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Evaluation of Gene Expression Level of Limonene and Flavone Synthase and Essential Oil Composition under Different Water Conditions in Cumin

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ABSTRACT

Cumin, *Cuminum cyminum* L., is the king of spices with a plethora of natural compounds with pharmacological features. Drought stress is a well-known factor that influences the production of some metabolites. We studied the impact of drought stress on gene expression and metabolite content in flower and leaf tissue organs of two ecotypes, Taybad and Ardakan populations. Plants were imposed into three water level conditions, control, moderate, and severe water deficit. Concerning the results, the expression of *Limonene synthase* in flower organ of the Ardakan genotype increased 2.2 times under 50% of field capacity, whereas, the expression of *Flavone synthase* in leaf tissue of the Ardakan ecotype, was the highest in this level of stress. Moreover, the majority of detected terpenoids were β-Acoradiene and γ-Terpinene in leaf and flower organs, respectively. Altogether, the monoterpenes content was decreased in both ecotypes, but sesquiterpenes increased only in the Ardakan population. Knowing the expression of key genes involved in the pathway of major metabolites in cumin under water stress conditions is important in the pharmaceutical industry and molecular researches.

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Introduction

Cumin, *Cuminum cyminum L.*, one of the well-known key species, is originated from Iran, Egypt, and Eastern Mediterranean. It is widely cultivated in Iran, China, Morocco, India, South Russia, Indonesia, Japan, Turkey, and Algeria (Tuncturk and Tuncturk, 2006). As far as the world market is concerned, Iran obtains about 52% of the world's cumin exportation, which is known as one of the major exporters of this valuable herb in the world (Kafi *et al.*, 2006). This plant shows some pharmacological features, like boosting appetite, abdominal distension, taste understanding, and lactation (Nostro *et al.*, 2005). Because of these beneficial characteristics, it is commonly used in food preparation as valuable specie. The seeds of this plant contain fixed oil (approximately 10%), protein, sugar, mineral elements, and volatile oil (Li and Jiang, 2004). It is known as anti-tumor, anti-inflammatory, diuretic, cytotoxic, antidiabetic, antifungal, antibacterial, antioxidant, and anti-spasmodic (Einafshar *et al.*, 2012). Traditionally, farmers add the cumin to animal nutrients as complementary feed (Elhamidi and Ahmed, 1966; Kafi *et al.*, 2006). Plant secondary metabolites are referred to as specialized compounds that do not aid in the maturation and evolution of plants; however, they are needed for the plant adaptation and

defense in its environment (Kafi *et al*., 2006). These specialized compounds can be divided into three major classes based on their biosynthetic origins: i) terpenoids; ii) flavonoids and allied phenolic and polyphenolic compounds, and iii) nitrogen-containing alkaloids and sulfur-containing compounds (Crozier *et al.,* 2008). The volatile oil of cumin mainly contains cumin aldehyde, cymene, and other terpenoids, which has antifungal, antibacterial, and antioxidant activities (Mohammadpour *et al.,* 2012; Oroojalian *et al.*, 2010). This oil helps plants to cope with environmental fluctuation stress and plant defense reactions. Despite the functional and structural variation of terpenes, they originated mostly from three simple ring-shaped precursors; the 10-C monoterpenes from geranyl diphosphate (GPP), the 15-C sesquiterpenes from farnesyl-diphosphate (FPP) and 20-C diterpenes from geranyl-geranyl diphosphate (GGPP). One of the simple ringing-shaped reactions is catalyzed by Limonene synthase. Different enzymes produce limonene as two enantiomers, (-) and (+) (Morehouse *et al.*, 2017). It has been proved that unfavorable conditions influence many aspects of plant physiology and biochemistry. Drought causes changes in the plant metabolism like enhancing some valuable compounds including essential oils and aroma, which plants grown in the semi-arid regions produce a high amount of these compounds (Al-Gabbiesh *et al.,* 2015; Bettaieb *et al.,* 2011; Osakabe *et al.,* 2014; Yadav *et al.*, 2014). Some researchers showed that the biochemical compositions of cumin aerial parts are greatly influenced by water constraints (Bettaieb *et al.,* 2010; Pandey *et al.,* 2015).

