentic compounds and confirmed by comparison of their retention indices with those of authentic compounds or with those reported in the literature (Adams 2001). Semi-quantitative data was obtained from FID area percentages without the use of correction factors. Essential oil yield in all the samples was quantified as percentage of leave dry weight (V/W), and methyl chavicol content in all the samples was quantified as µl/g DW.

RNA isolation

Total RNA was extracted from whole leaves of *Ocimum basilicum* by the RNX™-Plus Kit (CinnaGen, Iran) following the instructions of the manufacturer. Total RNA (10 μg) was treated with 20 units of RNase-free deoxyribonuclease I (DNase I) (Fermentas) at 37 °C for 15 min in 100 µl of mixture containing 40 mM Tris-HCl (pH 7.5) and 6 mM $MgCl_2$. The treated RNA was purified by extraction with Phenol/$CHCl_3$ (1:1, v/v) followed by ethanol precipitation. The quality and concentration of RNA samples were examined by agarose gel electrophoresis and spectrophotometer analysis.

cDNA synthesis and RT-PCR

The total RNA (2 µg) was used for synthesis of the first strand cDNA using a first-strand cDNA synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions.

PCR was performed in 20 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.3 μl of the first-strand cDNA, 0.4 U of recombinant Taq DNA polymerase (Fermentas), 200 μM of each deoxynucleoside triphosphate and 4 μmol each oligonucleotid.

All primers were designed by aligning many sequences from another plants and using Clustal-W software. Finally, we designed gene specific primers with using conserved segments and eight primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgibin/primer3/primer3/www.cgi) (Whitehead Institute, Cambridge, Massachusetts).

The primer sequences: forward C4H 5'TCCGCGGCAGGAAGTTCAAGCTG3' and reverse 5'AATCGGGATGAAATCGCCATAG3', for 4CL: forward 5'TCGCAA AACAGCCACTACCGAC 3' and reverse 5' AGGTGCAGCAAGTTTGGCTCTC 3', for EOMT: forward 5'GAAAACAACTCTAATAATCAAG3' and reverse 5'AACATGTCT CCTCCAATATAG 3', for CVOMT: forward 3' CCA ATT TCT TCA TAG AAG AAA ACT C reverse 5' GATAAGCCTCTATGAGAGACCTC, for tubulin: forward 5' GGGGCG TAGGAGGAAAGCA 3', and reverse 5' GCTTTCAACAACTTCTTCAG 3'.

For amplification of transcripts for each gene the initial denaturation was performed at 94 °C for 90 sec and the amplification was performed by 30 cycles for tubulin, as internal control and other genes. Denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 60 sec, followed by a final extension at 72 °C for 5 min. PCR products were subjected to agarose gel electrophoresis, and the band intensity on the gel stained with ethidium bromide was measured using UV Transilluminator (Bio Doc-It System). The RT-PCR results shown are from one experiment that was repeated at least three times with similar results. The identity of amplified fragment was confirmed by PCR product sequencing.

Statistical analysis

All analysis was conducted at least three times, each with three independent repetitions. The analysis of variance and the dunkan test *($P \leq 0.05$)* of mean comparison were performed using the MSTATC program ver. 1.4.

Results

Essential oils content and composition in two cultivars

The chemical composition of the essential oils of *Ocimum basilicum* L. was investigated by GC/MS. The content of essential oil in leaves of two cultivars was dependent on the leave development and maturity. In *Ocimum basilicum* L. cv green as shown in figure 1 the amount of essential oil in leaves was 0.35 ml/g dw at 10- leaves stage and it reach a maximum 0.75 ml/g dw at preflowering stage. The main constituent found in the oil of all stages of growth in two cultivars was methyl chavicol, It was 0.123 ml/gdw at 10 leaves stage and reach a maximum 0.208 ml/gdw at preflowering stage in *O. basilicum* L. cv green. The greatest essential oil content was also found at preflowering stage where, the main terpenoids detected were Z-citral and E-citral (Table 1).

In *O. basilicum* L. cv purple as shown in Fig. 2 chemical composition was differente at growth stages and the amount of essential oils reach a maximum level 0.9 ml/g DW in flowering stage. The results showed that an important component of purple basil at 4 stages was methyl chavicol; it was 0.183 ml/g dw at 10 leaves stage and reached to 0.488 ml/g dw at flowering stage as the maximum amount (Fig 4).

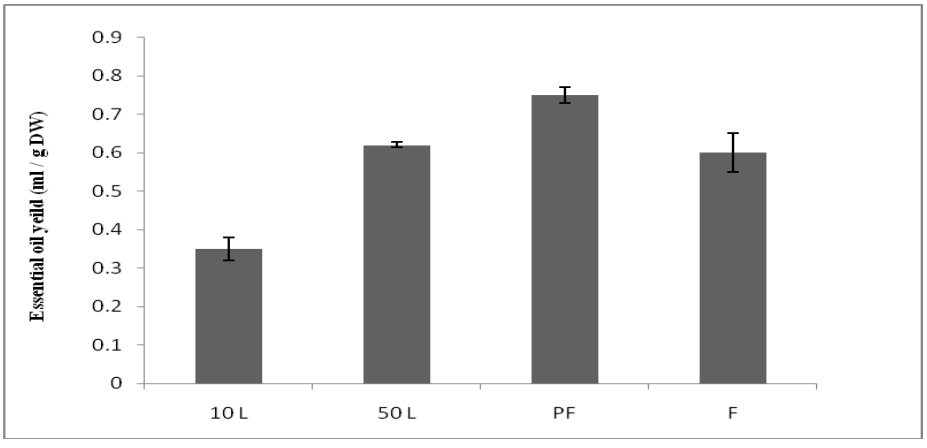

Fig 1. Total essential oils yield (ml/g DW) in various stages of growth in *Ocimum basilicum* cv. green (sweet basil). 10L: 10 – Leaves, 50L: 50 – Leaves, PF: Pre-flowering stage, F: Flowering stage.

