Gene expression and activity of phenyl alanine amonia-lyase and essential oil composition of *Ocimum basilicum* L. at different growth stages

Mahboobeh Ziaei[1], Mozafar Sharifi[1*], Mehrdad Behmanesh[2], Khadijeh Razavi[3]

[1]Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, P.O.Box 14115-154, Tehran, I.R. Iran [2]Department of Genetics, Faculty of Biological Science, Tarbiat Modares University, P.O. Box 14115-154, Tehran, I.R. Iran [3]National Institute of Genetic Engineering and Biotechnology, P.O. Box 14965/161, Tehran, I.R. Iran

Abstract

Phenylalanine amonia-lyase (PAL) is one of the most important enzymes that plays a key role in regulation of phenylpropanoid production in plants. It catalyzes the first step of the phenylpropanoid pathway in which L-phenylalanine is deaminated to trans-cinnamic acid. This step is significant for metabolic engineering and hyper-expression of the major phenylpropanoid, methyl chavicol. We followed gene expression and activity of PAL in *Ocimum basilicum* L. at different stages of growth including seedling, beginning and middle of growth phase, budding stage and flowering, and their correlation with final concentration of phenyl-propanoid compounds. The level of gene expression was monitored by semi quantitative RT-PCR and phenylpropanoid compounds were identified by gas chromatography/mass spectrometry (GC/MS). PAL activity was assayed using spectrophotometer. The results indicated that the level of gene expression and activity of PAL enzyme are altered during the plant development, where the highest expression and activity (0.851 μmol cinnamic acid/mg/min) was achieved at budding stage. In this experiment, changes of methylchvicol content were correlated to the transcription and activity of PAL enzyme.

Keywords: Essential oil; Methyl chavicol; *Ocimum basilicum* L.; phenylalanine amonia-lyase; phenyl-propanoid

*Correspondence to: **Mozafar Sharifi, Ph.D.**
Tel: +98 21 82884445; Fax: +98 21 82884717
E-mail: msharifi@modares.ac.ir

INTRODUCTION

The Lamiaceae family is a large plant family including the following species: thyme, lavender, sage, basil, coleus, hyssop, skullcap, pennyroyal, lemon balm and it is well recognized for the diversity of secondary compounds (Iijima *et al*., 2004). The genus *Ocimum* includes 50-150 species and subspecies (Labra *et al*., 2004; Javanmardi *et al*., 2002) that comprise annual and perennial herbs and shrubs, native to the tropical and sub-tropical regions of the world (Wang and Pichersky, 1998). *Ocimum basilicum* collectively called sweet basil (2n=48), is an important medicinal plant and culinary herb which is used in treatment of headaches, diarrhea, coughs, warts, worms and kidney malfunctions (Rai *et al*., 2004; Bais *et al*., 2002; Wang and Pichersky, 1998; Samuelsson, 1992). Sweet basil is widely cultivated for the production of essential oils, and be used as a fresh, dry herb or frozen (Samuelsson, 1992). Extracts of the plant especially essential oil have been shown to contain biologically active constituents that are insecticidal, nematicidal, fungistatic, antimicrobial and toxic activities (Wang *et al*., 1997) and contain high proportions of phenylpropanoids derivatives, such as eugenol, methyl eugenol, chavicol, methyl chavicol, myristicin, methyl cinnamat and elemicin, often combined with various proportions of linalool, a monoterpenes, and sesquiterpenes (Attieh *et al*., 1995). Despite the wide uses and the importance of sweet basil and its essential oils, little is known about

