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Research Article

Enhancement of Essential Oil Production and Expression of Some Menthol Biosynthesis-Related Genes in *Mentha piperita* Using Cyanobacteria

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Background: *Mentha piperita* L. is one of the most important aromatic crops and is cultivated worldwide for essential oils (EOs).

Objectives: The aim of the present study was to investigate the potential of two cyanobacteria, *Anabaena vaginicola* ISB42 and *Nostoc spongiaeforme* var. *tenue* ISB65, as biological-elicitors to improve the growth and essential oil production of *M. piperita*.

Materials and Methods: In this experiment, inoculation of *M. piperita* with cyanobacteria was performed by adding 1% cyanobacterial suspension to the soil of treated pots on the first time of planting and every 20 days thereafter. The experiment was performed in a randomized complete block design in an experimental greenhouse condition. After 90 days planting, the vegetative growth factors, the content of photosynthetic pigments, as well as the quantity and quality of EOs of treated and control plants were evaluated. Also, quantitative changes in the expression of some menthol biosynthesis-related genes were investigated.

Results: Cyanobacterial application led to significant increases in *M. piperita* growth indices including root and shoot biomass, leaf number, leaf area, node number and ramification, as well as photosynthetic pigments content. The statistical analysis showed a 41-75 % increase in some of these growth indices, especially in *Nostoc*-treated plants. *A. vaginicola* and *N. spongiaeforme* var. *tenue* inoculation led to a 13% and 25% increase in the EOs content of *M. piperita*, respectively. The EOs components were also affected by cyanobacterial treatments. According to the statistical analysis, *Nostoc*-treated plants showed the highest amount of (-)-menthone and (-)-limonene, with a 2.36 and 1.87-fold increase compared to the control. *A. vaginicola* and *N. spongiaeforme* var. *tenue* inoculation also led to 40% and 98% increase in transcript level of (-)-limonene synthase gene, respectively. The expression of the (-)-menthone reductase gene, was also increased by 65% and 55% in response to *A. vaginicola* and *N. spongiaeforme* var. *tenue* application, respectively.

Conclusions: Our data demonstrated that in addition to growth enhancement, these two heterocystous cyanobacteria improved the quantity and quality of EOs by up-regulating the key genes involved in the menthol biosynthetic pathway. Based on our results, these cyanobacteria can be considered valuable candidates in the formulation of low-cost and environmentally friendly biofertilizers in sustainable peppermint production.

Keywords: Anabaena vaginicola ISB42, Bioelicitor, Gene expression, Menthol, *Nostoc spongiaeforme* var. *tenue* ISB65, Peppermint,

1. Background

The increasing world population and higher demands for food, feed and plant-based products led to higher use of chemical fertilizers to improve the yield and quality of economic plants (1). The excessive application of chemical fertilizers is posing a serious threat to natural ecosystems as well as human health (2). Deterioration of soil pH, nutritional status, microbial community, and degradation of water resources as well as the introduction of heavy metals to the environment are among the deleterious effects of excessive usage of chemical fertilizer (3-4). In order to overcome this problem, there is an urgent need for innovative biotechnological approaches to explore the beneficial associations between plants and certain microorganisms as sustainable and environmentally friendly biofertilizers. These growth-promoting organisms, including bacteria, fungi and cyanobacteria with the ability to enhance plant growth and elicit the efficient production of plant-based valuable biomolecules, present a promising alternative to synthetic biofertilizers (5-6).

Cyanobacteria are a highly diverse group of Gram-negative photosynthetic prokaryotes found in different types of aquatic and terrestrial environments. Cyanobacteria are emerging organisms for sustainable agricultural and environmental practices, due to the production of valuable organic compounds (7). The presence of cyanobacteria in the soil can improve the aeration, water-holding capacity, and available nitrogen content of the soil (8). Most cyanobacteria can fix atmospheric nitrogen and convert it into the available form of ammonia (9). For example, heterocystous cyanobacteria such as *Anabaena* and *Nostoc* species, are surviving on the soil surface and can fix up to 20-25 kg. h^{-1} atmospheric nitrogen (7). Despite being suited for fully independent existence, cyanobacteria possess a unique ability to form associations with some plant species and promote their growth and productivity in variety of agricultural and ecological situations (10). In recent years, the use of cyanobacteria in agriculture has received increased attention, due to their ability to produce various biologically active compounds and biostimulants including phytohormones (auxins, gibberellins and cytokinins), amino acids and vitamins (vitamin B group) (11-12).

Most studies on the use of cyanobacteria as bio-elicitor and bio-fertilizer have focused on major agronomic species (9,13-15). For example, beneficial effects of cyanobacterial inoculation on plant species such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oat (*Avena sativa* L.), maize (*Zea mays* L.), soybean (*Glycine max* (L.) Merr.), bean (*Phaseolus vulgaris* L.) and cotton (*Gossypium hirsutum* L.) were reported (9,16). The improvement of plant growth and quantity of photosynthetic pigments, as well as the enhancement of carbohydrate and protein content in cyanobacterial-treated plants is part of the available reports in this field. Also, the increase of plants resistance to stress conditions as a result of cyanobacterial inoculation has been reported (16).

However, the application of cyanobacteria on aromatic and medicinal plants and targeting their impact on the production of secondary metabolites is still limited and under progress (17-18). Peppermint (*M. piperita* L.) is one of the most important aromatic crops and is cultivated worldwide for the production of its essential oils. *M. piperita* contains about 3% volatile oils consisting of 50 different compounds. The major EOs components of *M. piperita* are menthone and menthol, which determine the quality of the oil (18). The EOs distilled from *M. piperita* are of great economic importance and have been widely used in the food, cosmetic and pharmaceutical industries. It has been estimated that the international demand for EOs increases by up to 10% per year (19-20).