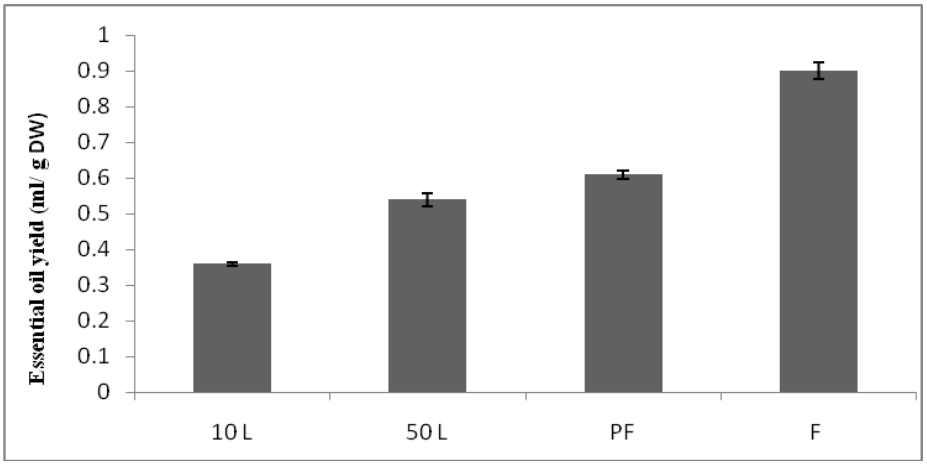

Fig 2. Total essential oils yield (ml/ g DW) in various stages of growth in *Ocimum basilicum* cv. purple (purple basil). 10L: 10 – Leaves, 50L: 50 – Leaves, PF: Preflowering stage, F: Flowering stage.

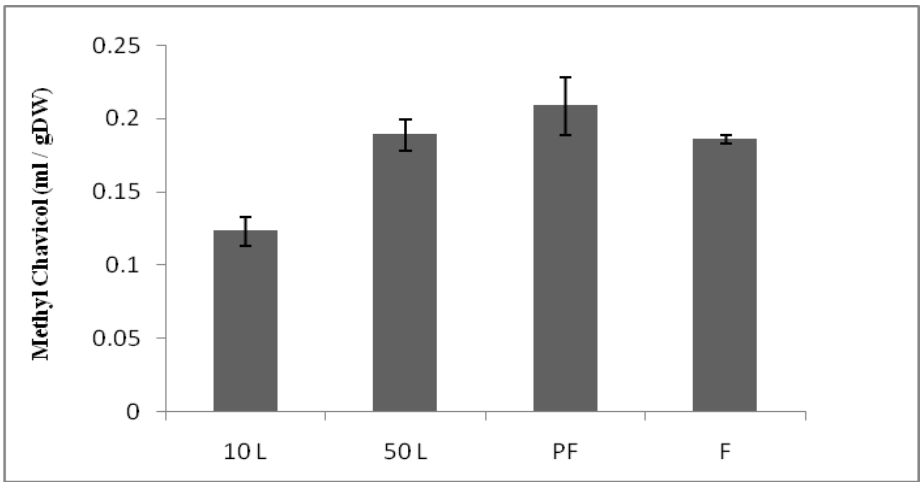

Fig. 3. Meythyl chavicol content (ml /g DW) in leaves of *Ocimum basilicum* cv. green (sweet basil) at different stages of growth according to using essential oil efficiency and GC-MS results.

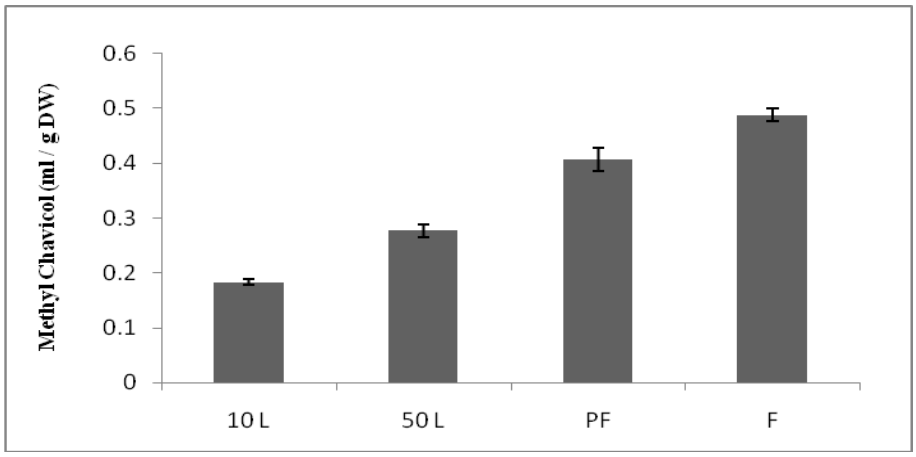

Fig 4. Meythyl chavicol content (ml /g DW) in leaves of *Ocimum basilicum* cv. purple (purple basil) at different stages of growth according to using essential oil efficiency and GC-MS results.