the biosynthesis and developmental regulation of the compounds responsible for the flavor quality of the fresh and dried herbs (Wang and Pichersky, 1998). These compounds synthesized and stored in peltate glands found on the surface of leaves, stems, and flowers (Achnine *et al*., 2004; Iijima *et al*., 2004). Biosynthesis of phenylpropanoid compounds that is important part of basil essential oil is passed from shikimate pathway and regulated by several groups of enzymatic reactions through metabolic channels in which these enzymes are loose or joint on cell membranes (Dixon *et al*., 1992). All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the deamination action of phenylalanine amonia-lyase (PAL) which is one of the most important enzymes and has a key role in regulation of phenylpropanoid production in plants (Achnine *et al*., 2004; Iijima *et al*., 2004). This enzyme is the branch point between primary and secondary (phenylpropanoid) metabolism (Adams, 2001) for the first time described by Koukol and Conn (Koukol and Conn, 1961) and has been extensively studied in plants. There are multiple isoforms of PAL in plants which is a homotetrameric enzyme whose subunits are encoded by a small multigene family (Cramer *et al*., 1989; Nagai *et al*., 1994; Wanner *et al*., 1995; Fukasawa-Akada *et al*., 1996). Metabolites resulting from PAL activity can be generally classified as phenolic derivatives and include coumarins, essential oils, flavanoids, lignin and tannins (Creasy, 1987). These compounds aid in disease, pest resistance and seed dispersal as well as pollination efforts. Many factors have some effects on synthesis of PAL and the resulting plants phenolics. These factors include age, concentration of growth factors (Bidlack and Buxton, 1995), herbivory, tissue wounding, pathogenic attack, UV irradiation and low temperature (Olson and Bidlack, 1997). The highest PAL activity is seen in young developing plants and often levels off or decreases with maturity (Olson and Bidlack**,** 1997). So based on present study, it is possible that these factors can affect the rate of gene expression and activity of PAL, but there is shortage of available data in relation with the PAL activity measurement and the level of gene expression under exposure of the factors alteration. To investigate the relationship between the changes of PAL activity, as a key enzyme in phenolic production, and phenylpropanoid production, chemical composition of essential oil in basil leaves at different stages of growth was also analyzed. Also in this study the rate of gene expression and activity of PAL enzyme was investigated in *Ocimum basilicum* at different stages of growth.

MATERIALS AND METHODS

Plant materials: Sweet basil seeds (*Ocimum basilicum* L. cv green), provided from the "Tehran University Experimental Research Station", and was grown in plastic pots containing a mixture of soil: sand (1:1), under natural light condition at greenhouse. The plants were irrigated every day and maintained at day/night temperature of 27-30ºC and 18-20ºC, respectively. The experiments took place from 1st April, to 31st July 2007 and the leaves were harvested by hand at five growth stages including seedling (S), 10-leaves plants (10 l), 50-leaves plants (50 l), budding stage (B) and flowering (F). A part of the samples were immediately frozen in liquid nitrogen and stored at -80°C for molecular analysis and the other part were dried at the shade and then were powdered for essential oil extraction.

Essential oil extraction: 250 gram of powder from dried plant material was mixed with 750 milliliter of water and then the mixture has been placed in a Clevenger type apparatus. The essential oil was extracted by hydrodistilation for 3 h. The essential oil was dehydrated with anhydrous sodium sulfate and stored at 4°C before use. Essential oil yield was calculated as percentage of leave dry weight (v/w).

Identification of essential oil components and methyl chavicol measurement: 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 60ºC to 250ºC at a rate of 5ºC 1/min. Injector and detector (FID) temperatures were kept at 250ºC and 280ºC, 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.

GC/Mass Spectrometry (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 250ºC. 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 this mass spectra was compared with those of the internal reference in the mass spectra library (Wiley 7.0) or with authentic compounds and confirmed by comparison of their retention indices 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. Then methyl chavicol content in all the samples was quantified as µl/g DW.

PAL activity assay: Phenylalanine ammonia-lyase (PAL) was extracted from 300 mg of fresh leaves (in triplicate) with 6.5 ml of 50 mM Tris-HCl buffer (pH=8.8) containing 15 mM of β-mercaptoethanol in an ice-cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged at 50,000 g for 30 min, and the supernatant was collected for enzyme assay. PAL activity was determined based on the rate of cinnamic acid production, according to Wang *et al.* (2006). Briefly, 1 ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine, 0.4 ml of double distilled water and 0.1 ml of enzyme extract were incubated at 37°C for 1 h. The reaction was terminated by the addition of 0.5 ml of 6 M HCl, and the product was extracted with 15 ml ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 M NaOH and the cinnamic acid concentration was quantified with the absorbance measured at 280 nm. One unit of enzyme activity was defined as the amount of PAL that produced 1 µmol of cinnamic acid within 1 min and was expressed as µmol cinnamic acid mg/protein/min.

Total protein was assayed with bovine serum albumin as the standard using the dye-binding method of Bradford (1976).