The content of EOs in aromatic plants is generally very low and fluctuates significantly depending on endogenous and exogenous factors (21). Efficient industrial exploitation of aromatic plants requires a higher percentage and quality of EOs in plants as well as higher plant biomass yield (22). Consequently, the enhancement of plant biomass, as well as improvement of quality and quantity of EOs are the main target traits for the improvement of aromatic crops (23). So, finding practical, innovative, and environmentally friendly strategies to boost aromatic plant's biomass production and improve EOs content is of a great importance.

Considering the importance of this issue, the optimization of plant growth and EOs production in some medicinal plants has been studied. For example, the optimization of EOs production using cyanobacterial elicitors in *Mentha longifolia* L. has been reported (24). According to the results, some medicinal metabolites such as menthol, eucalyptol and phytol were increased in the EOs of cyanobacterial-treated plants (24). Araújo *et al*. (2020) also reported an improvement of EOs yield

in *Rosa hybrid* L. as a result of plant growth-promoting bacteria inoculation (25).

In previous studies, *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65 were introduced as a plant growth promoting cyanobacteria (9,24). These two species of cyanobacteria are able to improve the vegetative growth of plants and increase the essential oil content of medicinal plants including German chamomile (*Matricaria chamomilla* L.) and mint (*M. lonifolia* L.). Shariatmadari *et al*. (2022), reported a 4-fold increase in menthol content in the *N. spongiaeforme* var. *tenue* ISB65 treated wild mints (24), which indicates the importance of cyanobacteria as a bio-elicitor, and shows their effect on menthol biosynthesis-related genes in mint species. The previous studies also reported a significant increase in the expression of most genes involved in menthol biosynthesis pathway such as menthone reductase (MR) after inoculation with elicitors (26). According to Bose *et al*. (2013), transcript level of menthol dehydrogenase/menthone reductase was found highly up regulated in calliterpenone treated *Mentha arvensis* L. plants with increased content of menthone and menthol in EOs (26).

2. Objective

The aim of the present study was to evaluate the plant growth-promoting potentials of two native heterocystous cyanobacteria, *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65, on *M. piperita.* The effect of cyanobacterial suspensions as bio-elicitors on various growth indices, photosynthetic pigments content, and expression of some key genes involved in menthol biosynthesis, as well as essential oil yield and constitutes in peppermint, were subjected in this research.

3. Materials and Methods

3.1. Purification and Identification of Cyanobacterial Species

For the isolation of cyanobacterial species employed in this study, soil samples were collected from the plant's bed soil. The sieved soils were transferred to sterile Petri dishes containing sterile liquid nitrate-free BG-11 medium (27). The Petri dishes were incubated in a culture chamber at 25±2 °C for two weeks of artificial light illumination (74 µmoL photons m^{-2} s^{-1}) with a 12/12 h light-dark cycle. After colonization, isolates were transferred to a solid nitrate-free BG11 medium for purification. For taxonomic determinations, the morphometric study was performed by light microscopy (Olympus, Model BH-2) based on the protocol described by Komárek (2013) (28). For molecular determination of the isolates, 16S rRNA gene sequencing was performed.

3.2. Preparation of Cyanobacterial Suspension

Two strains of cyanobacteria, *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65*,* were cultured in a solid nitrate-free BG11 medium. Algal suspensions were prepared by homogenizing 1 gr of cyanobacterial biomass after four weeks of culturing, in 100 mL of sterilized distilled water (17).

3.3. Plant Materials and Growing Condition

Rhizomes of *M. piperita* were obtained from Medicinal Plants Research Center, Institute of Medicinal Plants (ACECR). Healthy rhizomes (15 for each treatment) were grown in three liter pots (14 cm diam.), containing 60% peat, 25% sand and 15% normal soil, for 90 days. The rhizomes of each treatment were planted in five separate pots. The experiment was performed in a randomized complete block design in an experimental greenhouse condition. A temperature of 25±3 °C and natural daylight was considered during the growing period of plants cultivation. For cyanobacterial treatment, cyanobacterial suspensions (1 gr biomass in 100 mL distilled water) were added to each pot first day of planting and every 20 days thereafter (17). The control group received only water. At harvest, (90 days after planting), samples were collected simultaneously from control and treated plants.

3.4. Measurement of Growth Parameters

After 90 days of culture, plants were harvested and separated into root and aerial parts. Some quantitative parameters related to the growth of plants including root and shoot height (cm), dry weight of plants (gr), leaf number, leaf area (cm^2), node number, internode length (cm), and ramification were determined. The dimensions of the leaves were determined using ImageJ software (version 1.44P; US National Institutes of Health, Bethesda, Maryland, USA). The dry weight of plants was measured with a 0.001 gr accuracy digital scale (Sartorius, Germany), after placing roots and aerial parts in the oven at 70 °C for 24 hours (after the dry weight of the samples remains constant).

3.5. Determination of Chlorophyll and Carotenoids Content

Pigments were extracted from 500 mg of fresh leaves in 80% acetone. After filtration, the absorbance of each sample was measured at 470, 646 and 663 nm. The chlorophylls and carotenoid content were determined according to Lichtenthaler and Wellburn (1983) (29).

