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Increasing of Chavicol o-methyl transferase gene expression (CVOMT) and methyl Chavicol value of basil (*Ocimum basilicum*) by salicylic acid

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Abstract

Basil (*Ocimum basilicum*), is a medicinal plant mint family (Lamiaceae) which contains cyclic compounds and essential oil, and it has antibacterial and antioxidant properties. From Long ago, Basil has been used for treatment of headache, cough, diarrhea and fever, inflammation of the throat and stomach pain in Iran. Basil essential oil is mainly containing phenylpropanoids compounds, the biosynthesis path of these compounds are of course passed from the pathway of shikimate and the most important compounds of them, that can be cited, are chavicol and methyl chavicol. The role of salicylic acid is known as a bio- stimulant to improve the biosynthesis of secondary metabolites in many plants. In this study, the effect of salicylic acid on gene expression CVOMT and the amount of methyl chavicol was studied. Therefore, basil was treated with 2 mM solution of salicylic acid before flowering stage, and respectively the samples were harvested 1, 2, 3 and 5 days after treatment. Gene expression CVOMT which was under influence of salicylic acid, compared with controls, showed the highest activity of gene expression CVOMT. So, on the third day after treatment was reached to highest level and on the fifth day after treatment was reduced. Essential oil analysis also showed that the methyl chavicol influenced by

salicylic acid, in comparing with controls, like gene expression (CVOMT) was increased. In General, the changes in gene expression CVOMT and the amount of methyl chavicol that harvested at different stages were Conformity.

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Introduction

Basil by scientific name of *Ocimum Basilicum* (2n=48) is an annual herbaceous drug plant from the Lamiaceae family that, there are about 50 to 150 species of its herbaceous and shrub (Omid baigi, 2000; Zargari, 1993). It is naturally grows in tropical and subtropical regions of Asia, Africa, Central and South America. Basil essential oil is an aromatic source compounds and the anti-parasitic insect repellent, antibacterial, antifungal, antiviral and antioxidant (Juliani and Simon, 2002; Labra *et al*., 2004; Lewinsohn *et al*., 2000). From the distant past, basil have been used as a medicinal plant for treatment of headache, cough, diarrhea, parasites, warts and kidney disease (Labra *et al*., 2004).

The essential oils are secondary metabolites that are produced during vegetative and reproductive in medicinal plants (Lewinsohn *et al*., 2000). That in Basil is divided in to two groups: terpenoids (monoterpene and Sesquiterpene) and phenylpropanoids (such as eugenol, chavicol, methyl cinnamate and etc.). One of the main components of basil essential oil is methyl chavicol or estragole that is a derivatives of Allyl phenol non terpenoids and has aromatic odor. Experiments show that Allyl phenol derivatives are formed from phenylalanine and Cinnamic acid (Lewinsohn *et al*., 2000). Methyl chavicol is a colorless liquid that does not dissolve in water (<1.0 g per 100 ml) that Its own gravity is (0.965 g/l) and its boiling point is 215 to 216°C and its melting point is 81°C (Mc Donland *et al*., 1999). In the biosynthesis of methyl chavicol, phenylalanine is primary precursor. Two pathways are resulted, that from the first pathway, chavicol and methyl chavicol and from the second pathway, eugenol and methyl eugenol are derived. First, by deamination of phenylalanine by enzyme phenylalanine ammonia lyase (PAL), Cinnamic acid is formed. Then, para-coumaric is formed by adding hydroxyl, and the final result is chavicol. At the end of stage, after methylation of OH-4 chavicol is performed by chavicol o- methyl transferase enzyme (CVOMT), methyl chavicol is formed. At last step of methyl chavicol synthesis, catalyst is done by o-methyl transferase dependent to S-Adenosyl methionine, which can convert the precursor chavicol at the position of Para-hydroxy to methyl chavicol (Lewinsohn *et al*., 2000).

