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Effects of growth stage on essential oils and gene expression of terpene synthases in Mentha aquatica L.

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Abstract

In this work, the impact of different stages of plant growth on the composition of essential oils, the density of glandular trichomes, and gene expression of enzymes involved in terpenes production in *Mentha aquatica* were investigated. Our experiment was performed on 15-day-old plants (the initial stage of vegetative growth) and 45-day-old plants (the late stage of vegetative growth). Based on our investigation, the leaves of the *M. aquatica* plant were covered by non-glandular and glandular trichomes in the two growth stages. The density of glandular trichomes as the location of storage and biosynthesis of essential oils was higher in the late growth stage than in the early growth stage. The maximum harvest of essential oils was achieved at the late vegetative stage, representing that the generation of essential oils was boosted as the age of plants increased. Moreover, the growth stage influenced the essential oils composition in the *M. aquatica* plant. The main compounds of essential oils from *M. aquatica* plants in the early growth stage were menthofuran, limonene, germacrene D, pinene, viridiflorol, 1-terpinene, ledene, cymene, 3-carvon, terpinene, and cis-ocimene. The top compounds exit in the essential oils obtained from *M. aquatica* plants in the late vegetative stage were as follows: caryophyllene, cubebene, camphene, gurjunene, humulene, bicyclogermacrene, sabinene, 1-pinene, D-nerolidol, farnesene, 1,8-cineole, and cadinene. The ratio of sesquiterpenes to monoterpenes was enhanced as the plants developed. The expression level of the gene encoding enzymes that contributed to terpenoid production includes 1-Deoxy d-xylulose-5-phosphate synthase, geranyl diphosphate synthase, limonene synthase, isopentenyl diphosphate isomerase, and menthofuran synthase, which were also enhanced in the late growth stage. Gene expression studies supported our findings and demonstrated that the increased production of essential oils might be due to the stimulation of enzyme activity, contributing to their biosynthesis pathway. Overall, to obtain the maximum amount of essential oils, the late vegetative stage of *M. aquatic* is recommended.

1. 1. Introduction

Plants produce wonderful compounds called secondary metabolites. The generation of secondary compounds by plants has increased the commercial importance and value of plants. Due to their significant biological activities, these compounds have been applied in the food, pharmaceutical, cosmetic, and traditional medicine industries (Figueiredo et al., 2008; Picazo-Aragonés et al., 2020; Yang et al., 2018).

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Terpenes are secondary metabolites that play vital roles in plant growth and development and facilitate the interaction of plants with the environment (Jiang et al., 2019). Monoterpenes are generated mainly by the methylerythritol 4-phosphate (MEP) process in plants, which prepares terpene precursors dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Dudareva et al., 2013). DMAPPs are joined with different numbers of IPP units to manufacture farnesyl diphosphate, geranyl diphosphate, and geranylgeranyl diphosphate. (McGarvey & Croteau, 1995). Most essential oils are complex combinations of monoterpenes (C10H16) and sesquiterpenes (C15H24), and they are comprised of biogenetically related phenols (cinnamates and phenylpropanes) along with alcohols, carbohydrates, aldehydes, ethers, and ketones which are responsible for their features (Baser & Buchbauer, 2009).

Lamiaceae is one of the most important known plant families, with about 200 genera and more than 4,000 species. The family's importance is due to plants with biological and medical applications (Harley, 2004; Raja, 2012; Uritu et al., 2018). Mentha aquatica is a well-known aromatic and medicinal herb from the Lamiaceae family. This plant has biologically active compounds that are applied in traditional drugs (Tsai et al., 2013) and is prominent worldwide for its essential oils. Their essential oils are used to flavor foods in oral and dental products, fragrances, confectionary, cosmetics, and medicinal industries (Dhifi et al., 2011). Recently, M. aquatic has become a topic of scientific attention in view of other possible applications of their essential oils and extracts, for the most part, antioxidant and antimicrobial agents. The essential oils from M. aquatica mainly comprise terpenoid compounds that are usually produced, secreted, and stored by the glandular trichomes on the leaf tissues (Atsbaha Zebelo et al., 2011). M. aquatic is commonly used in traditional Iranian medicine, and characterizing its morphological and essential oil diversity is crucial for future breeding programs (Hassanpouraghdam et al., 2022).