3.6. Essential Oil Extraction and GC-MS Analysis

The plant materials were air-dried at ambient temperature. The volatile oils of dried samples (100 gr) were extracted by hydrodistillation method using a Clevenger-type apparatus. Each extraction was repeated three times. The extracted oils were desiccated by anhydrous sodium sulfate and were stored at 4 °C. Identification of EOs composition were conducted using a GC/MS instrument equipped with a fused silica DB-5 column (30 m 0.25 mm, film thickness 0.25 µm). The temperature program was adjusted to 60 °C for 1 min, rising to 250 °C at a rate of 4 °C min^{-1}, and then held at 250 °C for 10 min and the injector temperature was 250 °C. The carrier gas was helium with an ionization voltage of 70 eV and at 1.1 $mL.min^{-1}$. Identification of compounds was carried out based on direct comparison of the Kovats Indices (KI) and MS data. The Kovats retention indices (KI) were calculated for all identified compounds using a homologous series of n-alkanes (C8-C24) injected under the same chromatographic conditions described for samples. Then the constituents of EOs were compared by their mass spectra with the reference mass spectra library, Wiley 7.0, plus confirmation of their retention indices with reported indices in the literature (30). After identification the constituents of the EOs, the quantitative evaluating was performed by gas chromatograph coupled with FID (flame ionization detector) and DB-5 fused silica column (30 m 0.25 mm, film thickness 0.25 µm). The program oven temperature was adjusted as the same as GC/MS. The temperatures of the injector and detector were adjusted to 250 °C and 280 °C, respectively. Nitrogen was the carrier gas at a constant flow rate of 1.1 $mL.min^{-1}$.

3.7. RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent according to the protocol of the provider company (Invitrogen, USA). The concertation of isolated RNA and its integrity were analyzed via NanoDrop (IMPLEN, Germany) and agarose gel, respectively. RNA samples were incubated with RQ1 RNase-free DNase I (Promega) to remove possible DNA contaminations. To confirm the removal of DNA contamination, the extracted RNA samples were used as the template in PCR reaction with genomic DNA as control. the absence of PCR

Figure 1. Comparison between control and treated peppermint plants in growth parameters. Treatment conditions: 1% algal extracts (1.0 g fresh algal material in 100 mL of distilled water) were sprayed on soil of treated pots, **0)** Plant irrigated by distilled water (Control plant), **1)** Plant treated by *Anabaena vaginicola* ISB42, **2)** Plant treated by *Nostoc spongiaeforme* var. *tenue* ISB65, (bar = 5 cm).

products in DNase-treated RNA samples indicated the absence of genomic DNA. cDNA was generated using a cDNA Reverse Transcription kit (Viva 2-step RT-PCR kit). Specific primers for (-)-limonene synthase (*LS*), (-)-menthone reductase (*MR*) and actin (internal control) were designed by Primer Quest software **(Supplementary Table 1)**. The quantitative real-time PCR was carried out using Step One Plus™ (ABI 7500 Applied Biosystems). The RT-PCR program included an initial denaturation (3 min) at 95 °C, followed by 40 cycles of denaturation (95 °C, 30 sec), annealing (60 °C, 30 sec), and elongation (72 °C, 30 sec), and a final elongation step (72 °C, 5 min). After the determination of the amplification of efficiency (E value), the relative expression of samples was analyzed by the Livak method using the REST 2009 Software (31).

3.8. Statistical Analysis

One-way ANOVA statistical analysis was performed employing SPSS software version 16 (Package for the Social Sciences, SPSS Inc., Chicago IL). Means were separated using the Tukey HSD test at $p \leq 0.05$. The heatmap dendrograms were also performed using the CIMMiner online tool.

4. Results

4.1. Growth Indices

Inoculation of *M. piperita* plants by cyanobacteria led to remarkable improvement of plant growth indices **(Fig. 1)**. Application of both *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65 significantly ($p \leq 0.05$) increased root length, fresh and dry weight of plants, leaf