Reverse transcription polymerase chain reaction (RT-PCR): Total RNA from leaves of *Ocimum basilicum* was prepared using RNX™-Plus Kit (CinnaGen, Iran) based on the manufacturer's instructions. Total RNA (5 µg) was treated with 20 units of RNase-free deoxyribonuclease I (DNase I) (Fermentas) at 37°C for 15 min in 100 µl of reaction mixture buffer 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) followed by ethanol precipitation. The purified RNA was used for synthesis of the first strand cDNA using a first-strand cDNA synthesis Kit (Fermentas) 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 *i*M of each deoxynucleoside triphosphate and 4 µM each oligonucleotid.

PAL primers were designed by aligning PAL isoforms from *Arabidopsis*, *Robus*, *Glycine max*, *Nicotiana tobbacum, Populus* and etc. using Clustal-W software. A 480 base pair conserved segment was found in agree with the region related to basil in picheresky site. Finally we designed gene-specific primers capable of amplifying this segment in sweet basil PAL cDNA.

The sequence of oligonucleotid used for study was as follow: F: PAL (5´-GGCTACTCCGGCATAAGATTC-3´), R: PAL (5´-GTACGAGCTTCCGTCGAGGATG-3´), F: tubulin (5´-GGGGCGTAGGAGGAAAGCA-3´) and R: tubulin (5´-GCTTTCAACAACTTCTTCAG-3´). The initial denaturation was performed at 94°C for 90 sec and the amplification was performed by 30 cycles for tubulin, as external control, 35 cycles for PAL of 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 System). The RT-PCR results are shown from one experiment that was repeated at least three times with similar results. Finally to confirm the existence of amplified fragment, the PCR products were sequenced.

Statistical analysis: Three replicates of five growth stage samples were used for statistical analysis based on complete randomized block experimental design. Data were subjected to analysis of variance (ANOVA), and then means were compared by Duncan's method in MSTATC program ver. 1.4. Differences at $P<0.05$ were considered to be significant.

RESULTS

Essential oils content and composition: The content of essential oil in leaves was dependent on development and maturity stage of leave. The amount of essential oil in leaves was 3.5 µl/g DW at 10-leaves stage and it reach a maximum 7.5 µl/g DW at budding stage but it decreased at flowering stage (Fig. 1). The
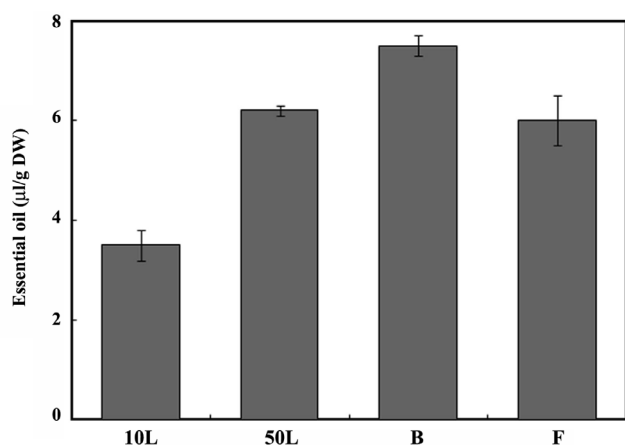
Figure 1. Total essential oils yield (μl/g DW) in leaves of *O. basilicum* at various stages of growth. 10 l (10-leaves plants), 50 l (50-leaves plants), B (budding stage) and F (flowering stage). [1]Error bars in the figure are standard deviations of three replicates.
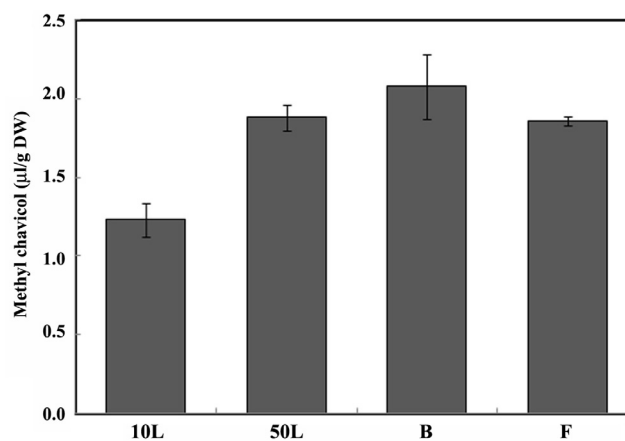
Figure2. Methyl chavicol content (μl/g DW) in essential oil of *O. basilicum* leaves at different stages of growth. 10 l (10-leaves plants), 50 l (50-leaves plants), B (budding stage) and F (flowering stage). [1]Error bars in the figure are standard deviations of three replicates.