Most secondary metabolites are produced from primary metabolites through various physiological alternations in plants. Thus, tissue and species specificity, determined by genetic aspects, influence the production and accumulation of secondary metabolites in medicinal plants. If we need to acquire better medicinal parts and the best yield time in a certain tissue, we must study the tissues and organ specificity of certain medicinal components of medicinal plants, discussing the connection between morphogenesis and production and accumulation of these components in medicinal plants. It is appropriate to select a longer growth period for the yield of woody medicinal plants and perennial herbs because these plants display stronger growth and metabolism in these growth periods, and their biomass increases with the increasing growth period, concurrent with the augmented content and yield of effective components. However, the biomass and compositions should be carefully studied for medicinal plants with certain components to determine the optimal collecting period for those specific metabolites (Li et al. 2020; Zandalinas et al. 2017).

Aromatic and Medicinal plants are seriously influenced by cultural practices such as planting dates, fertilization, irrigation, and harvest time. Many investigations have studied the impact of different factors on the quality features of the Mentha genus (Özyazıcı & Kevseroğlu, 2019; Yeşil & Özcan, 2021). Thus, the main aim of this work was to study the amount and composition of essential oil, alternations in the expression of genes that contributed to terpenes biosynthesis, and the density of glandular trichomes in M. aquatic in both early and later stages of vegetative growth, which are applied as an essential oil source in the cosmetic, pharmaceutical, and food industries. Identifying and studying these parameters can be useful in augmenting the generation of valuable compounds.

2. Material and methods

2.1. Plant material

Mentha aquatica seeds were obtained from the natural population in the city of Nowshahr in the Mazandaran province, Iran. The seeds were sanitized with 20% NaClO for 20 minutes and then washed with distilled water. In order to germinate, the seeds were kept in dark and humid conditions for three days. After germination, the seedlings were transmitted to plastic pots containing perlite to continue growing. Continued growth was performed under greenhouse conditions (16-h light/8-h dark, 25 °C, and 80% humidity). The seedlings were irrigated with Hoagland's solution every three days. In this study, the expression of candidate genes in the terpenes production and the density of glandular trichomes were analyzed to study the impact of different vegetative growth stages on the essential oil content; a time course of 45 days was considered. The comparison was performed on 15-day-old plants (initial stage of vegetative growth) and 45-day-old plants (late stage of vegetative growth). The experiment was performed completely randomly with three replications.

2.2. Glandular trichome density

The leaves were fixed with glutaraldehyde (3%) in sodium phosphate buffer (0.1 M), pH 7.2 for 4 h at 4 °C, then dehydrated by successive ethanol rising. The samples were transported onto scanning electron microscopy (SEM) stubs and covered with a layer of gold. Observations were performed at 15 kV on an SEM. Finally, the density of the glandular trichome was calculated using Image J software.

2.3. Essential oil extraction and analysis

Leaf tissue from plant samples collected from different stages of vegetative growth (early and late) was used to extract essential oils. The essential oil was extracted using the Clevenger apparatus with distilled water for 4 hours at 100 °C. Measurement of essential oils was done by a GC–MS system.

2.4. RNA extraction and gene expression

Whole RNA from leaves was extracted using an RNX-plus kit. The purity of RNA was identified by agarose gel electrophoresis, and its concentration was evaluated with spectrophotometry. cDNA was prepared as previously stated in Zarinkamar et al. (2012). Table 1 shows the primer sequences of the housekeeping (18S and actin) and target genes used for PCR analyses.

Table 1 Primer Sequences used in the qRT-PCR Assay

Accession	Gene	Primer sequences
AW255057	*Actin* (*ACT*, 130 bp)	Forward primer 5'-GCTCCAAGGGCTGTGTTCC-3' Reverse primer 5'-TCTTTCTGTCCCATGCCAAC-3'
NR_022795	*18s* (212 bp)	Forward primer 5'-ATGATAACTCGACGGATCGC-3' Reverse primer 5'-CTTGGATGTGGTAGCCGTTT-3'
AF019383	*1-Deoxy d-xylulose- 5-phosphate Synthase* (*DXS*, 171 bp)	Forward primer 5'-CCACCAGGCTTACCCACACAA-3' Reverse primer 5'-GCCACCGCCATCCCTAAAC-3'
EU108696	*Geranyl diphosphate synthase* (*GPPS*, 173 bp)	Forward primer 5'-ATGATAAGCGGGCTGCATAG-3' Reverse primer 5'-CCGAAATTCCTCAGCTTCTG-3'
AW255524	*Isopentenyl diphosphate isomerase* (*IPPI*, 124 bp)	Forward primer 5'-CTCTTGGGGTGAGAAATGCT-3' Reverse primer 5'-CATCTGAGGGGGCTTTGTA-3'
KY249928	*β-Caryophyllene synthase* (*CPS*, 271 bp)	Forward primer 5'-AACTCATCGATGCAATCCAACG-3' Reverse primer 5'-CAAGAATTTCCTCCCCATGCAC-3'
AW255536	*Limonene synthase* (*LS*, 150 bp)	Forward primer 5'-CGGTGGTGGAGAAATACTGGGTTT-3' Reverse primer 5'-CCGTAATCAGAGCGTGACTTTGC-3'
AF346833	*Menthofuran synthase* (*MFS*, 121 bp)	Forward primer 5'-GCAGAACGAGGTGCGAGAAG-3' Reverse primer 5'-TGCGAAAGGTGGATGTAGGC-3'