Limonene synthase is a cyclase enzyme in the biosynthetic pathway of some essential oil compounds. It is an intermediate factor that catalyzes the conversion of the GPP precursor to limonene (Muñoz-Bertomeu *et al.,* 2008). Monoterpene synthase regulates the first step of the formation of diverse monoterpene families. Thus, any fluctuations in the gene expression can change substance amounts in essential oil, or type of monoterpenes in organs which are not synthesized in normal condition (Muñoz-Bertomeu *et al.,* 2008). Hassanpour *et al.* (2014) assessed the expression of two genes involved in the monoterpene biosynthetic pathway in *Mentha pulegium* L. Expression of genes encoding isopiperitenone reductase (*iPR*) and pulegone reductase (Morehouse *et al.,* 2017) were enhanced under water deficit in contrast with control (Hassanpour *et al.,* 2014).

Flavone synthase (FNS) converts flavanones to flavone by insertion of a double bond between the C-2 and C-3 positions. There are two distinct FNS enzymes, FNSI and FNSII, which catalyze the conversion of flavanones to an identical product by different mechanisms (Jiang *et al.,* 2016). Wang *et al.* (2016) investigated the expression of 103 genes involved in flavonoid biosynthesis in *Camellia sinensis* leaves under drought stress. Hierarchical clustering analysis demonstrated that the level of *Flavone synthase* expression was enhanced under drought stress. Moreover, they showed a positive correlation between total flavonoid content and drought stress (Wang *et al.,* 2016).

Regarding the economic importance of essential oils, understanding the expression pattern of the genes involved in terpenoid and flavonoid biosynthesis pathways under different conditions helps to improve the production of these compounds in pharmaceutical and other related industries. There is no report on the effect of drought stress on *Lim* and *FS* gene expression in cumin. We elucidated the transcript level of Limonene synthase and Flavone synthase encoding genes under three levels of water deficiency in two ecotypes of cumin. Afterward, the amounts of some valuable compounds were measured using the GC-MS technique.

Materials and Methods

Plant growth and drought treatments

Two ecotypes (Ardakan-Yazd and Taybad-Khorasan) of *C. cyminum* L., were provided from a gene bank in College of Aburaihan, the University of Tehran (Geographical coordinates are shown in table 1).

Table 1. Geographical coordinates of studied ecotypes.

Origin	Latitude	Longitude	AMSL*
Ardakan, Yazd, Iran	32° 18′36″N	54°1′3″E	1005
Taybad, Khorasan, Iran	34°44′24″N	60°46′32″E	803

* Above mean sea level

Seeds were planted in pots in a controlled condition greenhouse (35°77′N 50°95′E). The experiment carried out in a CRD design with three levels (normal irrigation as control, 50% field capacity (FC) and 30% FC as severe stress) at three replications. Treatments were applied by the gravimetrical method after 20 days of planting (Chegah *et al*., 2013; Harb *et al*., 2010). Drought stress was applied for 16 days. Afterward, sampling from leaves and flowers performed in the plant reproductive phase.

Extraction of essential oils

In the reproductive phase, harvested samples were dried at room temperature and the essential oil was extracted using distilled water Clevenger-type apparatus by 10 gr of harvested samples for 3 hours. The extracted oils were dehydrated using sodium sulfate and were stored at 4ºC until future usages (Baydar *et al.*, 2004).

Gas chromatography-mass spectrometry

The gas chromatography analysis was carried out on an Agilent789, gas chromatography with a 5975C mass selective detector, and an HP5M5 column (30m×0.25mm×0.25µm). The operating conditions were as follows: The samples (2µL) were diluted to 1% with n-hexane, and the carrier gas was helium at a flow rate of 1.0 ml min^{-1}. The oven temperature was programmed 3 min at 60 °C, then increased to 150 °C (3 °C per min), after that reached to 260 °C (3 °C per min) then hold in this temperature for 10 min. The injector and the temperature of the detector were at 230 and 250 ºC, respectively. The components of the oil were identified by their retention indices relative to C8-C25 n-alkanes and commercial library (Willey) (Joulain and König, 1998; Thiem *et al.*, 2011).

RNA extraction and cDNA synthesis

Harvested samples of leaves and flowers were ground to a fine powder in liquid nitrogen. Meanwhile, total RNA was extracted using the BioZOL commercial kit following the manufacturer's instruction. The quality and quantity of extracted RNA were verified by agarose gel electrophoresis and NanoDrop spectrophotometer, NDS-2000 (Thermo Fisher Scientific, USA), respectively. Afterward, to avoid contamination of genomic DNA, extracted RNA was treated by DNase I (Invitrogen). PCR experiment was conducted by using *Tubulin α-3* housekeeping gene primer and RNA as a template.