Table 1 Essential oil components (ml/g DW) found in *Ocimum basilicum* cv. green (sweet basil) at different stages of growth RT

Component	RT	10L	50L	PF	F
Methyl chavicol	17.14	0.123	0.188	0.208	0.186
Z-Citral	18.21	0.072	0.112	0.155	0.122
E-Citral	19	0.069	0.115	0.165	0.140
cis-Caryophyllene	23.76	0.014	0.027	0.036	0.033
α-Humullene	24.59	0.010	0.022	0.020	0.012
Cis-α- Bisabolene	26.43	0.008	0.026	0.030	0.019
1-Octen-3-ol	10.86	0.005	0.006	0.005	0.015
Fenchone	13.98	0.004	0.006	0.003	0.001
trans-2-caren-4-ol	16.41	0.006	0.008	0.012	0.015
Methyl eugenol	22.23	0.004	0.011	0.010	0.001
Germacrene D	25.2	0.005	0.014	0.006	0.014
cis-Verbenol	15.92	0.002	0.004	0.009	0.009
Z,E-α-Farnesene	24	0.002	0.009	0.008	0.009
Limonene	12.58	0.002	0.003	0.002	0.001

(retention time), 10L: 10 – Leaves, 50L: 50 – Leaves, PF: Preflowering stage, F: Flowering stage.

Table 2 Essential oil components (ml/g DW) found in *Ocimum basilicum* cv. purple (purple basil) at different stages of growth

Component	RT	10 L	50L	PF	F
Methyl chavicol	17.14	0.183	0.277	0.407	0.488
Z-Citral	18.21	-	0.143	-	0.002
Linalool	10.85	0.022	-	0.082	0.167
cis- Caryophyllene	23.76	0.010	0.009	0.008	0.009
Z,E-α-Farnesene	24	0.016	0.002	0.023	0.045
1,8 Cineol	10.24	0.017	-	0.009	0.048
Methyl eugenol	22.23	0.007	0.005	0.006	0.001
Carvacrol	12.74	0.015	-	-	-
β-Ocimene	10.37	0.010	-	0.007	0.034
Germacrene D	25.2	0.009	0.003	0.007	0.016
Fenchone	13.93	0.006	-	-	

RT (retention time), 10L: 10 – Leaves, 50L: 50 – Leaves, PF: Pre-flowering stage, F: Flowering stage.

Expression of C4H, 4CL, EOMT and CVOMT genes in two cultivars at the different stages of growth

Semi quantitative RT-PCR was used to determine the expression of C4H, 4CL, EOMT and CVOMT genes at different stages of growth. Tubulin was used as internal control. The results showed that the highest transcription level of expression C4H and 4Cl genes in 2 cultivars was found at pre-flowering stage (PF) (Figs. 5-8). The levels of C4H transcript in leaves of purple basil were much higher than in sweet cultivar, while higher 4CL transcript levels were observed in sweet plants compared with purple plants.

Our results showed that EOMT transcript levels were significantly induced during the growth and development to receive a maximum at pre-flowering stage. After this induction, the transcript level fall at flowering stage (Figs. 9 and 10). Maximum CVOMT gene expression in sweet basil was at flowering stage but in purple basil maximum CVOMT gene expression occurred at preflowering stage (Figs. 11 and 12).