RT-PCR and assessments of the gel bands were performed, as previously demonstrated by Nazari et al. (2018). After PCR, samples were separated on agarose gel and discolored with ethidium bromide. Gel pictures were taken using a Gel-Doc Transilluminator. Subsequently, densitometric evaluations of the gel bands were done using the Image J software.

2.5. Statistical analysis

Each test was repeated three times, and the data were analyzed by one-way analysis of variance (ANOVA) using SPSS. The significance was checked at $P \leq 0.05$ using Duncan's multiple range test. The MetaboAnalyst web server was utilized to obtain heatmap and relationship analyses.

3. Results and discussion

3.1. Dry shoot weight and glandular trichome density

The dry shoot weight of M. aquatica plants augmented during the growth stages is shown in Fig. 1A. Vegetative tissues result from the activity of vegetative meristems, such as root and apex meristems and vascular cambium. As the plant grows and its meristems activity increases, its volume should also increase; hence, biomass volume is expected to increase in stress-free conditions as the plant age increases, as has been shown in previous studies. Increasing the activity of vegetative meristem or prolonging its activity period can lead to more vegetative tissues and, consequently, more biomass (Demura & Ye, 2010).

Comparison of the density of glandular trichomes in both stages of vegetative growth as well as on the abaxial and adaxial surfaces showed an increase in glandular trichomes density in the late stage on both surfaces compared to the early stage (Fig.1B, C). Yadav et al. (2014) also found that the glandular trichomes density in Artemisia annua was enhanced with development and growth.

In this work, leaves of the M. aquatica plant were enclosed by glandular and non-glandular trichomes at both growth stages. The glandular trichomes were capitate and peltate types. Previous studies on Lamiaceae also reported the presence of these two types of glandular trichomes (Kahraman et al., 2010; Werker, 1993).

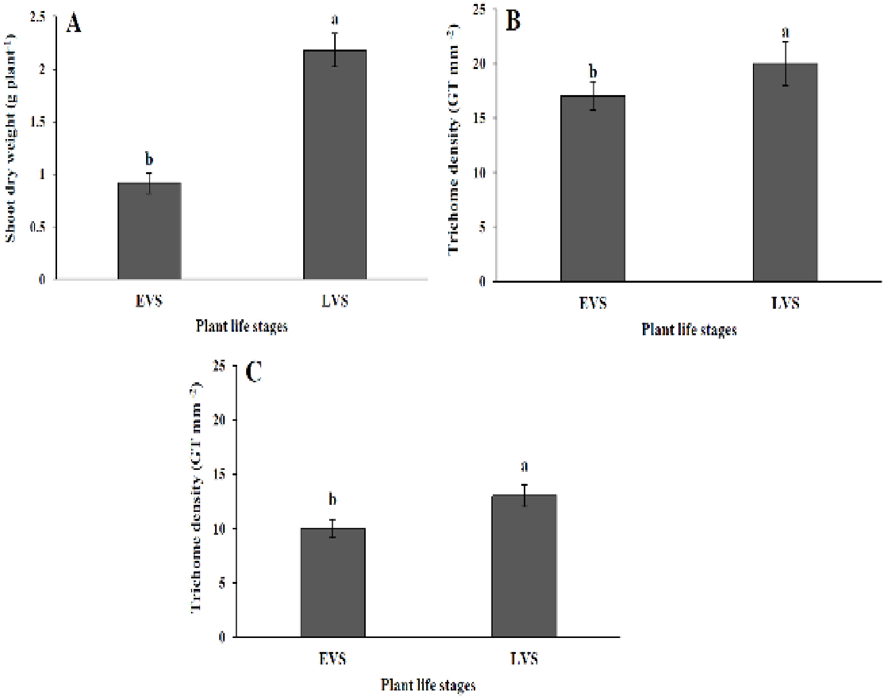

Figure 1: (A) Dry shoot weight, (B) density of glandular trichomes on abaxial, and (C) adaxial surfaces of leaves in *M. aquatica* in the early vegetative stage (EVS) and late vegetative stage (LVS). Means ± SE of three replicates are given. Different letters above columns indicated a significant difference at $P \leq 0.05$ using Duncan multiple range test. $P \leq 0.05$.

