

Biostimulants can alter the biosynthesis of secondary metabolites (or natural products) and expression of related genes in lemon balm (*Melissa officinalis*)

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Abstract

To evaluate the agronomic, physiologic, and biochemical responses of the medicinal plant Lemon balm (*Melissa officinalis*), an experiment was conducted with repeated measurements under greenhouse conditions. The treatments were four concentrations of smoke-water (SW) as a new family of plant growth regulators (including 1:5000, 1:1000, 1:500, and 1:100 (v/v)) accompanied by cytokinin (BAP), auxin (IAA), and gibberellic acid (GA3) (each of them with concentration 50 μ M) and control in two harvest stages with three replications. This work aimed to study the yield and compositions of essential oils in Lemon balm under foliar application of the above treatments. Moreover, in this regard, the expression pattern of important genes related to essential oil compounds' biosynthesis pathways was studied. The gene expression was monitored by microarray technology and was carried out by Zagros Bioidea Company. Microarrays were designed by Imaxio, which has been accredited by Agilent Technologies. The SW at concentration 1:100 (v/v) recorded a significantly greater yield of essential oils compared to the control. The results of GC-Mass and gene expression showed that GA3 and SW at concentration 1:100 (v/v) caused to up-regulate the expression rate of geraniol dehydrogenase and E-citral synthase that is increasing the amount of E-citral (26.53%) in lemon balm essence. However, overexpression of Z-citral synthase was not significant. The Z-citral level increased under all foliar treatments, especially cytokinin (28.34%). These findings suggest the possible use of SW for promoting the essential oils yield and accumulation of secondary metabolites in lemon balm.

1 Introduction

Previous studies on the use of smoke as an environmental-friendly organic matter have reported improved growth and quality of various crops and medicinal plants (Van Staden *et al.*, 2006; Sparg *et al.*, 2006). Bioactive compounds in plant-derived smoke (e.g., Karrikinolide or 3-methyl-2H-furo[2,3-c] pyran-2-one and other Karrikins) are involved in some physiological influence on the endogenous pool (quantity and quality) of phytohormones. Karrikins are considered a new family of plant growth regulators (Chiwocha *et al.*, 2009; Dixon *et al.*, 2009). Also, it has been shown that bioactive smoke compounds interact with other phytohormones (endogenous and exogenous pools) (Chiwocha *et al.*, 2009; Aremu *et al.*, 2016). Jain *et al.* (2008) reported that smoke-water (SW) and karrikinolide exhibited cytokinin- and auxin-like activities in the *Glycine max* and *Vigna radiata*, respectively. Singh *et al.* (2014) showed that these compounds had gibberellic acid-like activity in *Phaseolus vulgaris*.

Lemon balm (*Melissa officinalis* L.) is a medicinal plant belonging to the Lamiaceae family with various benefits for human health through a superlative pool of bioactive compounds (Abdel-Naime *et al.*, 2019). Among the medicinal effects of lemon balm can be mentioned as antimicrobial, antioxidant, anti-inflammatory, spasmolytic, sedative, antitumor, anti-hyperlipidemic, anti-hypertensive, hepatoprotective, and anti-Alzheimer's (Shakeri *et al.*, 2016).

The major components of lemon balm essential oil are mainly derived from three biosynthesis pathways, the mevalonate pathway, leading to sesquiterpenes (such as germacrene D, β -caryophyllen and β -caryophyllene oxide), the methyl-erithrytol-pathway leading to mono and diterpenes (such as citronellal, (Z)-citral, (E)-citral), and the shikimic acid pathway leading to phenylpropenes (such as eugenol, methyleugenol, chavicol, methyleylchavicol). The citronellal, (Z)-citral, (E)-citral, cause the lemon-like scent of lemon balm (Kittler *et al.*,

2018). These compounds are synthesized and stored in peltate glands found on the surface of leaves, stems, and flowers (Iijima *et al.*, 2004).

All biosynthetic pathways are regulated by several groups of enzymatic reactions through metabolic channels. Enzyme sets are, in turn, controlled by the expression of genes. In eukaryotes, transcriptional regulators control gene expression, and activated genes encode enzymes (Hall *et al.*, 2004). Phytohormones and phytohormone-like play roles in the stimulation and production of volatile compounds as well as other compounds in medicinal and aromatic plants (Ghasemi Pirbalouti *et al.*, 2019). It seems that biostimulants cause a change in the essential oil composition and expression rate of the genes involved in their biosynthesis.