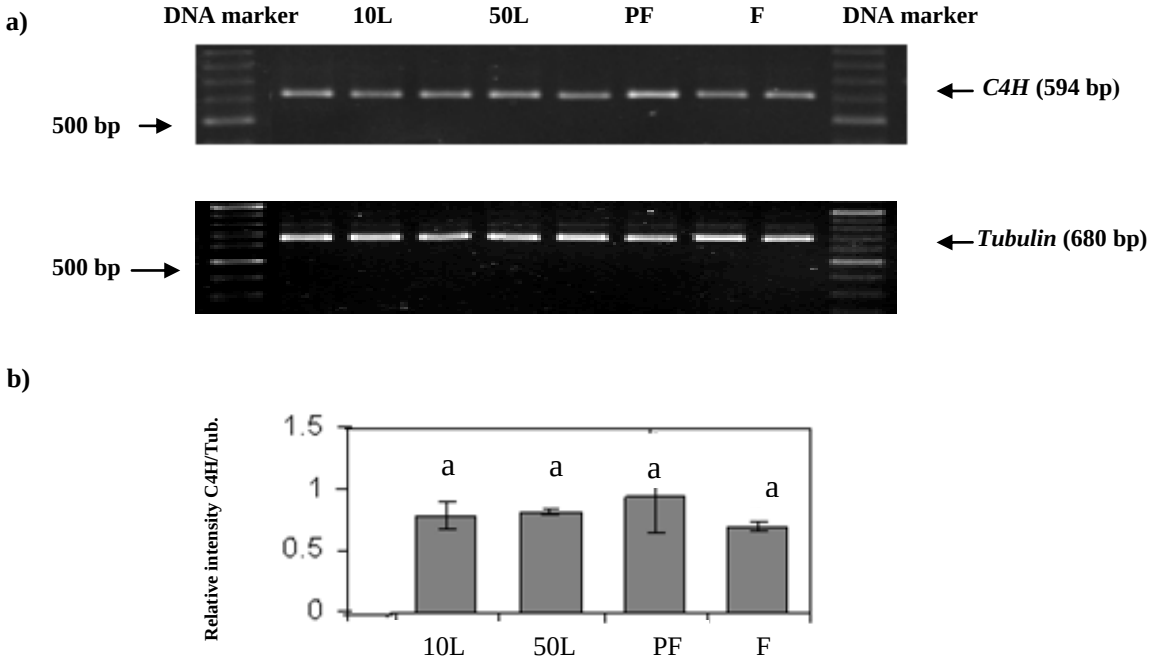

Fig 5. By Semiquantitative RT-PCR technique the accumulation of specific *C4H* transcripts in leaves of sweet basil at different stages of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *C4H*. b) The relative amounts of target (t; 594 bp) and control (c; 680 bp) amplification products were calculated. The amount of expression level was quantified using Image guage 4 software.

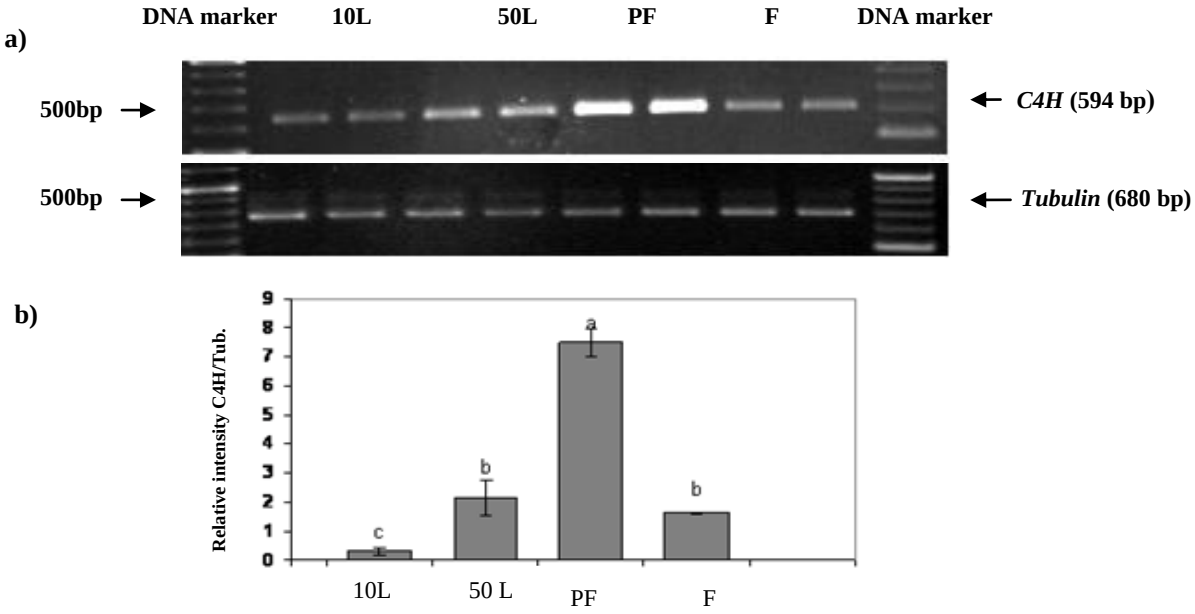

Fig 6. By Semiquantitative RT-PCR technique the accumulation of specific *C4H* transcripts in leaves of purple basil at different stages of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *C4H*. b) The relative amounts of target (t; 594 bp) and control (c; 680 bp) amplification products were calculated. The lowest and highest level of the *C4H* gene expression was seen in 10 leaves (10L) and preflowering (PF) stages. The amount of expression level was quantified using Image guage 4 software.