Salicylic acid (SA) or ortho hydroxy benzoic acid with the chemical formula ($C_7H_6O_3$) and a molecular weight of approximately 138.1, is a white, soft and crystalline powder. Salicylic acid is a phenolic compound that has an aromatic ring with a hydroxyl group and its derivatives have been found in plants as a hormone-like substance that plays a role in regulating plant growth and development (Kang *et al*, 2004; Coquoz *et al*., 1998). Salicylic acid plays a pivotal role in the regulation of physiological processes such as seed germination, stomata closure, plant ethylene biosynthesis inhibitor, increasing rates of photosynthesis and chlorophyll content, fruit production, production of heat and glycolysis (El-Tayeb, 2008; Popova *et al*., 2005). Salicylic acid regulates the expansion of division and cell death and the balance between growth and aging of the plant (Popova *et al.,* 1997). Salicylic acid is widely distributed in the plant hierarchy and in more than 34 species have been identified. This material is found in leaves and reproductive parts of plants. Salicylic acid is produced from plants in two ways by Cinnamic acid. The first method is through coumaric acid and the second method through benzoic acid (Popova *et al.,* 1997). the role of salicylic acid are well known on tolerance of plants to pathogens and other stressors and recently its role as a combined messenger to activate plant defense responses is highlighted (Hayat *et al*., 2009). The role of salicylic acid as a stimulus to Increase gene expression of secondary metabolites of the biosynthetic pathways that it has been specially considered (Pu *et al.,* 2009). Several studies have shown that salicylic acid is effective in stimulating the production of secondary metabolites such as terpenoids, coumarin derivatives, alkaloids and flavonoids (Kang and Wang, 2003). The aim of this study is Increasing of Chavicol o-methyl transferase gene expression (CVOMT) and methyl chavicol value of basil (*Ocimum basilicum*) by salicylic acid.

Materials and methods

To investigate the gene expression of CVOMT and mrthyl chavicol value influenced by salicylic acid, green basil seeds were prepared from Seed Company of Pakan Isfahan, and were cultivated in some pot in light soil, mixed equally of sifted sand, clay, humus and manure in early October 2013. After planting, the pots were transferred to the greenhouse and in the same conditions and daily at temperatures of 25 to 30 and nightly at 18 to 20 °C until the end of the flowering stage of growth. The pots were watered daily 2 times a week and were given 50 ml of Hoagland to them. The treatments were prepared, by Salicylic acid during before flowering with a solution of 2 mM salicylic acid. In this case, the required amount of salicylic acid were weighed by using heat, the volume of 1 ml Distilled water was gradually dissolved. Aerial parts of basil in 4 stages, consisting of 1, 2, 3 and 5 days after the treatment with salicylic acid were picked. After each harvest, plants after fixation with liquid nitrogen were maintained at freeze at -80°C until extraction and measurement. Finally, in order to extract the essence, some part of plants were dried at dark room temperature (25°C) for 20 days. For extracting the essence oil, was used a Clevenger apparatus with water distillation. Extraction operation continued for 3 hours and the resulting oily liquid was dry with absorbent material (sodium sulfate). Obtained essential oil were weighed carefully and kept in the dark dishes in a refrigerator until analysis (Shibamoto *et al.,* 1987). For the analysis of essential oils were used a gas chromatograph connected to a mass spectrometer Thermo quest-Finnegan, Trace Model DB-5 column with a length of 60 mm and an inner diameter of 0.25 mm and 0.25 mm thick. Oven from 60°C to 250°C at a rate of 4°C per minute for 10 minutes increased and was kept at 250°C. The helium carrier gas with a flow rate of 1.1 mm per minute was used, and also the ionization energy of 70 eV was used. Identification of compounds were done by using various parameters such as time and retention index (RI), mass spectra and comparison of these spectra with the combination of standard and computerized library and information in the GC-MS system (Adams, 2001). The relative percentage of each constituent of the essential oil composition were obtained according to GC chromatogram area under the curve in the normal way and ignoring the response factors (Shibamoto *et al.,* 1987).