GC-MS analysis of the essential oils revealed 45 compounds, representing 99% of the total volatiles in most samples. The average contents of the major constituents (accounting for approx. 90% of the total) of developing and mature leaves are shown in Table 1. Methyl chavicol was the predominant phenylpropanoid compound which ranged from 27.81 to 35.16% of the essential oils at different stages of growth. It was 1.23 μl/g DW at 10-leaves stage and reached to 2.08 μl/g DW at budding stage as the maximum amount. Methyl chavicol alterations were comparable with essential oil efficiency results (Fig. 2). On the other hand, the main terpenoids were Z-citral (approx. 20%) and E-citral (approx. 19.81%). There were only small, largely insignificant differences in the overall composition of essential oil at different stages of plant growth.

Determination of PAL activity: PAL activity (0.208 μmol cinnamic acid/mg protein/min) was observed in the 10-leaves plants which increased significantly (p≤0.05) in the leaves harvested from 50-leaves and budding stage (0.606 and 0.851 μmol cinnamic acid/mg/min, respectively). The PAL activity obtained from flowering stage was significantly lower only than budding stage (Fig. 3). These results are compatible with the results of essential oils and methyl chavicol analysis.

Expression of PAL gene at different stages of growth: To examine the expression level of PAL at different stages of growth semi quantitative RT-PCR

was used and as external control the expression of tubulin gene was utilized. The lowest and highest level of the PAL gene expression were in 10-leaves stage and budding stage of growth (Fig. 4). The sequence of amplified fragment submitted to Gene Bank (http://www.ncbi.nlm.nih.gov/genbank/) under AB436791 accession number for PAL gene.