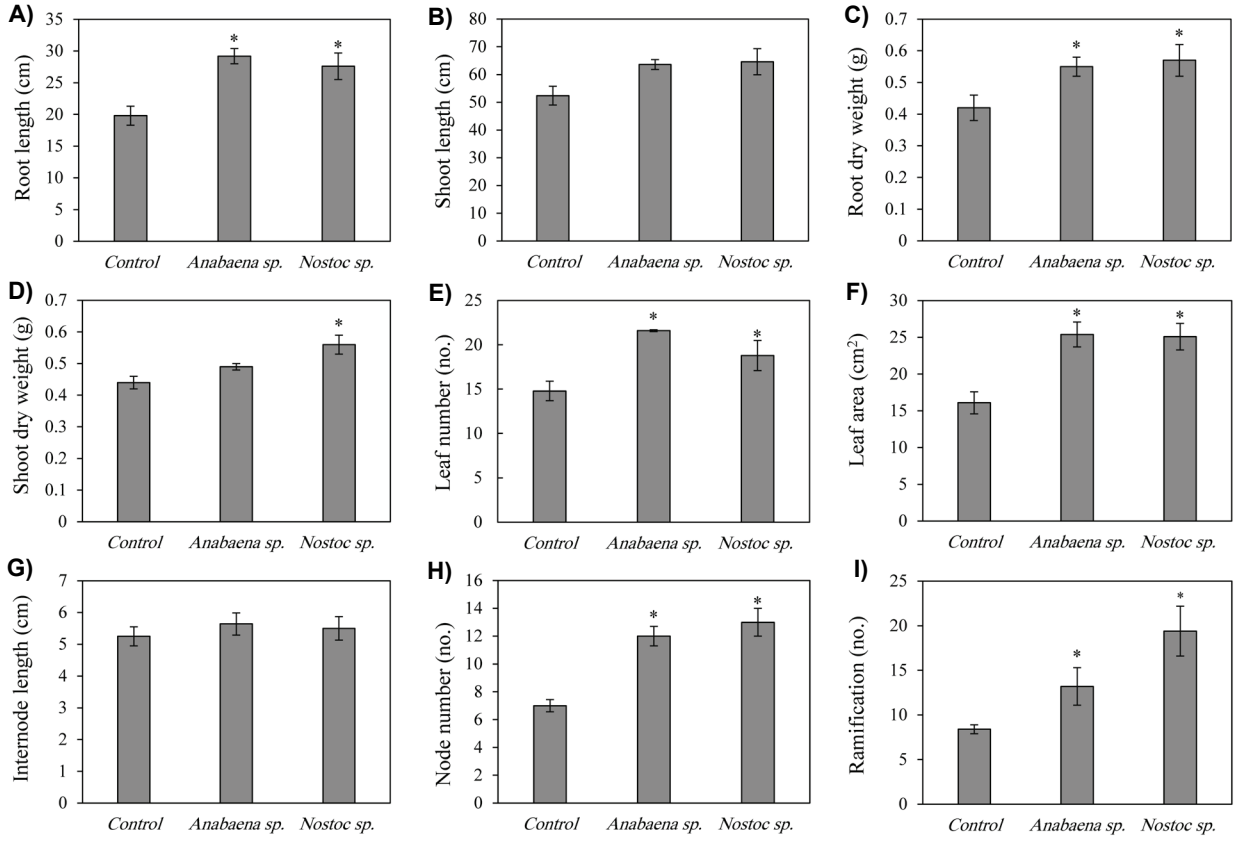

Figure 2. Effects of *Anabaena vaginicola* ISB42 and *Nostoc spongiae*forme var. *tenue* ISB65 inoculation on growth indices of *Mentha piperita* compared to the control. A) root length; **B)** shoot length; **C)** root dry weight; **D)** shoot dry weight; **E)** leaf number; **F)** leaf area; **G)** internode length; H) node number; **I)** ramification. values are average of three independent replicates ± standard error. Significant difference according to Tukey's test ($p \leq 0.05$).

area, number of nodes, and ramification. Other growth indices such as shoot and internode length also improved in response to inoculation of both taxa **(Fig. 2)**.

The obtained results showed that plant size increased in cyanobacterial-treated plants, especially in root section. The highest root length was recorded for plants treated with *A. vaginicola* ISB42 with 47% increase compared to the control. The *Nostoc*-treated plants also showed a 41% increase in the root length compared to the control. The highest increase in the length of aerial parts of plants was also recorded for *Nostoc*-treated plants with a 23% increase compared to the control plants, followed by those inoculated with *Anabaena* (21% increase).

The results of this study also showed a significant increase in the fresh weight of aerial parts and roots of *Nostoc* treatments compared to the control plants (75% and 67% increase, respectively). Similar to *Nostoc*-treated plants, the *Anabaena* treatments also showed a significant increase in the fresh weight of aerial parts and roots (67% and 55% increase, respectively). The obtained results also showed 33% and 43% increase in the dry weight of root in the *Anabaena* and *Nostoc* treatments compared to the control, respectively. The dry weight of aerial parts of these two groups of treated plants also showed an 11-27 % increase, respectively.

An increase in the number and size of leaves was another change observed in plants treated with cyanobacteria. The highest leaf number was recorded for plants treated with *A. vaginicola* ISB42 with 46% increase compared to the control plants. The leaf area also was significantly increased in two cyanobacterial treatments with a 56% increase compared to the control groups. A 2.3-fold increase in ramification was one of the other results obtained from the treatment of peppermint plants with *N. spongiaeforme* var. *tenue* ISB65 **(Fig. 2)**.

4.2. Chlorophylls and Carotenoid Content

The quantity of photosynthetic pigments increased in cyanobacterial-treated plants **(Fig. 3)**. In particular, the use of *A. vaginicola* ISB42 as a bio-elicitor led to a significant ($p \leq 0.05$) increase in chlorophyll *a,* chlorophyll b, and total chlorophylls content of *M. piperita* compared to the control group. The highest chlorophyll *a* content was recorded for *Anabaena*-treated plants with 53% increase, and the highest chlorophyll *b* content was also recorded in this group of treatments with 63% increase compared to the control plants. Similarly, the carotenoid content of *M. piperita* was significantly increased upon *Anabaena* inoculation (55% increase compared to the control group), while the photosynthetic pigments content of *Nostoc*-treated plants showed a slight increase **(Fig. 3)**.

4.3. Essential Oil Yield and Constitutes

Cyanobacterial inoculation elicited the production of EOs in *M. piperita* compared to the control group **(Table 1)**. The yield of EOs of *M. piperita* increased by 13% and 25% as a result of inoculation with *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65, respectively.

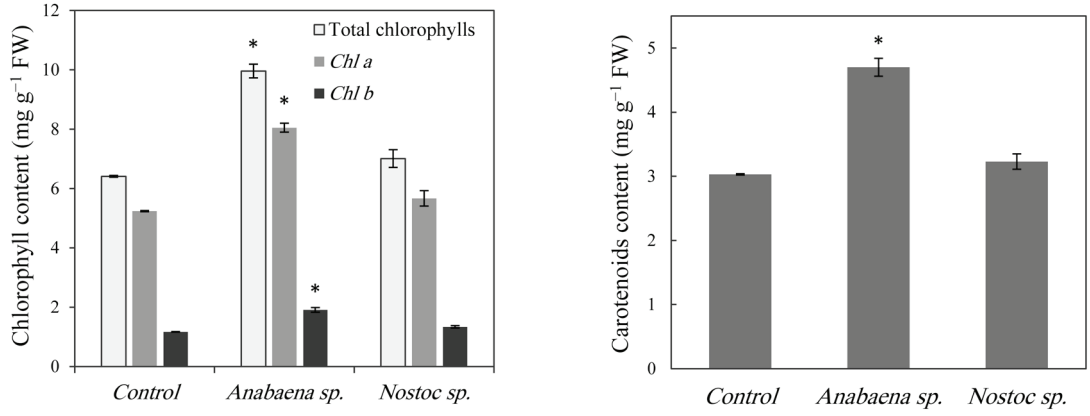

Figure 3. Effects of *Anabaena vaginicola* ISB42 and *Nostoc spongiaeforme* var. *tenue* ISB65 inoculation on chlorophylls and carotenoid content in *Mentha piperita* compared to the control. values are average of three independent replicates ± standard error. * Significant difference according to Tukey's test ($p \leq 0.05$)