According to our study, the density of glandular trichomes, such as peltate and capitate, was higher on the abaxial surfaces. Non-glandular trichomes were highly concentrated on the upper surface of the leaf. These simple trichomes, without branching, multicellular, or uniseriate, were more concentrated on the veins and leaf margins. The density of non-glandular trichomes was higher than that of glandular trichomes, as mentioned in prior research (Ascensão et al., 1995; Talebi et al., 2018; Wagner, 1991).

3.2. The effect of growth stages on yield and essential oils composition

This work studied yield and essential oils composition to identify the change of essential oils in M. aquatica plant at different growth stages. (Fig. 2 |) shows the essential oils yield of the *M. aquatica* plant during growth. The maximum yield of essential oils was achieved at the late vegetative stage, demonstrating that the generation of essential oils was enhanced as the age of plants increased. So, the importance of harvesting time associated with essential oil features varies depending on the species of interest (Yousefzadeh et al., 2022). The enhanced production of essential oils at the late vegetative stage rather than the early vegetative stage could be due to the improved number of glandular trichomes (the essential oils' main storage and biosynthesis location). These results recommend that the late vegetative stage could be considered appropriate for extracting essential oils from the *M. aquatica* plant.

As highlighted by numerous investigators, the amount of essential oils extracted from aromatic and medicinal plants depends on the development stage of the plants (Uyanık & Gürbüz, 2015; Açıkgöz and Kara, 2020). Arabacı et al. (2015) identified that collecting aromatic and medicinal plants should not be performed at an accidental stage, and the period for maximum essential oils extraction should be identified. Thus, the collecting time should be prudently selected to provide the optimum harvest of essential oils. Prior works on plants such as *Thymus vulgaris* L. (Badi et al., 2004) and *Hyptis suaveolens* (Oliveira et al., 2005) stated that the essential oils yield augmented during plant development. These results are further supported by the low level of essential oils biosynthesis during the early vegetative stage, possibly due to inhibiting enzyme activities required to produce these metabolites (Sellami et al., 2009).

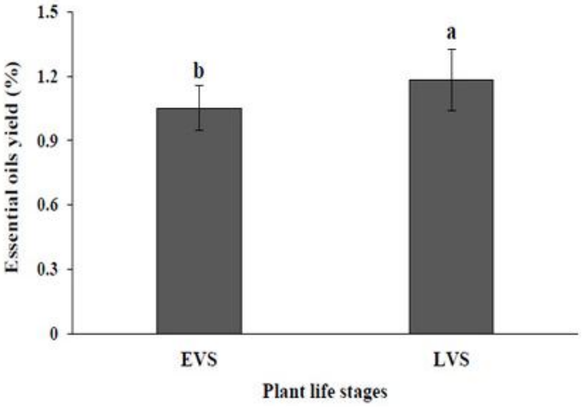

Figure 2: The amount of essential oil of the *M. aquatica* plant at different stages of growth. Means ± SE of three replicates are given. Different letters above columns indicated a significant difference at $P \leq 0.05$ using Duncan multiple range test. $P \leq 0.05$.

The essential oils from *M. aquatica* plants in different growth stages exhibited considerable variations in their composition (Fig. 3). Thus, the composition of terpene metabolites altered during vegetative growth in *M. aquatica* plants. GC-MS discovered a total of 23 chemical constituents from the essential oils in the two growth stages. The essential oils mainly included mono- and sesquiterpenes components at the two growth stages. The chief combinations of essential oils from *M. aquatica* plants at early growth stages were menthofuran, limonene, germacrene D, pinene, viridiflorol, 1-terpinene, ledene, cymene, 3-carvon, terpinene, and cis-ocimene. The top compounds existing in the essential oils gained from the *M. aquatica* plant in the late vegetative stage were as follows: caryophyllene, cubebene, camphene, gurjunene, humulene, bicyclogermacrene, sabinene, 1-pinene, D-nerolidol, farnesene, 1,8-cineole, and cadinene.