The cDNA synthesis kit (BioRad) was used to synthesize the first-strand cDNA. The mixture was prepared by adding one µg of RNA, 4 µL of 5x cDNA synthesis kit, and nuclease-free sterile water to a total of 20 µL. The prepared mixture was incubated at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and then at 4 °C. The final mixture was diluted with 10 mM Tris-HCl (pH 8.0) and 0.1 mm EDTA for storage at -20 °C until analysis. Two appropriate forward and reverse primers for real-time PCR were designed based on *Lim* (JN388566.1), *FS* (DQ683349.1), and TU (XM_002285685) gene sequences. Primers were designed to reproduce fragments smaller than 200bp (Table 2). These primers were designed by using Primer3 Plus and Reverse complement software and confirmed by the Oligo calculator (biotools.nubic.northwestern.edu).

Quantitative real-time PCR analysis

The quantitative real-time PCR (qRT-PCR) analysis was performed in a Rotor-Gene Q (USA) instrument. The following program was used to conduct the RT-qPCR, 300 nM of each primer, 100 ng of template cDNA, 5µL SyBR Green Supermix 2X from iQTM SYBR® Green Supermix (Bio-Rad, USA) in a total volume of 10 µl for each reaction. The RT-qPCR was programmed at 95 °C for 3 min, followed by 40 cycles at 95 °C (15 sec), 58 °C (40 sec) and 72 °C (5 sec). The melting curves were adjusted as 55-95 °C, 5 °C for 5 sec. The non-RT and non-template preparations were used as controls. The linear range of template concentration to Ct value (threshold cycle value) was determined by preparing a dilution series of cDNA from three independent RNA extractions analyzed in three technical replicates. For quantifying transcription levels, *Tubulin α-3* was used as an internal control to ensure that equal amounts of RNA were used from each sample. Relative gene expressions were determined according to the Livak method (Livak and Schmittgen, 2001).

Results

Analysis of gene expression

The responses of *C. cyminum* ecotypes exhibited considerable variation in the transcript abundance levels of *Limonene synthase*. Results showed that the expression levels of *Limonene synthase* in Ardakan-Yazd ecotype was more than Taybad-Khorasan Razavi ecotype under stress in both evaluated tissues (Fig. 1).

Table 2. Designed primers for real-time PCR.

Gene	Primer sequence (5'→3')	Tm (°C)	Product size (bp)
Limonene synthase	Forward: AGATTGCTTTCGACGTCCTC Reverse: CTATCGAAGGCCCCGTTATAC	58	180
Flavone synthase	Forward: TTGAGGCCTTTGAAGACTGG Reverse: CGTCACCCTGTTGATGAGTG	59	186
Tubulin α-3	Forward: CAGCCAGATCTTCACGAGCTT Reverse: GTTCTCGCGCATTGACCATA	59	119

In stressed conditions, the expression of the Limonene synthase encoding gene was reduced in Taybad genotype. In the Ardakan ecotype, the moderate-stressed condition led to the up-regulation of the *Limonene synthase* gene in leaf and flower tissues. However, under severe stress conditions, *Limonene synthase* showed reduced expression in leaf and flower tissues (Fig. 1).

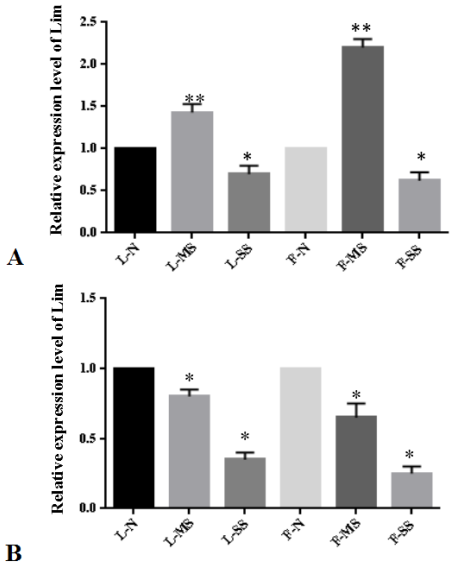

Fig. 1. The relative gene expression: The expression of the *Limonene synthase* gene under drought stress condition relative to control condition in leaves and flowers of Ardakan (A) and Taybad (B) ecotypes. L: leaf; F: flower; N: normal irrigated condition; MS: moderate stress; SS: Severe stress.