None of the studies have been carried out to investigate the effects of foliar application of SW and growth phytohormones on the content of essential oils, chemical components profile of essential oil, and key-genes expression rate of medicinal plants. Therefore, this research aimed to evaluate and compare the quality and quantity of lemon balm's essential oil and key-genes expression levels under application of SW as a phytohormone-like accompanied with cytokinin, auxin and gibberellic acid.

2 Materials and methods

2.1 Plant materials and treatments

The experiment was conducted on the lemon balm and its seeds purchased from "The Iranian Biological Resource Center Seed Bank". The experiment was set up in May 2017 in the Research Greenhouse of the Agricultural and Natural Resources Campus, Razi University. The plant bed was filled with a soil mixture (1 sand: 1: garden soil: 1 manure, by volume), and seeds were sown at 1 cm depth. Plants were irrigated until they reached field capacity. Foliar treatment consisted of spraying four concentrations (v/v) of SW (1:5000, 1:1000, 1:500, and 1:100) accompanied by cytokinin (6-benzylaminopurine), gibberellic acid (GA3), and auxin (Indole-3-acetic). Each of the treatments was sprayed with a concentration of 50 µM, and the control in two harvest stages with three replications. Plant growth regulators were obtained from Sigma Chemical Company, and to get the proper concentration of them, they were first dissolved in their own solvent. Before spraying, one drop of Tween®20 was added as a surfactant to each 250 mL of all treatment solutions, including control (distilled water). Three weeks after establishing plants, treated by foliar application (by handheld sprayer) for four weeks and each week for two consecutive days. The plants were harvested three days after the last foliar-application treatments (as harvest 1). Then allowed to grow for two weeks, and they were treated as before (as harvest 2).

2.2 Plant-derived smoke application

The SW produced by burned *Papaver boeas* in a system according to the modified method of Van Staden *et al.* (2004). The primary stock was prepared by bubbling plant-derived smoke in distilled water until it was completely saturated. The modified method in the previous system was to I: improve the prevention of wasted plant-derived smoke, II: increase the time of smoke passed throughout the water, III: more reduction smokes' temperature to its dissolution in water, IV: increase the contact level of smokes' particles with water,

and in general improvement the production accuracy and prepared a standard base solution. For foliar application treatment, the extracts were filtered through Whatman No. 1 filter paper before use. Then the base solution diluted with distilled water to obtain concentrations (1:5000, 1:1000, 1:500 and 1:100 (v/v)).

2.3 Essential oil extraction

Fifty grams of shadow-dried plant materials and 500 mL of distilled water have been placed in a Clevenger-type apparatus. The essential oil was extracted by hydrodistillation for three hours. The essential oil is stored at 4 °C before use. The essential oil yield was calculated according to the biological yield of plants.

2.4 Identification of essential oil components

Essential oil components were analyzed using a Gas Chromatography (GC) Agilent 6890, equipped with a BPX5 column (30 m×0.25 mm i.d., film thickness 0.25 µm). The oven temperature was raised from 50 °C to 300 °C at a rate of 15 °C/min. Injector temperatures were kept at 250 °C. Helium was used as the carrier gas at the constant flow of 0.5 ml/min. The split ratio was 1/35. GC-Mass Spectrometry (MS) Agilent 5973 is equipped with the same column as mentioned for GC. With ionization voltage 70 eV and EI ionization method and transfer line temperature was 220 °C. Quantitative data were obtained from Chemstation software. Identification of individual compounds was made, and this mass spectrum 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).

2.5 Study of gene expression

Autoclave apparatus and DEPS were used to extract RNA during the following steps. For this purpose, 100 mg of sample tissue was poured into a mortar and powdered with liquid nitrogen. Transfer the prepared powder to a 2 ml microtube and immediately (before freezing the powder) add one ml of extraction buffer (RNx-plus) (gently stirring the microtube for 5 to 10 seconds to buffer thoroughly mixed with powder).