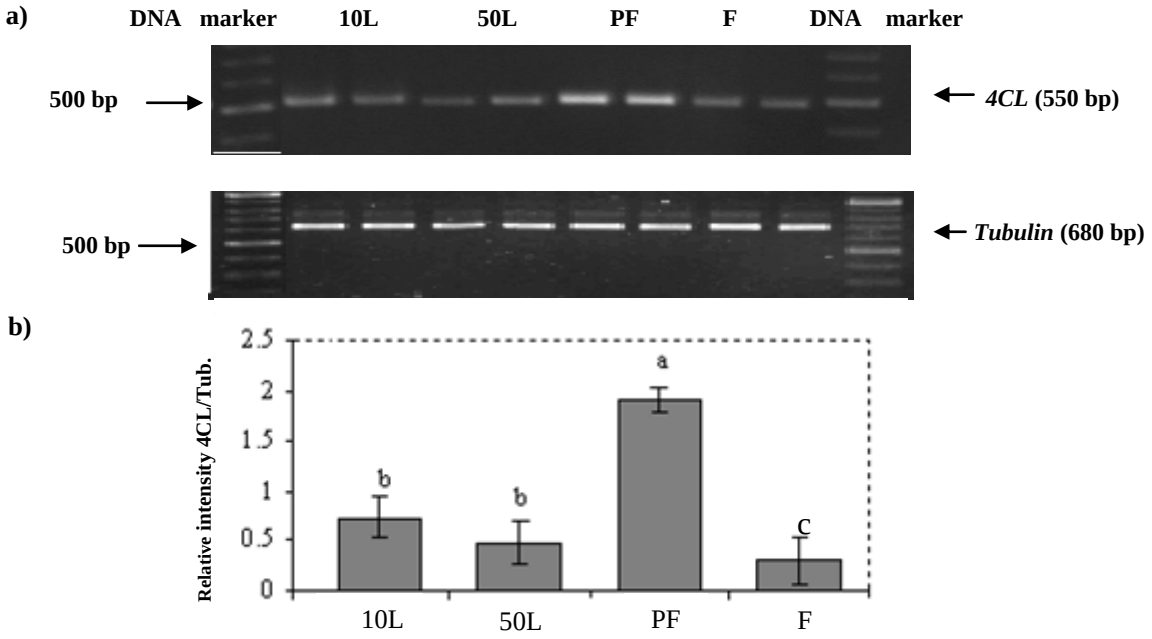

Fig 7. By Semiquantitative RT-PCR technique the accumulation of specific *4CL* transcripts in leaves of sweet basil at different stages of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *4CL*. b) The relative amounts of target (t; 550 bp) and control (c; 680 bp) amplification products were calculated. The lowest and highest levels of the *4CL* gene expression were seen in flowering (F) and pre-flowering (PF) stages of growth. The amount of expression level was quantified using Image guage 4 software.

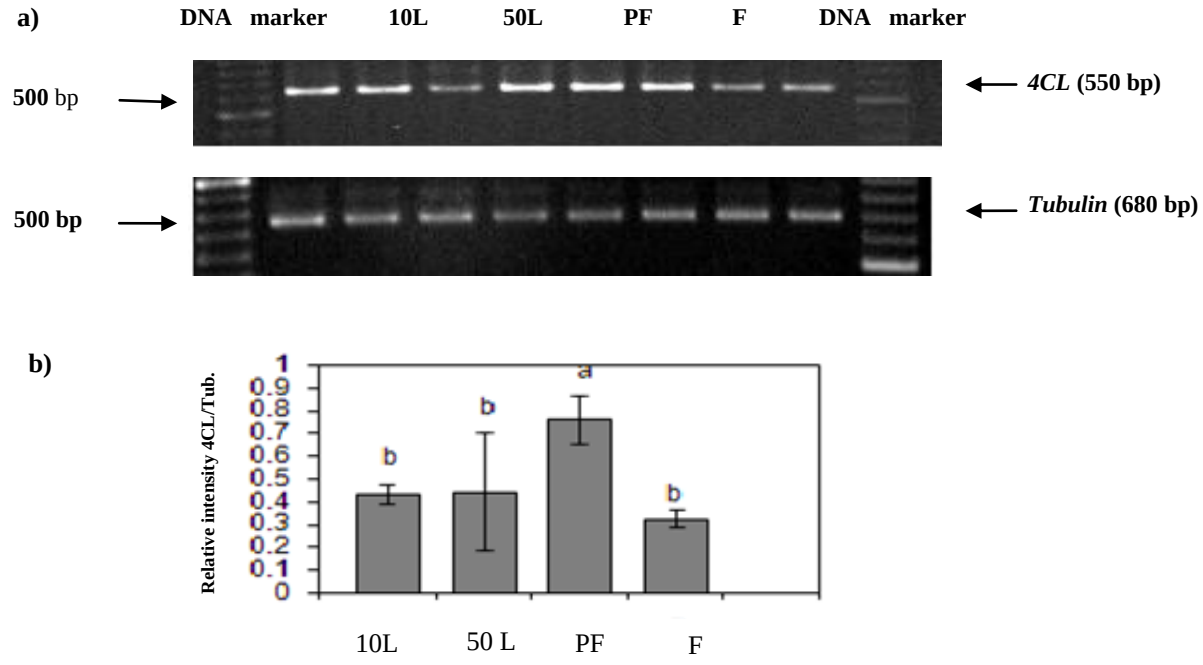

Fig 8. By Semiquantitative RT-PCR technique the accumulation of specific *4CL* transcripts in leaves of purple basil at different stage of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *4CL*. b) The relative amounts of target (t; 550 bp) and control (c; 680 bp) amplification products were calculated. The highest level of the *4CL* gene expression was seen at preflowering (PF) stage of growth. The amount of expression level was quantified using Image guage 4 software.