Many researches has been done in this area. Ziaei *et al.*were investigated the Gene expression and activity of Phenyl alanine ammonialyase and essential oil composition of *Ocimum basilicum* at different growth stage.Chalchat and ozen have Compared the essential oil composition of flower, leaves and stems of basil. And also Tahsili *et al.* also investigated the 4, C4H, 4CL, EOMT and CVOMT and their relationship with phenylpropanoid content in basil. Pourbozorgi *et al.* investigate Quality and quantity of oil essential and its relation to CVOMT gene expression at different growth stages basil.Tahsili *et al.* investicated Gene expression of EOMT and components of essential oils in basil at different stages of growth.

Molecular analysis

Total RNA was extracted from the leaves of basil, by using the RNXPlus solution of Cinagene Company with chloroform, isopropanol, ethanol and 75% water DEPC (diethyl carbonate follower) protocol was proposed Cinagene Company. After extraction the Total RNA, its quality was investigated by using agarose gel electrophoresis. In the next step for synthesis of cDNA from RNA extracted was used 2-Step RT-PCR kit that was product by Vivantis Company. For designing of primers for CVOMT and Tubulin gene were used software and web Oligo Therapeutics Oligonucleotide properties Calculator (Table 1).

Table 1. The primers designed for CVOMT and Tubulin gene.

Gene name	Primer sequence (5'-3')	T_m (°C)	GC (%)
F: CVOMT	GATCCCACTTTCACAAATCC	58.4 °C	50.0
R: CVOMT	GAGTACATGTGCCACAACCC	60.5 °C	55.0
F: Tub	CTCCTTGAGCTAGTCGTCGC	62.5 °C	60.0
R: Tub	AACAAGGCAAAAACATTCCG	54.3 °C	40.0

Duplication of CVOMT and tubulin genes for measurement of gene expression by Real-Time PCR reactions were relatively performed according to standard methods. The relative quantity in Real-Time PCR was performed by measuring the fluorescence radiation resulting color connection (EvaGreen) by using (RG-3000 Corbett Research). The reaction components of Real-Time PCR in a final volume of 20 micro liter were mixed together 4 micro liter Master mix, one micro liter of cDNA and 0.5 micro liter of each primer and 14 micro liter RNAse free water. Thermal program for duplication of both genes by using Real-Time PCR as were shown in Tables 2 and 3. After the reaction duplication by Q Real-Time PCR method the raw data as Ct (Threshold cycle) were extracted from the device software. For each sample taken three times, and data processing was performed by using ΔΔCt method.

Table 2. Real-Time PCR Table temperature and reaction time for CVOMT gene.

Step	Temperature and time used
Initial activation of the enzyme	95°C for 15 minutes
40 cycles of the following steps	
Denaturing	95°C for 30 seconds
Primers connection	58.8°C for 45 seconds
Extended mix	72°C for 45 seconds
Melting curve	Raising the temperature from 50 to 99 degrees every 5 seconds 1°C

Table 3. Real-Time PCR Table temperature and reaction time for *Tubulin* gene.

Steps	Temperature and time used
Initial activation of the enzyme	95°C for 15 minutes
40 cycles of the following steps	
Denaturing	95°C for 30 seconds
Primers connection	60°C for 45 seconds
Extended mix	72°C for 45 seconds
Melting curve	Raising the temperature from 50 to 99 degrees every 5 seconds 1°C

Statistical analysis

This experiment was performed in a factorial randomized complete block design, with 4 harvest and 3 replications. For analysis and comparison of means was used from SAS software version 9.2 and Duncan's multiple range test at Level of 5%, possibility.

Results and discussion

Gene expression CVOMT

The results showed that the gene expression of CVOMT influenced by salicylic acid at different times of harvest after treatment have increased than the control samples. The gene expression in the third day after treatment of Salicylic acid had reached to its highest level, and on the fifth day after treatment, decreased (Fig. 1).

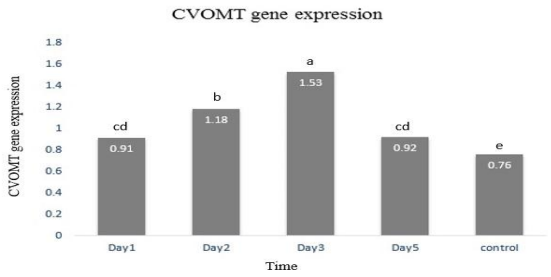

Fig. 1. CVOMT gene expression influenced by salicylic acid.