The samples were incubated at room temperature for 5 min. Then 200 µl chloroform was added to the mixture and shaken slightly for microtubes to mix thoroughly. The microtubes were placed on crushed ice for 5 minutes. The microtubes were centrifuged at 12,000 rpm and 4 °C for 15 min. After the microtube was removed from the centrifuge, the supernatant was separated and transferred to a new 1.5 ml microtube and 1 ml of isopropanol was added. Shake the microtube slightly until the ingredients are mixed and finally placed in a freezer at -20 °C for 20 minutes. The samples were centrifuged at 12,000 rpm and 4 °C for 15 min. The supernatant was poured, and 1 ml of 75% ethanol was added to the precipitate. The microtube was slightly vortexed to dissolve the sediment formed at the bottom of the microtube in ethanol. The samples were centrifuged at 7500 rpm and 4 °C for 8 minutes. Pour the supernatant and place it slightly at ambient temperature to dry slightly. Fifty microliters of sterile distilled water treated by DEPS were added to the sample. The microtube was incubated at 60 °C for 5 min to dissolve the precipitate formed in the water. RNA was ready for use at this stage. After this step, the sample was mixed with the buffering material and transferred to electrophoretic wells, and the electricity was passed through the resulting bands were photographed with a gel docking device. To construct single-stranded cDNA, the Vivantis two-step kit from

Sina Clone was used according to the following steps: 10,000 ng of the RNA sample was poured into the tube. One microliter of oligo dT primer was added to the RNA sample. Then one microliter of dNTP was added to the previous volume. The volume of the solution was deionized with deionized distilled water and nuclease-free to 11 µl.

The solution was heated at 65 °C for 5 minutes, then placed on crushed ice for 2 minutes. To synthesize cDNA, 2 µL of M-Mulv buffer, 0.5 µL of M-Mulv R-transcriptase, and 16.5 µL of Nuclease-free Water were added to the sample and incubated at 42 °C for 60 min. It was then incubated at 85 °C for 5 minutes to stop the reaction. The resulting product was a single-stranded cDNA that can be directly used for the PCR reaction.

To study gene expression, the leaf samples were first treated with liquid nitrogen and then sent to the Zagros Bioidea Company to study the effect of plant growth regulators on gene expression. Gene expression was performed by microarray technology. The design of the microarrays was done by Imaxio, which is accelerated through Agilent technologies. These technologies have been approved for microarrays. For this purpose, 60 nucleotide probes were used to evaluate gene expression. All statistical cases, such as data distribution, were followed for statistical analysis of gene expression. The normal distribution of the medians allowed for statistical analysis.

2.6 Statistical analysis

Three replicates of two growth stage samples were used for statistical analysis based on repeated measurements. Data were subjected to analysis of variance (ANOVA), and then means were compared by LSD in the SAS program ver. 9.4.

3 Results and discussion

3.1 Content and yield of essential oils

The results showed that the effect of foliar-application treatments and harvests on the content and yield of essential oil was significant ($P \leq 0.01$) (Table 1). Plants treated with SW at 1000-dilution had the highest content of essential oils (0.29 %) compared to the control treatment (0.20 %). The highest essential oil yield was obtained in SW at 500-dilution (1.18 mL m⁻²) and the lowest amount of mentioned trait (0.56 mL m⁻²) was obtained in control. However, there is not a significant difference in most treatments. The highest essential oil content (0.28 %) was obtained in harvest one, and the lowest (0.23 %) amount of the mentioned trait was obtained in harvest 2. Whereas the second harvest showed a 120.98 percent increase in the essential oil yield compared to the first harvest (Table 2).

All levels of foliar application increased the essential oil content. Plants treated with SW at 1000- and 500-dilutions, as well as cytokinin, showed no significant difference together. These observations indicate the similarity of the mechanism of action of bioactive smoke compounds with the cytokinin. The decrease in the essential oil in the second harvest compared to the first harvest was probably due to an increase in the stem/leaf weight ratio in this harvest (data not shown). It could be due to the number of secretory trichoma in the lemon balm's leaves being much more than its stems. Phytohormones and SW stimulate the

accumulation of primary and secondary metabolites by stimulating cell division and thereby increasing the number of biosynthetic units (Aremu *et al.*, 2012; Verma and Sen, 2008).