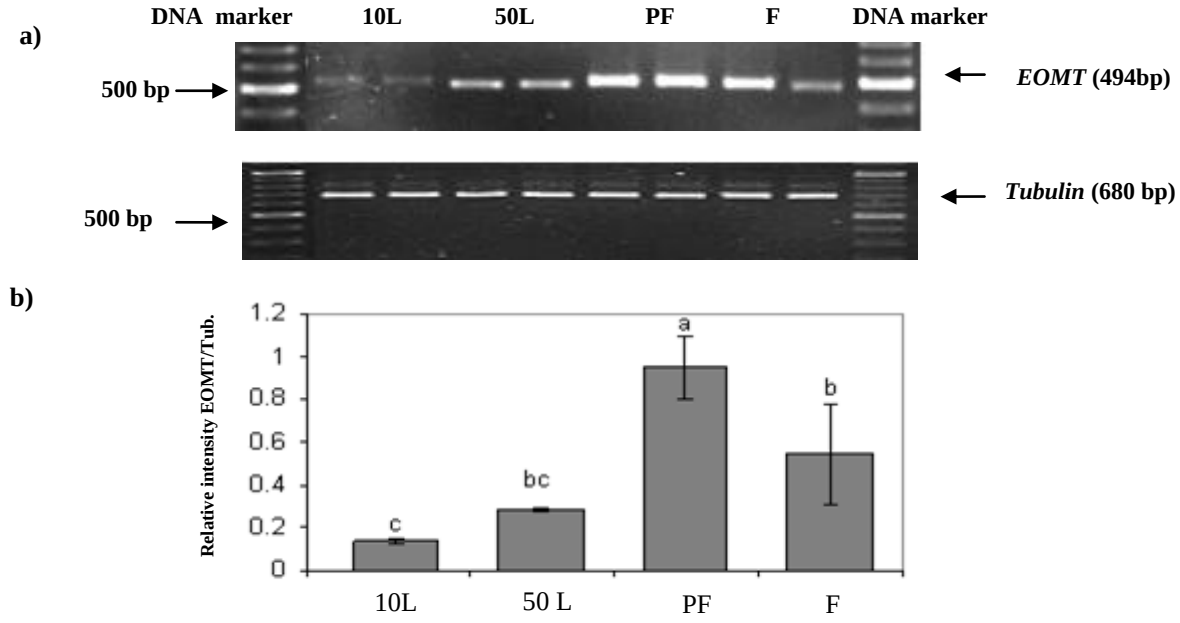

Fig 9. By Semiquantitative RT-PCR technique the accumulation of specific *EOMT* transcripts in leaves of sweet basil at different stage of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *EOMT*. b) The relative amounts of target (t; 494 bp) and control (c; 680 bp) amplification products were calculated. The highest level of the *EOMT* gene expression was seen at pre-flowering (PF) stage of growth. The amount of expression level was quantified using Image guage 4 software.

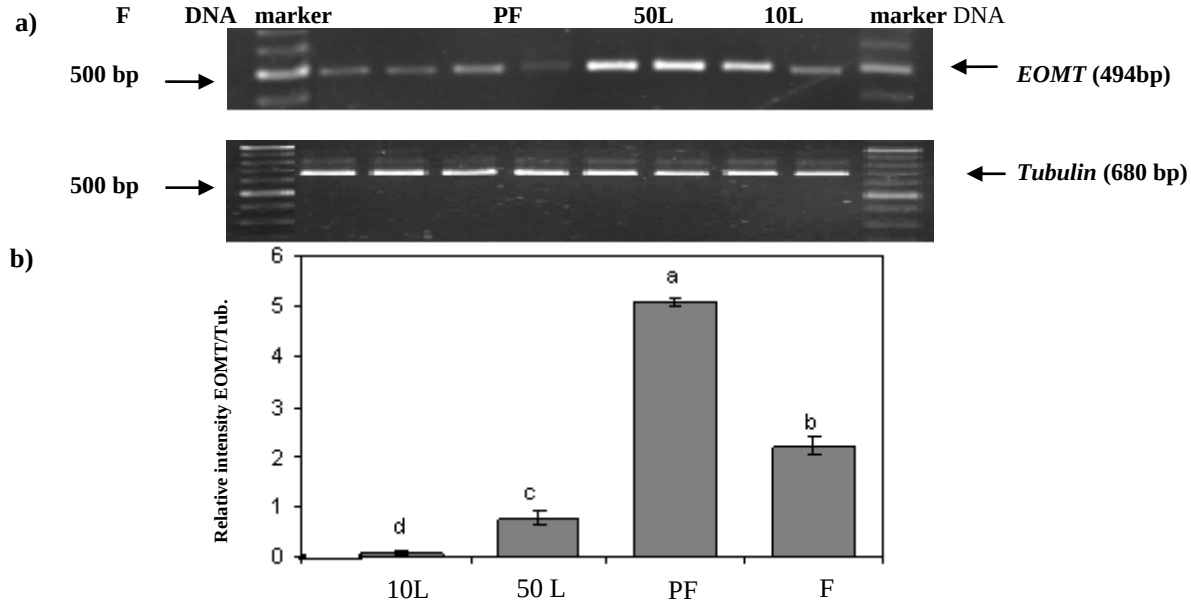

Fig 10. By Semiquantitative RT-PCR technique the accumulation of specific *EOMT* transcripts in leaves of purple basil at different stages of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *EOMT*. b) The relative amounts of target (t; 494 bp) and control (c; 680 bp) amplification products were calculated. The highest level of the *EOMT* gene expression was seen at pre-flowering (PF) stage of growth. The amount of expression level was quantified using Image guage 4 software.