Compared to the control conditions, the highest reduction percentage of *Lim* expression (75 and 56%) was observed in flower and leaf tissues of Taybad under severe stress conditions (Fig. 1). *Lim* expression in flower tissue of Ardakan under moderate stress showed the highest fold change (120%) (Fig. 1). Under medium stress conditions and in leaf tissue, the Flavone synthase expression significantly increased in Ardakan and Taybad ecotypes. In the severe stress conditions, FS expression was significantly reduced in flower and leaf tissues of the Ardakan and Taybad ecotypes (Fig. 2). Moderate stress decreased FS expression level in flower tissue of Ardakan ecotype while it was elevated in the Taybad samples (Fig. 2).

The highest and the lowest rate of *FS* gene expression occurred in leaf tissues of Ardakan genotype in moderate stress and flower tissues of the Ardakan ecotype in severe stress, respectively.

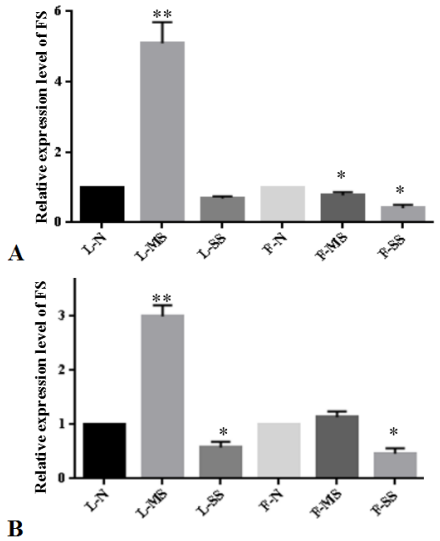

Fig. 2. The relative gene expression: The expression of *Flavone synthase* gene under drought stress condition relative to control condition in leaves and flowers of Ardakan (A) and Taybad (B) ecotypes. L: leaf; F: flower; N: normal irrigated condition; MS: moderate stress; SS: Severe stress.

Analysis of metabolite contents

Altogether, in normal irrigated plants, nine compounds were detected in leaf tissue from each genotype. Eight compounds were common in both ecotypes (Table 3). In flower tissue, 13 compounds were found in Ardakan and six compounds identified in Taybad ecotypes under normal irrigated conditions (Table 3).

Table 3. Chemical composition of leaf and flower tissues of two ecotypes under drought stress.

		Relative content components (%)											
		Ardakan						**Taybad**					
		Leaf			**Flower**			**Leaf**			**Flower**		
Compounds	RI	N.	M.S	S.S	N.	M.S	S.S	N.	M.S	S.S	N.	M.S	S.S
α-Pinene	928	-	-	-	0.9	-	-	-	-	-	-	1.1	-
β-Pinene	970	2.2	1.2	1	11.6	5.4	-	-	1.8	-	18.1	15.8	8.2
β-Myrcene		-	-	-	-			-	-	-	-	0.7	-
l-Phellandrene	1000	16.5	9.9	13.1	26.2	11.4	12.1	15.1	13.6	10.7	18.1	13.8	10.7
α-Terpinene	1016	-	-	0.6	-	-	-	-	-	-	-	-	-
O-Cymene	1022	7.5	7.2	6.5	8.7	11.8	4.3	9.4	6.3	3.2	10.0	16.0	9.6
dl-Limonene	1023	1.8	2.2	0.9	1.6	3.6	1.1	2	1.6	1.1	1.7	1	0.8
β-Phellandrene	1026	-	-	1.1	2.6	-	-	2.5	1.9	-	-	0.6	-
γ-Terpinene	1052	12.2	15.8	15.8	28.4	38.4	4.8	6.3	9.7	6.6	36.1	33.4	36.1
β-Caryophyllene	1416	5.6	2.7	2.2	2	-	-	5.4	3.7	1.7	-	0.9	-
β-Farnesene	1451	-	0.9	2.2	0.9	-	2.8	-	1.7	2	-	0.6	-
β-Acoradiene	1468	37.3	33.1	41.8	-	-	61.5	50.2	50.4	64.1	10.0	6.3	22.8
γ-Curcumene								-	-	-	-	-	-
Germacrene D	1486	-	-	0.5	0.4	-	-	-	-	-	-	-	-
γ-Cadinene	1515	-	-	0.3	-	-	-	-	-	-	-	-	-
Hexadecane	1605	2.4	6.7	3.9	1.8	3.1	2.1	1.2	3.3	1.3	-	0.8	-
Octadecane	1790	2.7	7.9	1.7	1.5	1.1	1.4	2	-	1.4	-	1.4	3.5
Eicosane	1986	3.2	7.9	0.4	1.3	0.9	1.3	4.4	1.8	2.2	5.8	1.1	2.5
Monoterpene hydrocarbons		40.2	35.1	39	35.3	34.9	22.6	35.3	34.9	21.6	84.2	82.4	65.4
Sesquiterpene hydrocarbons		42.9	36.7	47	55.6	55.8	66.8	55.6	55.8	67.8	10.0	7.8	22.8
Other		8.3	22.5	6	7.6	5.1	4.9	7.6	5.1	4.9	5.8	3.3	6
Total		91.4	94.3	92	98.5	95.8	943	98.5	95.8	94.3	100	93.5	94.2