Application of SW at 1000-, 500-, 100-dilutions, as well as cytokinin and gibberellic acid, resulted in higher essential oil yield than other treatments. Biosynthesis of terpenoids and phenylpropanoids is restricted to glandular trichomes. Whenever the number of trichomes increases under the application of biostimulants, especially cytokinin and cytokinin-like, the accumulation of these volatile metabolites also increases (An *et al.*, 2011; Verma and Sen, 2008). Increasing the amount of biomass in the second harvest (data not shown) increased the essential oil yield compared to the first harvest.

3.2 Essential oils composition

The GC-MS analysis of the essential oils revealed 20 compounds, representing 93.73% of the total volatiles in most samples. The average contents of the control sample are shown in Table 3. E-citral (37.28 %), Z-citral (27.81 %), methyl chavicol (11.26 %), linalool (3.89 %), geranyl acetate (3.58 %), methyl hepten (2.55 %) and caryophyllene oxide (2.24 %) were the predominant compounds (Table 3). The application of biostimulants, in addition to the amount of essential oil, also affected its chemical composition.

The results of GC-MS showed that the volatile profile was influenced by foliar-application levels (Table 4). The present study found except for the Application of SW at 5000 dilutions, Methyl hepten increased under all levels of foliar application compared to the control treatment. All levels of treatments caused to decrease in the content of methyl chavicol and linalool, compared to the control treatment. Whereas all levels of treatments except for SW 1:5000 (v/v) caused a considerable increase in the content of two monoterpene isomers: E-citral and Z-citral, compared to the control treatment. All levels of treatments except for SW 1:500 and 1:1000 (v/v) caused to increase in the content of geranyl acetate, compared to the control treatment. Increase in the amount of the compound by application of SW at 100-dilution more than the other treatments. The amount of caryophyllene oxide increased under all levels of foliar application except for cytokinin.

In this study, the basil chemotype was a geranial/neral cluster (Sharopov *et al.*, 2013). The increase in the number of citrate isomers under the application of growth phytohormone in our study was consistent with the results of other researchers. According to Silva *et al.* (2005), the culture medium supplemented with auxin and cytokinin (IAA 11.42 $\mu\text{mol L}^{-1}$; BA 8.87 $\mu\text{mol L}^{-1}$ and IAA+BA) caused to increase of 1.4 fold on nerol (Z-citral) and 4.1 fold on geraniol (E-citral). Because the phytohormones added to the culture medium could have inhibited reactions of reduction from alcohol to aldehydes (Silva *et al.* 2005).

3.3 Study of gene expression

The foliar-application growth biostimulants had a significant ($P \leq 0.01$) effect on gene expression. The abundance of gene expression variations compared to the control sample is shown in Table 5. In summary, it can be stated that except for SW 1:5000 (v/v), all levels of foliar-applications were increased significantly ($P \leq 0.01$) level of the geranial dehydrogenase and E-Citral synthase genes expression compared to control. The expression of geranyl acetate (geraniol acetate synthase) gene under all treatments increased, but the increase was not statistically significant. Applying SW at 100 dilutions caused to increase in gene expression

of 1.828 fold on geranial dehydrogenase and 1.871 fold on E-Citral synthase. These observations were very similar to those obtained from the application of cytokinin. Levels of foliar application decreased coding gene expression of biosynthetic enzymes, including linalool synthase, chavicol O-methyltransferase/estragole synthase, methyleugenol synthase, trans-alpha-bergamotene synthase, epicubenol synthase, and α -bulnesene synthase.

Metabolism and gene regulation are two fundamental biological processes, and there is a strong link between them. It has been observed that control of gene expression during cell development allows a cell to respond to signals and environmental changes (Van der Knaap and Verrijzer, 2016). Several studies supported the evolution of secondary metabolism by the recruitment of enzymes and pathways from primary metabolism (Carrington *et al.*, 2018). Therefore, one of the factors presumably necessary for the large diversity of secondary metabolism is its high level of catalytic systems. Specialized transcription factors are activated in response to changes in metabolism and elicit the appropriate changes in gene expression (Van der Knaap and Verrijzer, 2016). In this experiment, cell metabolism was strongly influenced by the different levels of foliar application. It means that metabolites and metabolic enzymes regulate each other by a double duty. On the other hand, their shared effect can modulate gene expression.