Methyl chavicol value

Investigating of methyl chavicols value influenced by salicylic acid showed that after the treatment with salicylic acid, methyl chavicol was increasing trend, on the third day after the treatment had reached to the highest level and it fell on the fifth day (Fig. 2).

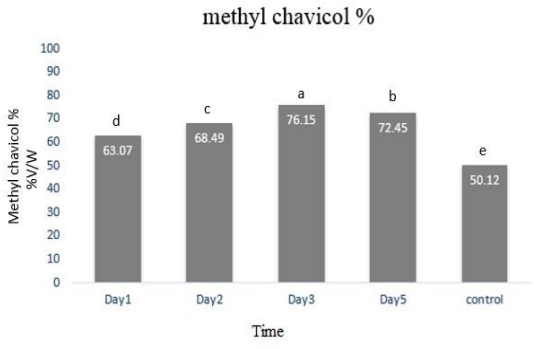

Fig. 2. Methyl chavicol present influenced by salicylic acid.

Discussion

Green basil like other mint family plants such as mint (*Mentha*), sage (*Salvia*), marjoram (*Origanum*) and thyme (*Thyme*) are widely cultivated due to its essential oils (Lewinsohn *et al*., 2000). Essential oil synthesis in plants are changed by influencing factors such as immature plant, essential oil production, photosynthesis, photo periodic changes, the effects of light intensity, seasonal variation, climate, relationships nutrition, plant growth regulators and environmental stresses such as drought, salinity and temperature (Werker *et al*., 1993). The ecological and climate situations have a huge impact on the efficiency and variety of essential oils. On the other hand, the results of the total amount of essential oil, have shown that mature and immature leaves or leaf age dramatically effect on the essential performance. The yields of essential oil from green and purple basil during vegetative growth before flowering is increasing and it decreases during flowering. In other words, when the plant is in its final stages of vegetative growth, has the most of basil essential oils (Poor bozorgi Roudsari, 2007; Ziaei *et al*., 2012). In this study, the gene expression levels of methyl chavicol CVOMT in basil was investigated and studied. The results of gene expression (CVOMT) and the amount of methyl chavicol before flowering at different times of harvest after the treatment with salicylic acid indicates that the activity and gene expression in the various stages of the process is increased, so that, gene expression CVOMT On the third day was increased, and on the fifth day was declined in its lowest level. Salicylic acid also caused increasing the amount of methyl chavicol in basil essential oil ,so that its amounts on the third day after treatment was reached to the highest level, but its level in five days was declined. This reflects that there is positive correlation between gene expression CVOMT and the amount of methyl chavicol so, along the increasing of gene expression CVOM, the increasing of the amount of methyl chavicol were observed during the different times of harvests. This indicates that the amount of methyl chavicol, by increasing gene expression CVOMT is increased and by reducing gene expression the amount of methyl chavicol is also reduced. According to the past survey, several factors including: temperature, PH environment, substrate concentration, types of inhibitors of the enzyme, the levels of gene expression and regulatory mechanisms are influenced on the activity of an enzyme. Consequently, these evidences indicate that the activity of chavicol O-methyl transferase enzyme (CVOMT) is regulated at the level of gene expression of these enzymes. In other researches, methyl chavicol, linalool, Methyl cinnamate, camphor, methyl eugenol and geraniol most essential components has been reported in different cultivars of basil (Marotti *et al.,* 1996; Sajjadi, 2006; Sangwan *et al.,* 2001; Vina and Murillo, 2003). Results of this study shown that the gene expression CVOMT before flowering is corresponded with the results of the yields of methyl chavicol. Thus, the salicylic acid can use as an efficiency stimulus, through the induction of the immune system ,in order to increase the biosynthesis of secondary metabolites and can be used in many medicinal plants.

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