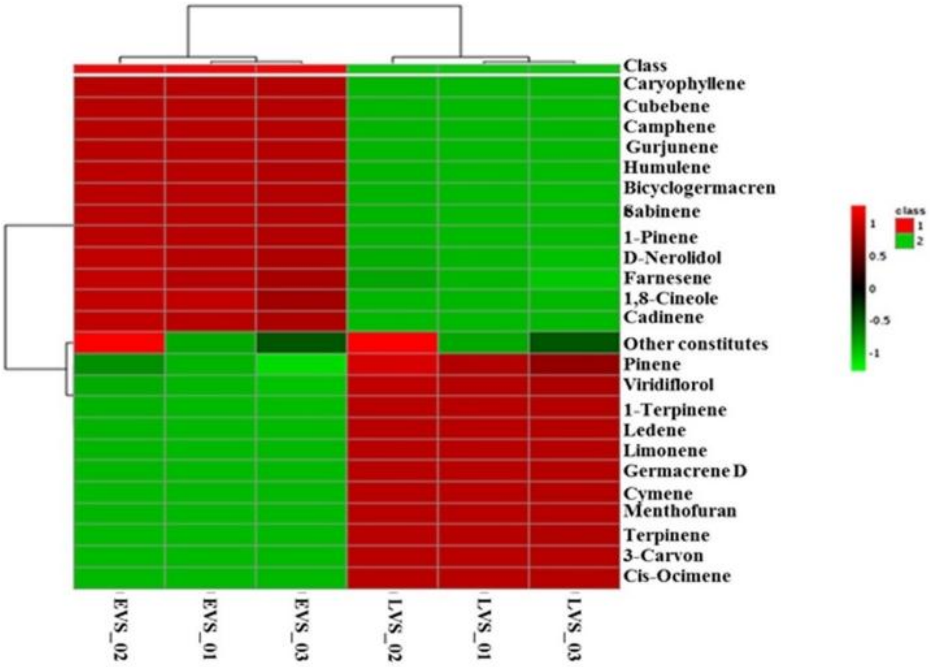

Figure 3: Heatmap according to the relative levels of essential oils analyzed by GC–MS in *M. aquatica* plant at the two growth stages.

Plants use essential oils to defend themselves against biotic and abiotic agents, herbivores, and pathogens and attract insects and pollinators. Essential oil is broadly used in the cosmetic, perfume, pharmaceutic, and food industries. As the plant approaches the end of vegetative growth and enters the reproductive phase, the amount of these compounds and their content changes. In this study, the maximum quantity of essential oil was gained in the final stage of vegetative growth (Fig. 3). Also, the essential oil composition was different in different stages of growth, which shows that the essential oil composition generated by the plant is affected by growth stages. This conclusion confirms the work of Verma et al. (2014), who found variations in essential oils composition in different months/seasons. Previous studies have investigated the impact of various factors on essential oil compounds (Farzadfar et al., 2018; Nazari et al., 2017; Yang et al., 2018). For example, differences in the composition of the essential oil gained from different organs of plants can be attributed in part to the presence of different secretory structures that are heterogeneously dispersed on the plants (Figueiredo et al., 2008). The work of Daghbouche et al. (2020) highlighted the difference in composition and content of essential oil in *Cytisus triflorus* L'Her during growth stages. They found flowering to be an interesting stage for gathering, with a more specific essential oil composition. Also, the proportions of pulegone, menthofuran, and limonene declined as the plant aged in this study, while constituents such as cineole, neomenthol, and menthol were enhanced in *Mentha piperita* according to the developmental changes (Abdi et al. 2019).

According to our results, most essential oils compounds were monoterpene and sesquiterpene in *M. aquatica* plants at early and late growth stages, respectively. The ratio of sesquiterpene to monoterpene of essential oils showed an increasing trend during the growth of *M. aquatica* plants. Among the other compounds, caryophyllene is often used as a food flavoring and has antimalarial and anti-inflammatory characteristics (Vuerich et al., 2019). Humulene also revealed anti-inflammatory features. Thus, collecting *M. aquatica* plants at the late vegetative stage is suggested to obtain the highest amount of caryophyllene and humulene.

3.3. The impact of the growth stage on the expression of genes related to terpene production

The relative expression of six candidate genes, geranyl diphosphate synthase (GPPS), (1-Deoxy d-xylulose-5-phosphate synthase (DXS), β-caryophyllene synthase (CPS), isopentenyl diphosphate isomerase (IPPI), menthofuran synthase (MFS) and limonene synthase (LS), encoding the enzymes contributing in the biosynthetic pathway of essential oils in *M. aquatica* plants was studied to discover the impacts of different growth stages on the terpenoid biosynthesis pathway.