In this study, the results of RNA microarray technology were in complete agreement with the results obtained from GC-MS. In this study, the content of citrate isomers in essential oil was significantly increased, which could be due to various reasons. The significant increases in the content of germinal and neural monoterpenes indicate that the biostimulants increased the methylerythritol 4-phosphate pathway in the cells. The increased activity of biosynthetic pathways is dependent on the expression of genes encoding key enzymes in the pathway. The overexpression of three key enzymes, including the geranial dehydrogenase, E-Citral synthase, and geranyl acetate (geraniol acetate synthase), caused an increase in E-citral and Z-citral in essential oils compared to the control treatment.

Geranyl dehydrogenase catalyzes the reaction of dehydrogenation of geraniol and then converts it to geraniol. Up-regulation of gene expression of geraniol synthase that catalyzes the conversion of geranyl diphosphate to geraniol (a substrate for the germinal dehydrogenase) was not significantly affected by foliar application levels. A significant part of Z-citral biosynthesis has been through the transformation of its trans-geometric form due to an increase in the expression of genes encoding isomerase enzymes. On the other hand, the application of growth phytohormones and phytohormones-like compounds increased cell division (data not shown) and consequently increased the number of plastids (monoterpene synthesis sites). The synthesis pathways of methylerythritol 4-phosphate occur in plastids. So this may be another reason for the increase in essential oil content and citrate content.

The results showed a down-regulation in the expression of the linalool synthase gene and the content of linalool in essential oils. Linalool synthase catalyzes the conversion of geranyl diphosphate to linalool in the isoprenoid pathway. A similar trend was also observed for chavicol O-methyltransferase (estragole synthase) gene expression and content of phenylpropanoid methyl chavicol (methylheptenone) in essential oils. All levels of foliar application up-regulated the gene expression of sulcatone/methylheptenone synthase, and enhanced the content of methylheptenone (sulcatone) in essential oils. The positive correlation between

levels of gene expression and metabolite profiling in this study was consistent with many other studies (Kahrizi *et al.*, 2017; Ghaheiri *et al.*, 2019; Esmaeili *et al.*, 2018; Iijima *et al.*, 2004; Young *et al.*, 2012).

4 Conclusion

Several studies show that smoke extracts exhibit hormone-like responses and interact with phytohormones. It can be involved in some physiological aspects of the life-cycle of plants. The present investigation revealed that SW concentrations and growth phytohormones can qualitatively and quantitatively influence the volatile composition of lemon balm. Foliar application of SW, especially in 500- and 100-dilution similar to synthetic phytohormones, caused to increase citral content as a significant and commercial constituent of essential oils (more than 60 percent). Citral is a racemic mixture of two isomers (geranial and neral) responsible for the lemon-like olfactory character. The application of biostimulants increased the expression rate of genes encoding key enzymes in the biosynthetic pathway of this compound compared to the control. The use of smoke technology can compete with chemical growth phytohormones as well as improve the quantity and quality of essential oil in the medicinal plant lemon balm.

Abbreviations

SW
Smoke-water
IAA
Indole-3-acetic
BAP
6-benzylaminopurine
GA3
Gibberellic acid

Declarations

Acknowledgment

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Author contribution

Saeid Jalali Honarmand supervised the experiment. Faride Noroozi Shahri conducted the experiments, collected and evaluated the data. Mohsen Saeidi, Farzad Mondani, Danial Kahrizi served as the study's research consultant.

Data availability

We declare that the submitted manuscript has not been published anywhere before and is not currently under publication elsewhere.

Conflict of interests

The authors explicitly state that they have no conflict of interest.

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Tables

Table 1
Analysis of variance Essential oils content and yield in lemon balm

Source of variation	d.f	Mean of Squares	
		Essential oils content	Essential oils yield
Block (R)	2	0.003 ^{ns}	0.90 ^{**}
Foliar-application levels (A)	7	0.006 [*]	0.29 ^{**}
Error A	14	0.001	0.02
Harvests levels (B)	1	0.03 ^{**}	6.64 ^{**}
A*B	7	0.0006 ^{ns}	0.10 ^{ns}
R*B	2	0.01 ^{**}	1.28 ^{**}
Error B	14	0.0006	0.02
CV		9.81	15.48
Where CV (coefficient of variation), df (degree of freedom), ns (non-significant differences),			
*and** are significant at 1% and 5%, respectively.			