Table 1. Effects of cyanobacterial application on essential oil yield and selected essential oil constitutes (%) of *Mentha piperita* L. (Mean±SE)

	Treatment		
Components	**Control**	***Anabaena vaginicola***	***Nostoc spongiaeforme***
(-)-Limonene	1.41± 0.22	2.41± 0.43 *	2.65± 0.36 *
(-)-Menthone	8.61± 1.20	18.52± 1.80 *	20.37± 0.9 *
(+)-Mentofuran	1.90± 0.31	3.24± 0.51 *	5.33± 0.86 *
(+)-iso-Menthone	1.27± 0.60	2.19± 0.52 *	2.18± 0.71 *
(+)-neo-Menthol	3.25± 0.13	3.73± 0.18	4.76± 0.05*
(-)-Menthol	38.00± 0.12	37.90± 1.16	37.60± 1.44
(+)-iso-Menthol	1.06 ± 0.03	0.78 ± 0.25	0.80 ± 0.37
(+)-Pulegone	1.38 ± 0.21	2.05 ± 0.12 *	4.11 ± 0.45 *
Essential oil yield	1.00 ± 0.08	1.13 ± 0.06	1.25 ± 0.03 *

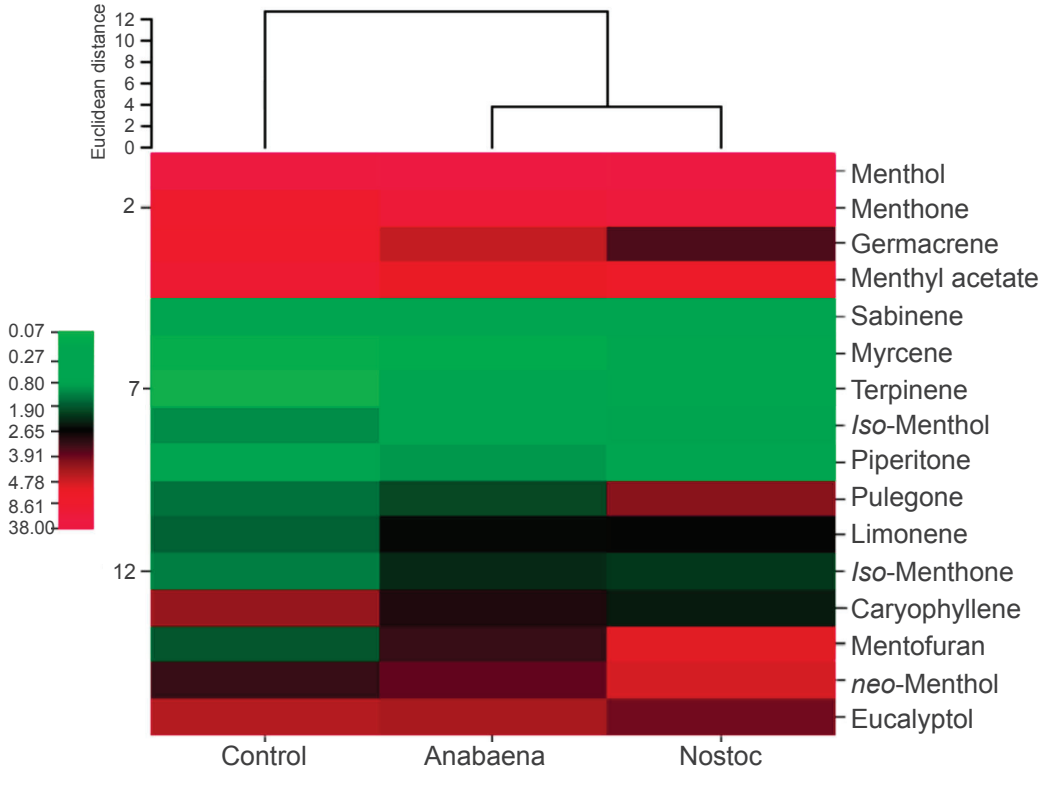

Figure 4. Clustering Heatmap analysis of essential oil constitutes in the treated and control peppermint plants. Each colored cell on the map corresponds to different values of metabolite composition.

The highest EOs content was recorded for the *Nostoc*-treated plants (1.25±0.03 %), and the lowest quantity of EOs was recorded for control plants (1.00±0.08 %). The GC/MS analyses of chemical composition of EOs revealed a variation in several compounds of *M. piperita* EOs after cyanobacterial treatment **(Fig. 4)**. The correlation between the amount of the major detected EOs compounds and the treatments is shown through a Heatmap dendrogram **(Fig. 4**). According to this dendrogram, qualitative and quantitative changes of some of these metabolites in cyanobacterial-treated plants was considerable. These results showed that some of the main compounds of peppermint EOs such as menthol, menthone, neo-menthole, iso-menthone, limonene, and pulegone significantly changed in the treatment conditions. For example, inoculation of *A. vaginicola* and *N. spongiaeforme* var. *tenue* increased the (-)-menthone content of peppermint 2.15 and 2.36-fold, respectively **(Table 1)**. Furthermore, the plants inoculation with *Nostoc* and *Anabaena* increased (+)-iso-menthone content by 71% and 72% compared to the control, respectively. The obtained results also showed a significant increase of (-)-limonene in *Nostoc* and *Anabaena* treatments with 1.87 and 1.7-fold increases compared to the control group, respectively. The 1.7 to 2.8-fold increase of (+)-mentofuran was observed in cyanobacterial-treated plants. Also, 14 to 46 % increase of (+)-neo-menthol for *Anabaena* and *Nostoc* treated plants recorded in this study.