DISCUSSION

Sweet basil, like similar to many members of the Lamiaceae family such as *Mentha*, *Salvia*, *Origanum*, and *Thyme* spp. have been also cultivated to be used as herbs and as a source of essential oils (Sangwan *et al.*,
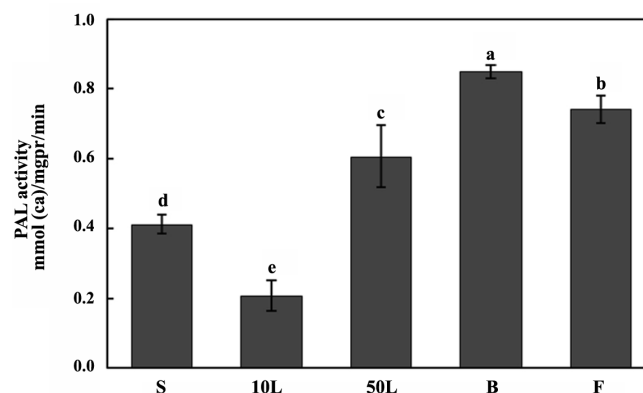
Figure 3. Phenylalanine ammonia lyase (PAL) activity of sweet basil determined in various stages of growth. S (seedling stage), 10 l (10-leaves plants), 50 l (50-leaves plants), B (budding stage) and F (flowering stage). [1]Error bars in the figure are standard deviations of three replicates.
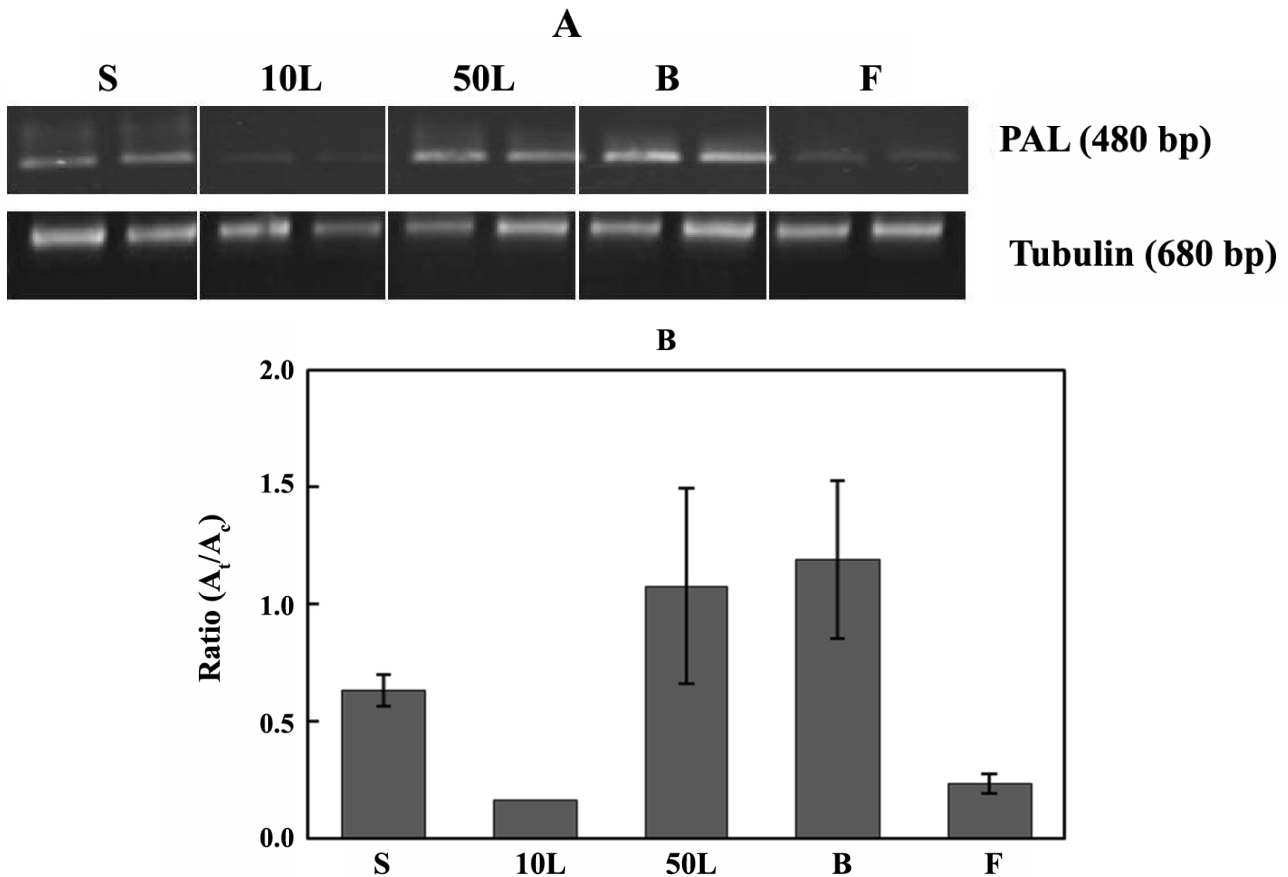
Figure 4. Accumulation of PAL m-RNA at transcripts in different stages of growth in sweet basil. S (seedling stage), 10 l (10-leaves plants), 50 l (50-leaves plants), B (budding stage) and F (flowering stage). A: RT-PCR was performed using equal amounts of cDNA from tubulin as external control and PAL. B: The relative amounts of target (t; 480 bp) and control (c; 680 bp) amplification products were calculated.

Table 1. Essential oil components (average percentage of total volatile content) found in leaf samples of *Ocimum basilicum* plants at different stages of growth.

Component	[a]RI	Composition in oil %			
		[b]10L	[c]50L	[d]B	[e]F
Methyl chavicol	17.14	35.16	30.45	27.81	31.01
Z- Citral	18.21	20.58	18.87	20.35	20.49
E- Citral	19	19.75	18.62	22.13	18.74
Cis-Caryophyllene	23.76	4.25	4.48	4.92	5.55
α -Humullene	24.59	3.11	3.69	2.78	2.07
cis α-Bisabolene	26.43	2.48	4.28	4.05	3.29
1- Octen- 3- ol	10.86	1.56	1.01	0.68	2.5
Fenchone	13.98	1.33	0.98	0.5	0.19
Trans- 2-caren-4-ol	16.41	1.36	1.37	1.68	2.5
Methyl eugenol	22.23	1.15	1.89	1.37	0.26
Germacrene D	25.2	1.67	2.48	2.27	2.38
Cis- Verbenol	15.92	0.85	0.82	1.01	1.63
Z,E- α -Farnesene	24	0.83	1.66	1.44	1.66
Limonene	12.58	0.66	0.59	0.33	0.24
Percentage of total included		94.74	91.19	91.32	92.51

[a]RI: Retention indices calculated against n-alkanes on DB-5 fused silica column, [b]10L (10-leaves plants), c50L (50-leaves plants), [d]B(budding stage) and [e]F (flowering stage).