N= Normal irrigated condition; MS= Moderate stress; SS= Severe stress; RI= Retention indices of EO components as tested on the HP-5column

Then, more compounds were detected in flower tissue compared to leaf tissue. Ten different compounds were detected in leaf tissues of each genotype during the moderate stress (50% of FC). Applied severe stress (30% of FC) increased components of leaf essential oil to 14 compounds and decreased to 8 components in flower tissue in the Ardakan genotype. Considering seed yield reduction, the Ardakan genotype is more susceptible, which responded to stress intensity more than the Taybad genotype in the numbers of metabolite compounds. An increase and decrease in components of essential oil were shown in flower and leaf tissues of the Ardakan genotype by a decrease in soil water availability. These changes have a semi-constant trend in the Taybad drought-tolerant genotype (Table 3). β-myrcene was detected just in moderate-stressed flower tissues of the Ardakan and the Taybad genotypes, respectively. Then, change in metabolite constitution during stress implementation is related not only to genotype but also to evaluated tissue. In sunflower, the sesquiterpenes and diterpenes content was increased from 0.4% to 0.79% in the moderate water deficit.

Analysis of these ecotypes revealed that the major leaf compounds belong to sesquiterpenes in all conditions. Under the moderate stress, the highest amounts of sesquiterpenes were identified in severe stress of the Taybad genotype (67.8%) and the lowest amounts were found in the Ardakan genotype (36.7%). Considering flower tissue, except the Ardakan genotype under severe stress, monoterpenes constitute the main components of essential oil. The highest amount was found in the Taybad genotype cultivated in normal irrigated condition (84.16%) and the lowest content was in the Ardakan genotype under severe stress (21.2%).

γ-terpinene constitutes the maximum amount of essential oil content in flower tissue under all conditions except the Ardakan ecotype in severe

stress. Moreover, β-accoradiene is the main component in the essential oil of leaf tissue of all ecotypes at all conditions. Consequently, the changing trend of these two components was considered in both ecotypes under stress rather than normal irrigated conditions (Table 4). Except for a moderate stress situation in the Ardakan ecotype, we observed an increase in β-Accoradine contents in leaf tissues and a decrease in γ-terpinene contents in flower tissues in stressed-condition (Table 4). This result can be explained by more sensitivity of the Ardakan ecotype rather than Taybad to drought stress conditions. We found γ-terpinene only in leaf tissue of cumin but in the early flowering developmental stage.

Table 4. Reduction or increment percentage of β-Acoradiene and γ-Terpinene relative to normal (control) condition in leaf and flower tissues, respectively.

Water condition	Genotype	Organ	
		Leaf	Flower
Moderate stress	Ardakan	-13.0	+34.0
	Taybad	+0.3	-7.4
Severe stress	Ardakan	+9.1	-83.6
	Taybad	+27.7	-1

Discussion

A study on the peppermint biosynthetic pathway has shown that any increase in the biosynthesis of monoterpenes was regulated in transcription level, and the relative gene expression of key enzymes involved in mono, di and sesquiterpenes biosynthesis was varied among different developmental stages. Furthermore, it has been shown that these genes express just in a short period during leaf development (McConkey *et al.*, 2000).