Typically, DXS converts glyceraldehyde-3-phosphate and pyruvate to 1-deoxy-D-xylulose 5-phosphate (Querol et al. 2002). However, the *DXS* gene showed a different expression pattern in *M. aquatica* plants at different growth stages, with the growth stage strongly changing the expression of *DXS* (Fig. 4A). The expression rate of this gene in the late growth stage was more than in the early growth stage. DXS is the first and rate-limiting enzyme in the MEP process, which generates terpenoid biosynthesis precursors (Zhang et al., 2020).

In addition to increasing the expression of *DXS*, we saw an increase in glandular trichome density on both lower and upper levels of leaves in the final stage of growth compared to the early stage of growth. By examining the glandular trichome density and information obtained from *DXS* gene expression, it was determined that there is a positive correlation between the expression of the *DXS* gene and the increase in the glandular trichome density in the *M. aquatica* plant in the late growth stage. Carretero-Paulet et al. (2013) observed the role of the *DXS* gene in the production of glandular trichomes in tomato plants. In a similar study on *Artemisia annua*, the maximum expression of the *DXS* gene was reported in apical buds with higher trichome density (Lu et al., 2013).

GPP is a common precursor to all monoterpenes made by the GPPS enzyme, which is found in plastids (Ueoka et al., 2020). The expression level of the GPPS gene was significantly boosted as the plant age increased. Accordingly, the lowest expression level of GPPS was identified in M. aquatica plants in the early growth stage (Fig. 4B). GPPS, the main enzyme for monoterpene production, presents as homodimeric and heterodimeric GPPS in plants (Burke et al., 1999). Overexpression of GPPS boosts the monoterpene content in Litsea cubeba (Zhao et al., 2020).

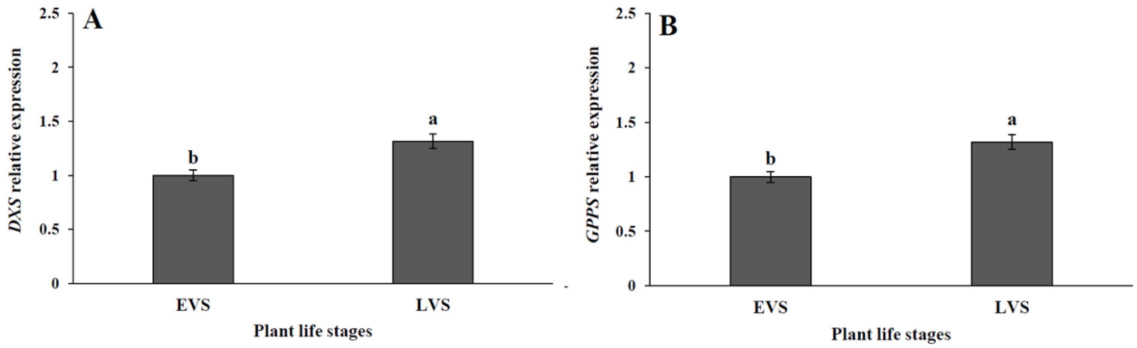

Figure 4: Expression levels of (A) *DXS* and (B) *GPPS* genes in *M. aquatica* leaves at the two stages of growth. Means ± SE of three replicates are given. Different letters above columns indicated a significant difference at $P \leq 0.05$ using Duncan multiple range test. $P \leq 0.05$.

Another gene examined in this study was LS. This enzyme catalyzes the cycling of GPP, a common precursor to monoterpenes, to limonene (Turner et al., 2000). The expression level of LS presented significant alterations associated with the growth stage in M. aquatica plants. The highest expression level of LS was recorded in the late vegetative stage, whereas the lowest expression was detected in plants in the early vegetative stage (Fig.5A).

IPPI catalyzes the isomerization between the terpene precursor substances IPP and DMAPP during terpenoid biosynthesis (Zhang et al. 2015). In this study, there was a considerable modification in the IPPI expression level between the M. aquatica plants at the two growth stages (Fig. 5B). The expression level of IPPI was more in M. aquatica plants in the late vegetative stage than in the early vegetative stage.