Table 2

Effect of foliar-application treats and harvests on essential oils yield of lemon balm

Treatments	Treatment levels	Essential oils content (%)	Essential oils yield (mL m ⁻²)
Foliar-application	Ctrl	0.20	0.56
	SW 1:5000	0.22	0.85
	SW 1:1000	0.29	1.09
	SW 1:500	0.28	1.18
	SW 1:100	0.26	1.16
	BAP	0.28	1.17
	GA3	0.27	1.10
	IAA	0.25	0.84
	LSD 0.05	0.04	0.21
Harvests	Harvest 1	0.28	0.62
	Harvest 2	0.23	1.37
	LSD 0.05	0.01	0.09

Table 3

Essential oil components (average percentage of volatile content) found in the control sample of lemon balm plants

No	Components	%	Type	Kovat's retention Indices	Retention Index
1	1-Octen-3-ol	0.29	Other	989	14.41
2	6-Methyl-5-Hepten-2-ONE	2.55	Other	994	14.67
3	Linalool	3.89	Oxygenated Monoterpenes	1108	20.60
4	Citronellal	0.94	Oxygenated Monoterpenes	1162	23.26
5	Rosefuran epoxide	0.23	Oxygenated Monoterpenes	1180	24.15
6	Estragole	11.26	Other	1212	25.74
7	Z-Citral	27.81	Oxygenated Monoterpenes	1252	27.63
8	Geraniol	0.23	Oxygenated Monoterpenes	1261	28.06
9	E-Citral	37.28	Oxygenated Monoterpenes	1282	29.06
10	Trans-Granic acid methyl ester	0.35	Oxygenated Monoterpenes	1331	31.26
11	Geranyl acetate	3.58	Oxygenated Monoterpenes	1386	33.72
12	β -Elemene	0.38	Sesquiterpene Hydrocarbones	1396	34.17
13	(4 α , 7 β , 7 α)-Nepetalactone	0.29	Other	1413	34.88
14	Methyleugenol	1.01	Oxygenated Monoterpenes	1417	35.04
15	Caryophyllene	1.38	Sesquiterpene Hydrocarbones	1429	35.54
16	Trance- α -Bergamotene	1.29	Sesquiterpene Hydrocarbones	1439	35.97
17	α -Bulnesene	0.20	Sesquiterpene Hydrocarbones	1511	38.97
18	γ -Cadinene	0.80	Sesquiterpene Hydrocarbones	1525	39.51

No	Components	%	Type	Kovat's retention Indices	Retention Index
19	Caryophyllen oxide	2.24	Oxygenated Sesquiterpenes	1598	42.41
20	Epicubenol	1.73	Oxygenated Sesquiterpenes	1660	44.73
	Total Identified	97.73			

Table 4

Essential oil components (average percentage of volatile content) found in samples in lemon balm plants at foliar-application treatments

No	R.I.	Components	Compounds percent/ Foliar levels							
			Ctrl	1:5000	1:1000	1:500	1:100	CK	Ga	IAA
1	14.41	1-Octen-3-ol	0.29	0.29	0.42	0.38	0.35	0.36	0.36	0.37
2	14.67	6-Methyl-5-Hepten-2-ONE	2.45	2.22	3.05	3.82	3.48	2.67	2.98	2.85
3	20.60	Linalool	3.66	1.28	0.66	0.42	0.44	0.38	0.37	0.39
4	23.26	Citronellal	0.86	0.62	0.86	0.68	0.79	0.81	0.97	0.87
5	24.15	Rosefuran epoxide	0.36	0.41	0.42	0.34	0.32	0.28	0.3	0.34
6	25.74	Estragole	9.07	2.98	0.95	0.31	0.21	0.18	0.11	0.13
7	27.63	Z-Citral	26.42	25.21	31.96	32.9	32.93	33.91	33.07	32.92
8	28.06	Geraniol	0.13	0.12	0.45	0.43	0.54	0.62	0.49	0.58
9	29.06	E-Citral	36.33	35.85	43.64	44.5	45.97	45.1	45.02	44.15
10	31.26	Trans-Granic acid methyl ester	0.24	0.34	0.13	0.17	0.18	0.65	0.51	0.15
11	33.72	Geranyl acetate	2.83	3.04	2.78	2.82	3.17	2.9	3.05	3.05
12	34.17	β -Elemene	0.74	8.61	2.75	0.94	0.25	0.13	0.12	-
13	34.88	(4aa, 7 β , 7aa)-Nepetalactone	-	-	-	-	-	-	-	-
14	35.04	Methyleugenol	0.82	0.46	0.16	-	-	-	-	-
15	35.54	Caryophyllene	1.41	0.9	2.12	2.09	1.66	3.65	4.62	3.93
16	35.97	Trance- α -Bergamotene	1.48	0.45	0.2	-	-	-	0.27	-
17	38.97	α -Bulnesene	0.31	0.31	0.17	0.11	-	-	-	-
18	39.51	γ -Cadinene	0.81	0.34	-	-	-	-	-	-
19	42.41	Caryophyllen oxide	2.17	2.96	2.41	2.46	2.43	2.03	2.43	3.51
20	44.73	Epicubenol	1.49	0.54	0.28	0.1	0.13	0.13	0.11	0.15