Constant reduction of *Lim* expression level in leaves rather than flower tissue indicates the higher activity of *Lim* related biosynthetic pathway in reproductive tissues. It is well known that there is an intelligently regulation system in which the expression of some key genes has some critical role. Sometimes, there is an obvious correlation between gene expression level and the product amount. Ghannadnia *et al.* (2014) showed that the expression of *Lim* does not occur in the root, leaf, and medium-large sized flowers in the reproductive phase. Their results showed that this gene is expressed only in flowers with small and very small size. Given the qRT-PCR results in our study, this gene can express not only in small flowers but also in the leaf tissues. Gershenzon *et al.* (1984) reported that the biosynthesis of monoterpene is confined to some specific stages of development and tissue in plants, for instance, it is limited to the leaf tissue in *Majorana hortensis* and *Salvia officinalis*, to the fruit in *Carum carvi* and the stem in *Pinus pinaster* (Gershenzon, 1984). Redha *et al*. (2012) demonstrated that tolerance to stresses (drought, salinity, and high temperature) in *Conocarpus lancifolius Engl*. is correlated with the synthesis of flavonoids (Redha *et al.*, 2012). Flavones have diverse functions such as antioxidative activity and cancer and coronary heart disease prevention (Martens and Mithöfer, 2005). Recently, an increase in the expression level of several flavonoids biosynthesis genes under drought stress conditions have been reported at two cultivars of wheat (*Triticum aestivum*) (Ma *et al.*, 2014), and in *Scutellaria baicalensis* (Yuan *et al.*, 2012). Moreover, it has been reported that flavonoids biosynthesis genes expression is correlated to polyphenol content and it is dependent on the cultivars under severe drought conditions (André *et al.*, 2009). In the current investigation, we found that the relative expression of *FS* is remarkably higher than *Lim*.

Bettaieb *et al*. (2011) examined the effect of water deficit on essential oil classes and revealed that the proportion of the major class, terpenic hydrocarbons, was significantly increased under moderate stress by 10.04%. They proved that it was mainly mediated by the increase of γ-terpinene percentage (Bettaieb *et al.*, 2011). Stress intensity causes a change in components profile, for instance, in 30% of FC α-terpinene and α-accoradiene are produced in leaf and flower tissues of the Ardakan genotype.

A short vegetative period in cumin, about two weeks, can be linked to high cell efficiency in monoterpene and other metabolite production involved in drought stress tolerance (Ghannadnia *et al.*, 2014). A high level of terpene precursor in the young leaves can accelerate the production of monoterpene and therefore, plants can respond quickly to the applied stress. Also, this abundance of precursors is not a restricted factor

in terpene production (Fisher *et al.*, 2000). The high-level biosynthesis of monoterpene is associated with the activity of storage structures such as secretory glandular trichomes and secretory cavities that are frequently available in young tissues like small flowers that we found in our study (Gershenzon *et al.*, 2000). It was shown that preventing leaf growth leads to this increase, however, the number of terpenoids in the same biomass did not change (Gershenzon, 1984). Change in metabolite volume under stress conditions is dependent on the species. For example, the production of terpenoids increases in some medicinal plants such as peppermint, sunflower, daisies, and cumin under stress, whereas, the metabolites decrease in the trees, particularly in softwoods tree during stress (Gershenzon, 1984). In another experiment, the manganese treatments at both vegetative and blooming stages cause to increase the monoterpene concentrations of the flowers more than treatment at the blooming stage alone (Ghannadnia *et al.*, 2014). According to the study conducted by El-Sawi and Mohamad (2002), 21 compounds were detected in the stem, leaf, and seed of cumin altogether that sesquiterpenes were just found in the stem and leaf in the low amount. They also reported that cumin seeds contain β-Acoradiene and β-Pinene, while, stem and leaf showed 4-hydroxybenzoic acid and (1-methylethyl) benzoic acid is highly content (El-Sawi and Mohamed, 2002). Given Tables 3 and 4, the amount of β-Acoradiene and β-Pinene in the current study is similar to El-Sawi and Mohamad experiment. Concrete evidence suggests that in different stages of life, the production of limonene gradually declines during the development of the fruits (Bouwmeester *et al.*, 1998). It seems that low detection of limonene in this study could explain by the afro-mentioned phenomenon.

In conclusion, our study illustrated a large production of mono and sesquiterpenes, which is consisted of a large part of the essential oils in cumin. These terpenoids can be used as a natural anti-bacterial and pesticides. Moreover, we realized that the amount and type of these compounds are influenced by tissue, sampling time, genotype, and environmental conditions. Given to GC-MS results and applied stress, we conclude that some precious secondary metabolites were increased during stress. However, the production of these compounds is handled by plant and depends on substrates and products. Drought stress can accelerate the alteration of compounds to each other and also accumulate some of them. On top of that, the expression of genes encoding Limonene synthase and Flavone synthase differently increased in flowers and leaves of two ecotypes, respectively, under drought conditions. In the pharmaceutical industry, it is important to know in which step there is a high amount of interesting products and how it is possible to induce as much as accumulation by environmental stimuli.

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Conflicts of interest

The authors have no competing interests.

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