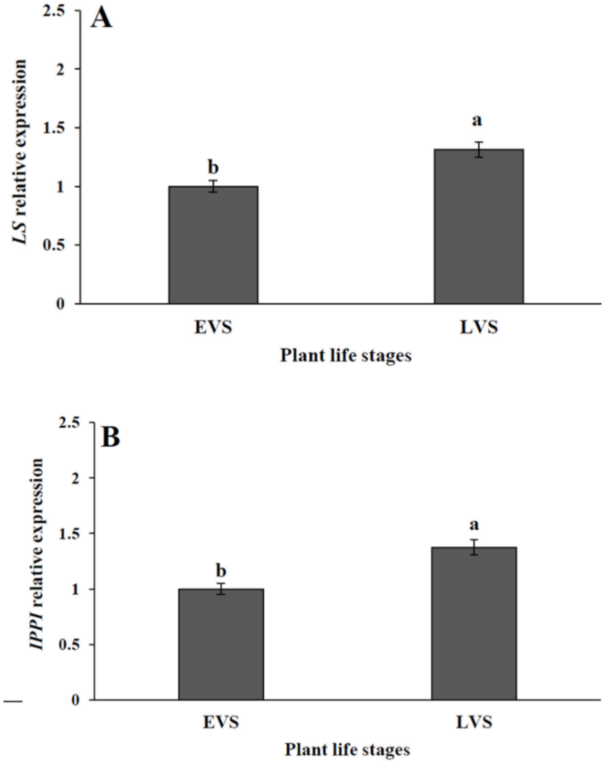

Figure 5: Relative expression of (A) *LS* and (B) *IPPI* genes in *M. aquatica* leaves at the two stages of growth. Means ± SE of three replicates are given. Different letters above columns indicated a significant difference at $P \leq 0.05$ using Duncan multiple range test. $P \leq 0.05$.

Based on the acquired data, the expression level of MFS was enhanced in M. aquatica during vegetative growth (Fig. 6A), and the maximum expression level of MFS was shown in plants in the late vegetative stage. In contrast to the other five genes, expression of CPS significantly reduced with increasing plant age, and the lowest expression of this gene was observed in the late stage of growth (Fig. 6B). CPS enzyme catalyzes the conversion of FPP to b-caryophyllene. The CPS gene is expressed in most plant tissues during early development (Cai et al., 2002).

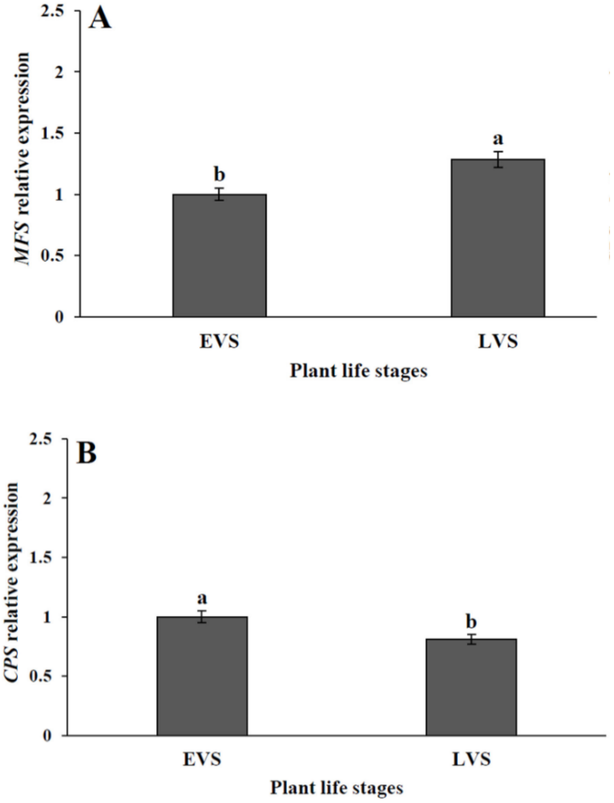

Figure 6: Relative expression of (A) *MFS* and (B) *CPS* genes in *M. aquatica* leaves at the two stages of growth. Means ± SE of three replicates are given. Different letters above columns indicated a significant difference at $P \leq 0.05$ using Duncan multiple range test. $P \leq 0.05$.