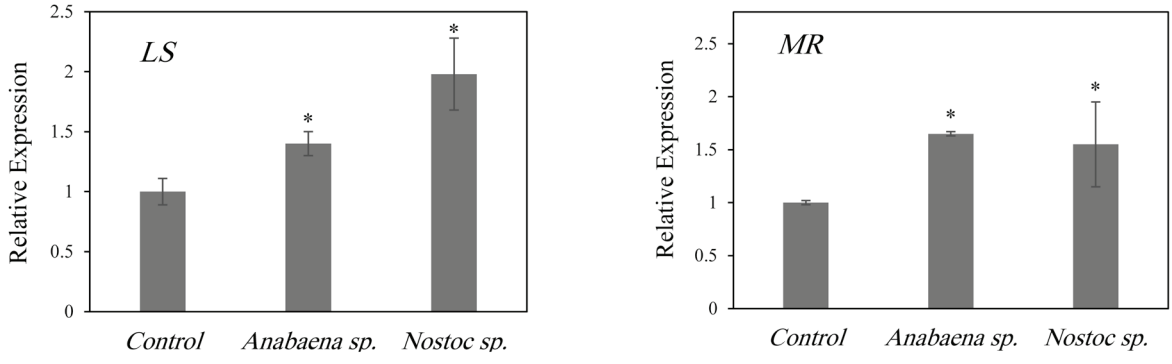

Figure 5. Changes in expression patterns of (-)-limonene synthase *(LS),* (-)-menthone reductase *(MR)* genes in *Mentha piperita* inoculated with *Anabaena vaginicola* ISB42 and *Nostoc spongiaeforme* var. *tenue* ISB65. Values are average of three independent replicates ± standard error. * Significant difference according to Tukey's test ($p \leq 0.05$)

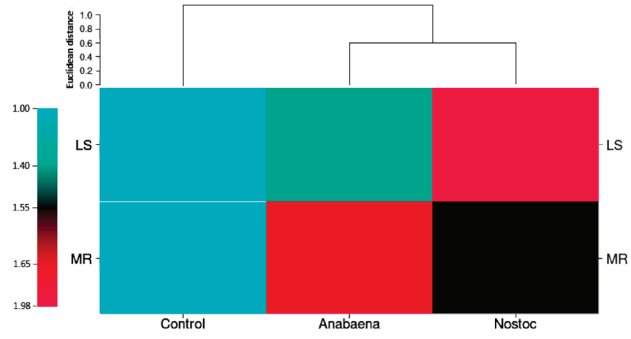

Figure 6. Clustering Heatmap analysis of gene expression patterns of (-)-limonene synthase *(LS)* and (-)-menthone reductase *(MR)* in the treated and control peppermint plants. Each colored cell on the map corresponds to different values of metabolite composition.