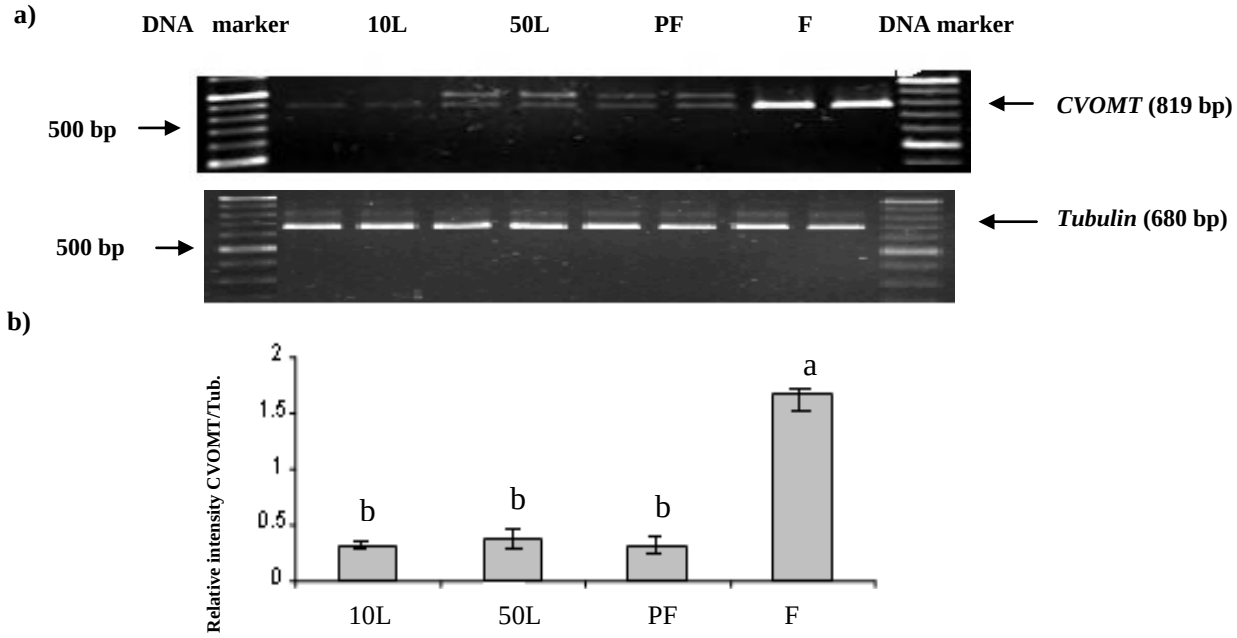

Fig 11. By Semiquantitative RT-PCR technique the accumulation of specific *CVOMT* transcripts in leaves of sweet basil at different stages of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *CVOMT*. b) The relative amounts of target (t; 819bp) and control (c; 680 bp) amplification products were calculated. The highest level of the *CVOMT* gene expression was seen at flowering (F) stage of growth. The amount of expression level was quantified using Image guage 4 software.

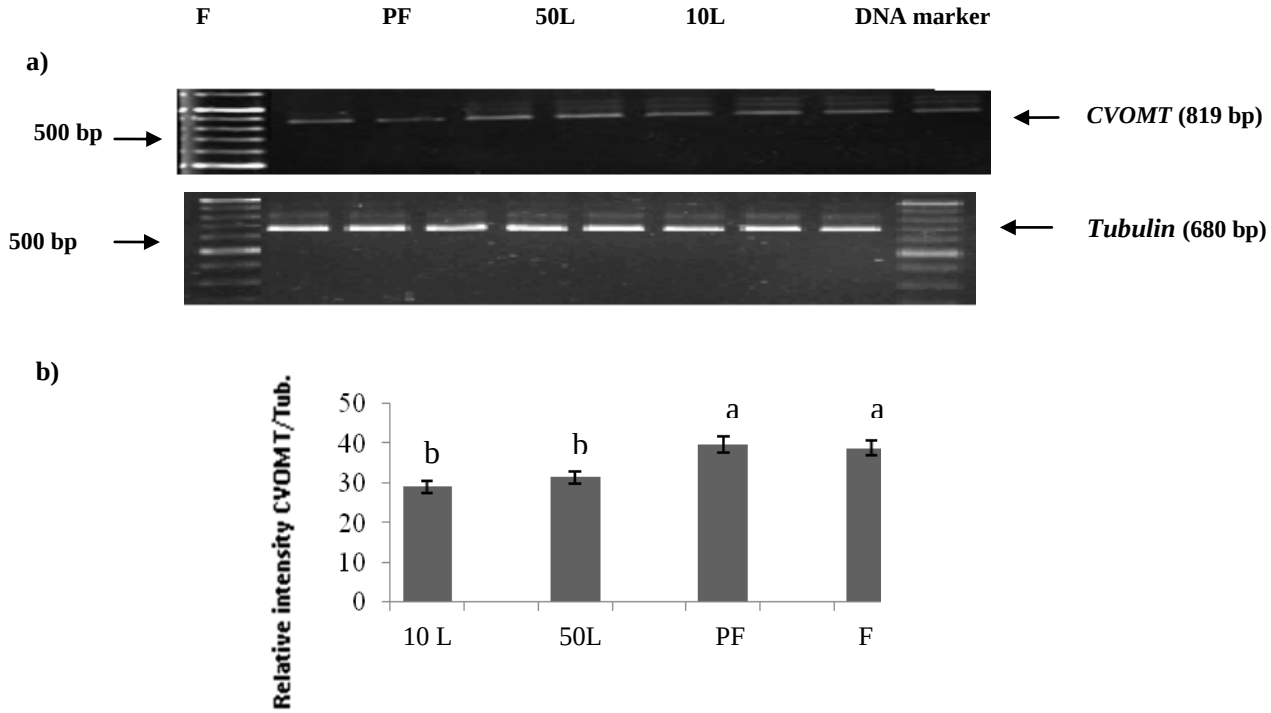

Fig 12. By Semiquantitative RT-PCR technique the accumulation of specific *CVOMT* transcripts in leaves of purple basil at different stages of growth was examined. a) RT-PCR was performed using equal amounts of cDNA from tubulin as internal control and *CVOMT*. b) The relative amounts of target (t; 819bp) and control (c; 680 bp) amplification products were calculated. The highest level of the *CVOMT* gene expression was seen at flowering (F) and preflowering (PF) stages of growth. The amount of expression level was quantified using Image guage 4 software.