The data from our work clearly showed that the expression level of key enzyme genes contributing to terpenoid production in M. aquatic plants was enhanced in the early vegetative stage. Thus, the expression of these genes is dependent on the age of the plants. As reported in recent studies, the expression level of genes that contribute to terpenoid production can be controlled by post-transcriptional and transcriptional mechanisms during the growth period (Abbas et al., 2017). Meanwhile, the results of the analyzed enzymes-encoded genes essential in terpenoid production highlighted the point that the generation of terpenoid components is boosted by the age of plants. A previous relationship has been reported between essential oil yields and the expression level of enzyme genes contributing to their production (Atsbaha Zebelo et al., 2011), confirming our findings. According to the results, a close connection was witnessed in the essential oils amount, terpenes composition, and expression level of genes contributing to terpenoid production in M. aquatic plants during the growth period. This shows that transcript control of terpene synthase genes is the most important regulatory mechanism adjusting the composition and content of essential oils of M. aquatic. The biosynthesis of secondary metabolites depends on the development stage because the developmental details affect the differentiation and initiation of specific cellular structures contributing to the storage and biosynthesis of secondary metabolites (Broun et al., 2006). A close relationship between biosynthesis and accumulation of essential oils and leaf development has been proved in many aromatic plants of the Lamiaceae family (Croteau et al., 1981) and several Poaceae (Singh et al., 1989) and Asteraceae (Mallavarapu et al., 1999) plants.

3.4. Organization of studied parameters

The relationships between the analyzed parameters and expression level of analyzed genes from the M. aquatica terpenoid production process in the early and late vegetative stages are presented in (Fig. 7). These results could expand our information on the relationships between the morphological, molecular, and physiological responses of M. aquatica plants at the early and late vegetative stage. The potential connections between the studied parameters were evaluated according to Pearson's correlation coefficient. This analysis ordered the studied parameters into two main groups, which were then separated into several minor groups. These data revealed a positive connection between essential oils amount and expression level of enzyme genes involved in the M. aquatica terpenoid production at various stages of plant growth and hence were grouped together. These results suggest that the growth stage affects morphological, molecular, and physiological processes in M. aquatica plants, dependent or independent of each other.

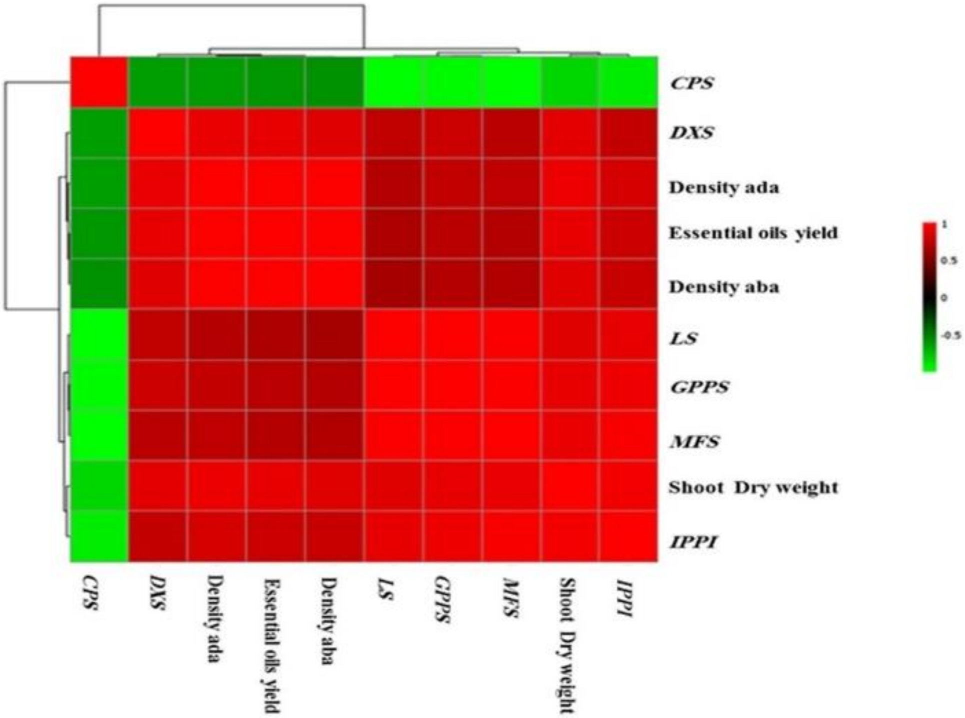

Figure 7: The relationship (Pearson correlations coefficient) between the studied parameters in the *M. aquatica* plant.

4. Conclusion

The current study proved that the composition and amount of essential oils in *M. aquatica* plants are affected by developmental stages. According to the data, biomass and essential oil yields were enhanced in *M. aquatica* as the plant age increased. This could be due to the increasing quantity of glandular trichomes and the fact that most of the analyzed expressed genes belonged to the terpenoid biosynthesis process. So, our results recommend that plant age can have a progressive impact on the generation of essential oils in *M. aquatica*. Due to the metabolites' pharmacological and economic importance, these results can be useful for industrial and medical aims.

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Compliance with Ethical Standards

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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