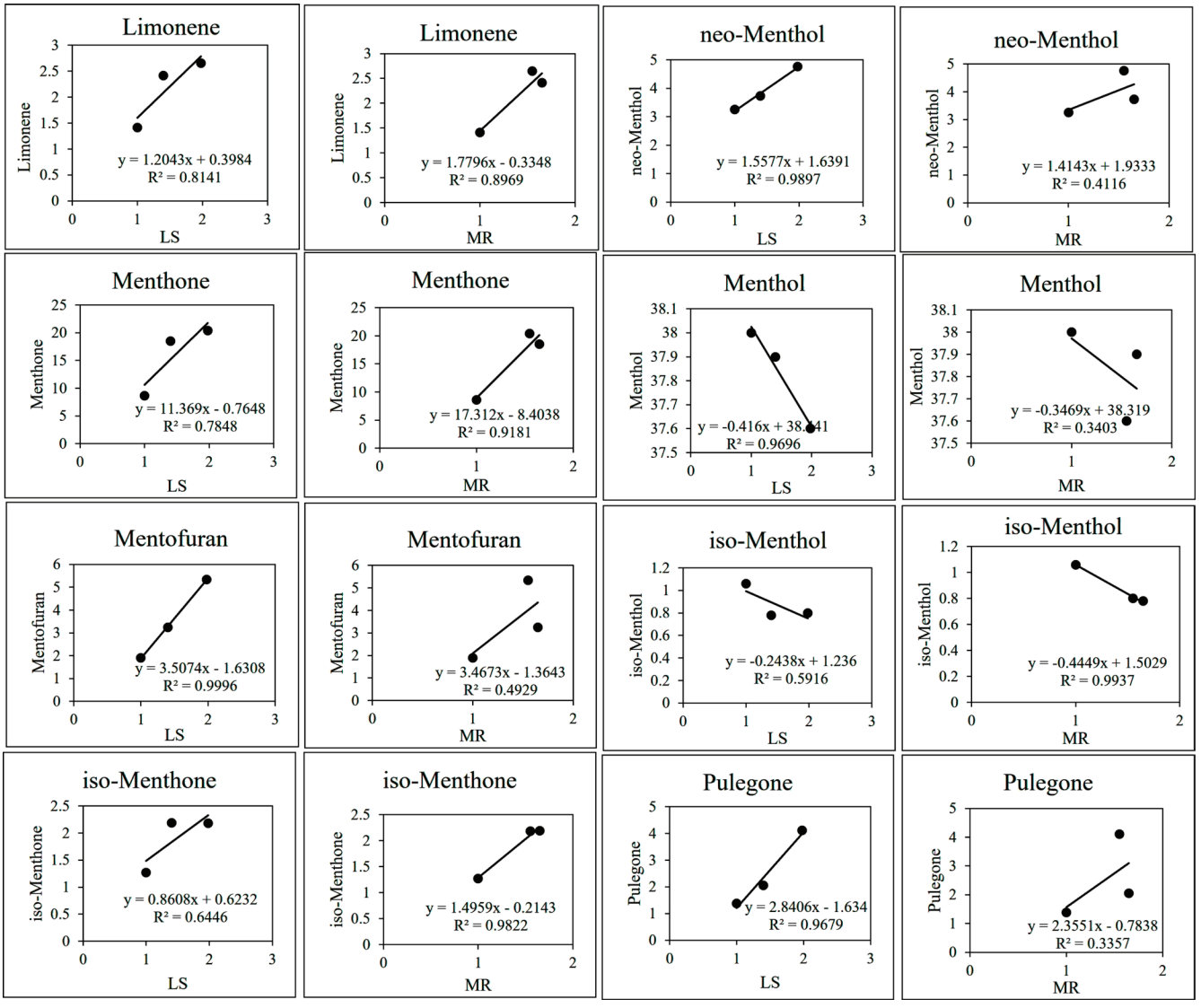

Figure 7. The correlation between changes in expression levels of the studied genes and the percentage of essential oil constituents of control and cyanobacterial-treated plants. *LS*: (-)-limonene synthase, *MR*: (-)-menthone reductase.

Contrary to the mentioned metabolites, the quantity of metabolites such as (-)-menthol and (+)-iso-menthol slightly decreased in cyanobacterial-treated plants compared to the control groups **(Table 1)**.

4.4. Expression of Menthol Biosynthetic Genes

Quantitative real-time PCR results revealed that the transcript levels of *LS* and *MR* genes in *M. piperita* significantly increased in response to both cyanobacteria inoculums compared to the control group **(Fig. 5)**. Inoculation with *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65 led to 40% and 98% increase in transcript level of *LS* gene in *M. piperita*, respectively. The expression of the *MR* gene, also increased by 65% and 55% in response to *Anabaena* and *Nostoc* treatment respectively.

The clustering Heatmap analysis of gene expression patterns of *LS* and *MR* in the treated and control peppermint plants shows two treatment groups in a single cluster and separate from the control **(Fig. 6)**.

The correlation between changes in expression levels of the studied genes (*LS* and *MR*) and the percentage of essential oil constituents of control and cyanobacterial-treated plants also recorded as **Figure 7**. In these graphs, we can clearly see the high correlation between the level of *LS* and *MR* genes expression and the increase in the amount of some metabolites such as menthone, limonene, iso-menthone, and neo-menthol in treated plants.

5. Discussion

The presence and activity of microorganisms in the rhizosphere positively contribute to the growth and productivity of plants (32). Cyanobacteria are one of the most important constituents of the soil microflora with a significant ability to promote plant growth. The mechanisms of growth enhancement vary depending on cyanobacterial strain as well as plant species and may include both direct and indirect mechanisms (7). Several cyanobacterial strains, including *Anabaena* and *Nostoc* species have specialized cells called heterocysts that are able to fix atmospheric nitrogen and increase soil fertility (8). Furthermore, it has been well-documented that the production of phytohormones and other growth-stimulating substances such as proteins, vitamins, carbohydrates, and amino acids by cyanobacteria are among the most important factors influencing plant growth (33). In addition, cyanobacteria enhance plant growth through the improvement of soil structure by secretion of mucilage compounds and exopolysaccharides (EPS) (10). The EPS increases soil water holding capacity through the aggregation of soil particles and accumulation of organic matter in the soil (34). In the present study, inoculation of *M. piperita* plant with both cyanobacterial specimens led to an increase in almost all growth parameters, some of which were significant. The plant biomass also was significantly increased as a result of both treatments compared to the control group. This effect appeared to be due to an increase in leaf number, leaf area, as well as node numbers, and plant height in cyanobacterial-inoculated plants. Root weight and length were also affected by the cyanobacterial application. An increase in root growth corresponds to higher root surface area and nutrient uptake capability to support the water and nutrient demands of the growing shoot. Improved growth and development following the application of cyanobacterial strains have been reported in several plant species. Consistent with our findings, Zarezadeh *et al*. (2020) reported an increase in several growth indices of the chamomile plant in response to three cyanobacterial strains including *Wollea vaginicola*, *Nostoc carneum*, and *Nostoc punctiforme* (35). Similarly, Chookalaii *et al*. (2020) investigated the effects of heterocystous cyanobacterial extracts on *Plantago major* L. plant (36). They observed that the application of cyanobacterial extracts led to a significant increase in several growth parameters of *P. major* including leaf number, leaf area, number and length of inflorescence, as well as root and shoot biomass. Inoculation of *Zea mays* L., *Sorghum bicolor* (L.) Moench, *Oryza sativa* L., *Vigna unguiculata* (L.) Walp. and *Paspalum scrobiculatum* L., with *Anabaena variabilis* and *Nostoc calcicola* also enhanced the germination rate and growth parameters of these plants (14).

The chlorophyll and carotenoids content of plants is an important indicator for the evaluation of the photosynthetic status and efficiency in different physiological and environmental scenarios (37). In the present study, we observed higher content of chlorophylls and carotenoid content in *M. piperita* plants inoculated with *A. vaginicola* ISB42 compared to the control group. *N. spongiaeforme* var. *tenue* ISB65 treatment also led to an insignificant increase in chlorophyll *a* and total chlorophyll content. Higher content of photosynthetic pigments correlated to higher photosynthetic activity and consequently higher biomass in cyanobacterial-treated plants. It has been well-documented that the increase in photosynthetic pigments can be attributed to a probable higher rate of water and nutrient uptake in the inoculated plants along with the synergic effects of cyanobacterial growth-promoting substances (37-38). Furthermore, plant growth-promoting bacteria can induce the upregulation of photosynthesis-related genes and increase the pigment content of inoculated plants (39). Our results are in accordance with the findings of Cappellari *et al*. (2015), which reported a significant increase in chlorophyll *a*, chlorophyll *b,* and total chlorophylls in *M. piperita* plants inoculated with several plant growth-promoting rhizobacteria including *Bacillus subtilis*, *Pseudomonas fluorescence* and *Pseudomonas putida* (40).