2001). There is relationship between some factors such as light quality, seasonal and climatic variations, nutritional relationships, plant growth regulators, some abiotic stresses, leaf length and essential oils synthesis in plants (Werker *et al*., 1993). The essential oil yield (0.58%) in the present study was comparable to what reported 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% (Bozin *et al*., 2006; Telci *et al*., 2006) while the yield of essential oil from *O. basilicum* grown in Serbia and Montenegro has been reported to be 0.37% (Klaus *et al*., 2008). These variations might be attributed to the varied agroclimatic conditions of the regions. On the other hand, total essential oils content in this study indicated that maturity and leaf development influence strongly the expression of essential oil compounds and maximum quantity of essential oils was in the last stage of vegetative growth in this plant. Our result was in agreement to those studies reported that essential oil yield generally has a positive correlation with biomass (Chang *et al*., 2008; Naghdi Badi *et al*., 2004). This tight integration of the oil production with the preset developmental program of the tissue is particularly interesting from the viewpoint of developmental dynamics of certain relevant primary biochemical activities (Sangwan *et al*., 2001).

The major aroma constituents of the essential oil found at 4 stages of growth were methyl chavicol, Z-citral and E-citral. Same results related to chemotype of *O. basilicum* with high methyl chavicol and citral contents have been reported from Turkey which is known as methyl chavicol/citral chemotype (Telci and Ceylan, 2006). European originated basil has the highest quality aroma, containing linalool as the major constituents (Simon *et al*., 1999).

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. According to the improved methyl chavicol content during the vegetative growth, where the maximum value was observed at budding stage, it might be conclude that the PAL, a known regulatory enzyme in phenylpropanoid pathway, plays a key role in regulation of methyl chavicol level in basil, scince the level of gene transcription and activity of PAL increased during the vegetative growth. PAL catalyzes the first step of the phenylpropanoids pathway in which L-phenylalanine is deaminated to trans-cinnamic acid and is considered as an important regulation

point between primary and secondary metabolism (Dixon and Paiva, 1995). Fluctuation in PAL levels are known as a key element in the regulation of phenylpropanoid synthesis whereas overexpression of AtPAL in citrus transgenic roots substantially altered the growth and phenolic production (Kostenyuka *et al*., 2002). Age, growth factors, herbivory, tissue wounding (Campos-Vargas *et al*., 2005; Peiser *et al*., 1998), pathogenic attack (Jones, 1984), UV irradiation (Teklemariam and Blake, 2004), ozone concentration, low temperature (Olson and Bidlack, 1997) and salicylic acid (Kiselev *et al*., 2010) have been identified as important factors affecting on *de-novo* synthesis and activity of PAL. The age factor up to budding stage induced the PAL activity in correlation with PAL transcript levels. It was remarkable that activity of PAL was regulated developmentally at the transcriptional level. No PAL expression was detected in flesh during grape berry development by Boss *et al*. (1996); we also found low gene expression and activity of PAL at flowering stage in *O. basilicum*. This reduction was probably due to leaf drop and dehydration, a happenstance of senescence (Olson and Bidlack, 1997) and it seems that the PAL gene expression plays a regulatory role in the abscission process (Bauer *et al*., 2011). Due to the nature and medicinal role and defense related function of these products, the activation of PAL under plant growth condition have been considered as a part of defense mechanism and production of essential oil compounds (Peiser *et al*., 1998).

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.

CONCLUSIONS

Essential oil of *O. basilicum* cv green from Iran is containing of mainly methyl chavicol and citral. Total essential oil content was correlated directly with biomass production. Maximum essential oil yield was achieved before of the flowering phase, while it decreased at flowering stage. These differences can be attributed to the different stage of plant metabolism. Our data indicated that gene expression and activity of PAL exhibit a similar pattern of regulation during the basil development. The highest level of PAL activity and gene expression was obtained at the budding stage. PAL is an important enzyme that has a regulatory role in the formation of lignin, flavonoids, benzoic

acids, tannins and stilbenes. Synthesis of these natural products may be governed by a set of internal or environmental stimulus that involve in regulation of key enzymes.

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