Table 5

Effect of foliar-application treatments on the gene expression related to some key biosynthesis enzymes of essential oil compositions compared to control in lemon balm (The numbers represent a manifold expression than the control. A negative number indicates a decrease in expression, and a positive number indicates an increase in gene expression (compared to the control)).

Gene_description	Gene expression/ Foliar levels						
	1:5000	1:1000	1:500	1:100	CK	Ga	IAA
Sulcatone/Methylheptenone synthase	1.019	1.016	1.140	1.073	1.001	1.004	1.003
P.value	0.636	0.673	0.002	0.004	0.598	0.765	0.845
linalool synthase	-1.404	-1.353	-1.296	-1.326	-1.305	-1.409	-1.451
P.value	0.003	0.006	0.005	0.007	0.003	0.009	0.006
Iridoid/Xcitronellal Synthase	-1.155	1.177	-1.163	-1.168	-1.214	-1.177	-1.186
P.value	0.384	0.384	0.384	0.384	0.280	0.753	0.753
chavicol O-methyltransferase/estragole synthase	-2.820	-2.778	-2.189	-2.185	-1.988	-2.684	-3.072
P.value	0.002	0.006	0.003	0.008	0.008	0.001	0.005
geranial dehydrogenase	-3.629	1.724	1.725	1.828	1.824	1.819	1.818
P.value	0.037	0.008	0.000	0.004	0.003	0.005	0.003
trans-3,7-dimethylocta-2,6-dienal/E-Citral synthase	-1.818	1.818	1.822	1.871	1.871	1.867	1.863
P.value	0.004	0.007	0.009	0.001	0.006	0.009	0.006
geraniol synthase	-1.400	-1.399	-1.399	-1.456	-1.456	-1.457	-1.457
P.value	0.472	0.563	0.676	0.466	0.440	0.563	0.425
eugenol/isoegenol/methyleugenol synthase	-2.256	-6.171	-6.070	-2.187	-1.812	-1.806	-1.501
P.value	0.002	0.007	0.011	0.007	0.046	0.009	0.003
Geranyl acetat/Geraniol acetate synthase	1.483	1.259	1.260	1.117	1.117	1.149	1.283
P.value	0.462	0.618	0.645	0.818	0.837	0.760	0.591
Z-Citral synthase	1.481	1.482	1.482	1.482	1.482	1.483	1.485
P.value	0.411	0.412	0.460	0.449	0.436	0.462	0.425

Gene_description	Gene expression/ Foliar levels						
	1:5000	1:1000	1:500	1:100	CK	Ga	IAA
Caryophyllene/beta-elemene synthase	1.867	-1.587	-1.699	-1.697	1.575	1.378	-1.377
P.value	0.001	0.006	0.002	0.005	0.007	0.005	0.006
trans-alpha-bergamotene synthase	-1.609	-1.608	-1.608	-1.470	-1.469	-1.468	-1.324
P.value	0.007	0.040	0.001	0.005	0.004	0.003	0.005
epicubenol synthase	-1.942	-1.687	-1.595	-1.312	-1.430	-1.331	-1.249
P.value	0.001	0.009	0.007	0.003	0.007	0.006	0.004
rose furan epoxide synthase	1.177	1.273	-1.17	-1.179	-1.211	-1.191	-1.198
P.value	0.002	0.001	0.721	0.720	0.704	0.704	0.704
α -bulnesene synthase	-1.245	-1.244	-1.200	-1.162	-1.127	-1.127	-1.097
P.value	0.000	0.004	0.020	0.003	0.001	0.001	0.006