The yield and composition of *M. piperita* EOs can be affected by various environmental factors including microorganisms (17,41). In the present study, cyano-bacterial inoculation improved the yield and composition of *M. piperita* EOs**.** The improvement of EOs

content in some species of Lamiaceae family (*Mentha arvensis* and *Ocimum basilicum* L.) was reported as a result of mycorrhiza treatment (42). The positive effect of plant growth-promoting bacteria (PGPB) on EOs production of peppermint was also reported by Cappellari *et al*. (2019) (18). Furthermore, the enhancement of plant biomass and EOs production in other plants such as sweet marjoram (*Origanum majorana* L.), wild marigold (*Tagetes minuta* L.), and Italian oregano by PGPB has been reported (43-44). Cappellari *et al*. (2019) suggested that inoculation of peppermint with PGPB increases the EOs yield by increasing the glandular trichome density, as well as increasing the jasmonate and salicylic acid concentrations (18). It has been reported that the improvement of total chlorophyll content and fresh weight of PGPB-treated plants can positively affect the EOs yield (40). These results are consistent with our finding that the higher chlorophyll content and fresh weight of the inoculated *Mentha* were associated with EOs content improvement.

An increase in metabolites such as limonene and neo-menthol in cyanobacterial-treated plants was observed in our study. Shariatmadari *et al*. (2022) also reported the change in the metabolite content of *M. longifolia* L. using plant growth promoting cyanobacteria (24). A significant increase of some economic and medicinal metabolites such as menthol, eucalyptol and phytol in the EOs of treated plants have been reported in this study (24). In the present study, several other metabolites such as (+)-mentofuran, (+)-pulegone and, (-)-limonene content increased in cyanobacteria-treated plants. An increase in the amount of these metabolites in stress conditions has been reported in other studies. For example, abiotic stress promotes the production of (+)-menthofuran and (+)-pulegone metabolites in peppermint. These two constitutes of EOs are considered stress metabolites whose production is favored in environmental stress conditions (45-46).

Both *A. vaginicola* ISB42, and *N. spongiaeforme* var. *tenue* ISB65 are able to produce bio-elicitors including plant growth regulators such as auxins, gibberellins, and other phytohormones. Significant content of auxins, especially IBA, has been reported in *Anabaena* and *Nostoc* species biomass (11). Several reports are available regarding the effect of plant growth regulators such as auxins on EOs yield in mint species (26). These studies indicate that phytohormones such as Indole-3-butyric acid (IBA) can change some metabolite content in EOs of peppermint. Soleymani *et al*. (2017) also investigated the effect of the exogenous application of chitosan, gibberellic acid, and methyl jasmonate on the gene expression of the menthol biosynthesis pathway, and introduced them as an efficient elicitor (47).

The biosynthesis of peppermint EOs takes place in highly specialized epidermal oil glands. The biosynthesis process of EOs begins with the ionization and cyclization of geranyl diphosphate (C_{10}) to (-)-limonene, by (-)-limonene synthase. (-)-limonene is the first cyclic intermediate in the biosynthesis of oxygenated monoterpenes. (-)-limonene synthase represents the committed step of EOs biosynthesis in *M. piperita* and other *Mentha* species (26). In the present study, the expression of *LS* gene significantly increased in response to both cyanobacteria inoculation. Elevated expression of *LS* gene contributes to higher (-)-limonene content as the first substrate of the EOs biosynthetic pathway and consequently higher yield of EOs observed in this study. In the EOs biosynthetic pathway, (-)-limonene is converted to (+)-pulegone through a series of enzymatic reactions. Newly formed (+)-pulegone can be converted (-)-menthone and (-)-menthol by the action of (+)-pulegone reductase and (-)-menthone reductase, respectively (48). In the present study, the expression of the *MR* gene significantly increased upon cyanobacterial application. Higher expression of *MR* gene along with a higher concentration of (-)-menthone reductase substrate or (-)-menthone was observed in the present study. This result is consistent with finding the previous studies, which reported a significant increase in the expression of most genes involved in menthol biosynthesis pathway after inoculation with elicitors (26). Similar to our findings, Guo *et al*. (2020), reported that inoculation *M. piperita* with *Trichoderma viride* led to an increase in the expression of (-)-menthol biosynthetic genes (including *MR* and *LS)* and higher EOs yield (20).

Although the menthol biosynthetic pathway is well-characterized, transcriptional regulation of genes involved in the pathway and the mechanisms of induction of the pathway in response to bio-elicitors are poorly understood. However, AP2-ERF and WRKY transcription factor families are quickly emerging as important regulators of terpene biosynthesis (18).

A complex balance of monoterpenes constitutes determines the quality of the *M. piperita* oil. Moderate percentage of (-)-menthone and (-)-menthol is

characteristic of high-quality oil. (-)-Menthone, as a food additive for human consumption, provides remarkable commercial value to *M. piperita* EOs. In the present study, the percentage of (-)-menthone significantly increased upon cyanobacterial inoculation.

Due to induction of biomass production accompanied by improvement of yield and quality of EOs, the application of *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65 represent a suitable candidate as biofertilizer in *M. piperita* cultivation.

5. Conclusion

The results obtained from this study elucidated the beneficial effects of *A. vaginicola* ISB42 and *N. spongiaeforme* var. *tenue* ISB65 application on the growth and EOs yield and quality of *M. piperita*. According to the results, cyanobacteria-treated plants have a significantly higher content of medicinal compounds such as menthone and limonene. Treatment of this medicinal plant by cyanobacteria also led to higher expression levels of some biosynthesis-related genes of menthol (*LS, MR*). The results of this study provided the promising basis for the potential application of cyanobacterial inoculums in *M. piperita* cultivation.

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