Discussion

Basil (*Ocimum basilicum* L.) is an important medicinal plant and culinary herb and is also marketed as an herb, fresh, dried, or frozen [26]. Basil similar to many members of the Lamiaceae family such as *Mentha*, *Salvia*, *Origanum*, and *Thyme* spp. is also cultivated to be used as herbs and as a source of essential oils [37]. The amount of total phenolic compounds in all tested basil accessions was higher than the other Lamiaceae plants [43]. The essential oil yield (0.58%) in our research was comparable to reports by Politeo et al. 2007, who found the yield of essential oils of *O. basilicum* to be 0.62%. In *O. basilicum* landraces from Turkey, the essential oil yields were from 0.4 to 1.5% [8, 41]. while it is reported that the yield of essential oil from *O. basilicum* grown in Serbia and Montenegro to be 0.37%. These variations might be attributed to the varied agroclimatic conditions of the regions. On the other hand, total essential oils content in our study indicates that maturity and leaf development influence strongly on the production of essential oil compounds and maximum quantity of essential oils is in the last stage of vegetative growth, pre-flowering, in this plant. Our result was in agreement with those of other researchers who reported that essential oil yield generally positively correlated with biomass [9, 29].

The major aroma constituents of the essential oil found at 4 stages of growth were methyl chavicol, Z-citral and E-citral. However, methyl chavicol, linalool, citral, eugenol, and methyl cinnamate are reported as major components of the oils of different chemotypes of *O. basilicum* [36]. In agreement with our result, a chemotype of *O. basilicum* with high methyl chavicol and citral contents have been reported from Turkey which is known as methyl chavicol/citral chemotype [41]. European originated basil was reported to have the highest quality aroma, containing linalool as the major constituents [39]. Our results indicated that phenylpropanoid compounds especially methyl chavicol compose an important part of essential oil of Iranian *O. basilicum* at all stages of growth. Instead of the enhanced total essential oil yield, phenylpropanoid ratio was slightly reduced during the basil growth and development.

To further investigation on the regulation of biosynthesis phenylpropanoids in basil and to determine what are relationships between C4H, 4CL and EOMT enzymes and phenylpropanoids levels, we used RT-PCR analysis with specific primers for C4H, 4CL, CVOMT and EOMT genes.

C4H catalyzes the conversion of cinnamate into 4-hydroxy-cinnamate, a key reaction of the phenylpropanoid pathway which leads to the biosynthesis of several secondary metabolites [27]. It has been reported C4H activity and gene expression levels is induced by a number of stresses, including wounding, chemical effectors and pathogen [35]. Our results suggest that C4H transcript levels are developmentally regulated in basil and it may has significant role in production of essential oil constituents.

In other hand, C4H catalyzes the first oxygenation step in phenylpropanoid biosynthesis, and the phenylpropanoid branch pathways lead to a wide array of secondary products [35]. This changes in gene expression was probably due to the defense related function of these products, the activation of C4H under plant growth condition have been considered as a part of defense.

In the biosynthesis of phenylpropanoids, 4CL has a pivotal role at the divergence point from general phenylpropanoid metabolism to several major branch pathways [14]. 4CL has been the subject of extensive study for many years, mainly in higher plants. It has been shown to occur in the form of multiple isoenzymes with either similar or distinct substrate affinities [14]. The results shown here demonstrate that 4CL gene expression were increased during pre-flowering stage indicating that the enhanced activity and expression of 4CL is correlated with formation of phenylpropanoids in response to plant growth and environmental condition. In previous studies northern blots analyses indicated that 4CL mRNA transcripts are highest in old stems and higher in the non-pigmented corolla tubes than in the pigmented limbs of tobacco flowers [25]. Our result showed that the age factor induced the 4CL transcript levels. We also found low gene expression at flowering stage in *O. basilicum.* This decrease was probably due to leaf drop and dehydration, a happenstance of senescence [28].

Previous studies have reported a significant correlation between methylchavicol accumulation and EOMT and CVOMT enzyme activities [17, 26]. They report that CVOMT and EOMT activity levels, CVOMT and EOMT transcript expression levels, which decrease with leaf age. Developmental regulation has also been reported for other plant methyltransferases [13, 20]. In this research EOMT gene expression in two cultivars was higher in pre-flowering stage than other stages and however EOMT transcript expression levels increase overtime as the leaves matured. This increase was probably related to the nature (an important insect pollinator attractant) role and defense function of methyleugenol but more work is necessary to elucidate the mechanism of regulation phenylpropanoid production mediated by developmental factors, which is of interest to be studied in the future.

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