UV-B modulates the interplay between terpenoids and flavonoids in peppermint (Mentha x piperita L.)

UV-B modulates the interplay between terpenoids and flavonoids in peppermint (*Mentha* × *piperita* L.)

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Abstract

Modulation of secondary metabolites by UV-B involves changes in gene expression, enzyme activity and accumulation of defence metabolites. After exposing peppermint (*Mentha* × *piperita* L.) plants grown in field (FP) and in a growth chamber (GCP) to UV-B irradiation, we analysed by qRT-PCR the expression of genes involved in terpenoid biosynthesis and encoding: 1-deoxy-d-xylulose-5-phosphate synthase (*Dxs*), 2-C-methyl-d-erythritol-2,4-cyclodiphosphate synthase (*Mds*), isopentenyl diphosphate isomerase (*Ippi*), geranyl diphosphate synthase (*Gpps*), (−)-limonene synthase (*Ls*), (−)-limonene-3-hydroxylase (*L3oh*), (+)-pulegone reductase (*Pr*), (−)-menthone reductase (*Mr*), (+)-menthofuran synthase (*Mfs*), farnesyl diphosphate synthase (*Fpps*) and a putative sesquiterpene synthase (*S-TPS*). GCP always showed a higher terpenoid content with respect to FP. We found that in both FP and GCP, most of these genes were regulated by the UV-B treatment. The amount of most of the essential oil components, which were analysed by gas chromatography–mass spectrometry (GC–MS), was not correlated to gene expression. The total phenol composition was found to be always increased after UV-B irradiation; however, FP always showed a higher phenol content with respect to GCP. Liquid chromatography-mass spectrometry (LC–ESI-MS/MS) analyses revealed the presence of UV-B absorbing flavonoids such as eriocitrin, hesperidin, and kaempferol 7-*O*-rutinoside whose content significantly increased in UV-B irradiated FP, when compared to GCP. The results of this work show that UV-B irradiation differentially modulates the expression of genes involved in peppermint essential oil biogenesis and the content of UV-B absorbing flavonoids. Plants grown in field were better adapted to increasing UV-B irradiation than plants cultivated in growth chambers. The interplay between terpenoid and phenylpropanoid metabolism is also discussed.

Keywords

- *Mentha piperita*;
- UV-B irradiation;
- Terpenoid gene expression;
- Quantitative real-time PCR;
- Essential oil composition;
- Flavonoids

1. Introduction

In natural conditions plants are continuously exposed to environmental stresses. One such inevitable stress factors is the exposure to ultraviolet-B radiation (UV-B, 280–320 nm). Even though it represents only a small fraction of the electromagnetic spectrum, UV-B radiation is known to influence all living organisms, including higher plants [1]. The level of UV-B radiation depends upon a number of factors such as season, time of the day, latitude, altitude, cloud cover and thickness of the ozone layer. The latter can be modified by seasonal, geographical and meteorological factors [2], [3], [4] and [5].

Plants have developed multiple defensive mechanisms against the challenge of direct exposure to solar UV-B during their evolution. Among the several effects induced by enhanced UV-B radiation on plants, the most common is modulation of secondary metabolism including the rise of phenolic compound concentrations in leaf tissue [6] and [7] and the increase of isoprene and terpenoid production [8], [9] and [10]. However, while the effect is clearer on flavonoids, not always supplementary UV-B leads to increased terpenoid production [11] and [12].

To evaluate whether an increase of UV-B radiation sets constraints on terpenoid biogenesis and flavonoid content in industrial plants, we supplied peppermint (*Mentha* × *piperita* L.) plants with additional UV-B irradiation. This plant belongs to the Lamiaceae family and the distilled essential oil is widely used for medicinal, cosmetic, flavoring and general commercial applications [13]. The phenolic profile and fingerprint of peppermint has been studied, describing developmental, taxonomical, environmental and physiological aspects [14], [15], [16], [17] and [18]. Furthermore, peppermint has also been used as a model plant for terpene metabolism [19] and [20].

Two distinct pathways have evolved in nature for the synthesis of the universal precursors of all terpenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In plant cells, the mevalonate (MVA) pathway produces cytosolic IPP, which is then isomerized to DMAPP by the activity of IPP isomerase. Alternatively, plastidial IPP and DMAPP are synthesized by the unrelated 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway [21]. However, in mint oil glands, it has been demonstrated that the classical MVA pathway for the biosynthesis of IPP is blocked [22] and [23].

Peppermint oil contains more than 200 compounds [24], most of which are monoterpenes and the eight-step biosynthesis pathway leading to the main component, (−)-menthol, includes a wide spectrum of enzymatic reactions [20]. There is a consistent interest to understand factors affecting the biosynthesis of peppermint oil in order to increase the content of the commercially important component (−)-menthol and (−)-menthone and decrease the presence of undesirable (+)-pulegone and (+)-menthofuran [20], [25] and [26].

Previous works have demonstrated the influence of environmental factors on essential oil composition in some mint species [27]. Among them, the quality of light revealed to be an important factor. In peppermint, supplementary blue light (450 nm) was shown to decrease (−)-menthol biosynthesis by shifting it to (+)-menthofuran production [28], and additional UV-B light had similar negative effect on essential oil quality [29]. UV-A radiation (360 nm) also affects peppermint oil composition, by increasing (+)-menthofuran content. (−)-Menthol biosynthesis was decreased after UV-A treatment during the night period and increased by UV-A with white light [30]. All these experiments were performed under controlled climatic conditions. However, the effects of enhanced UV-B radiation on plants grown in natural conditions are known to be different from those observed under laboratory tests. In fact, plants grown in field show enhanced resistance to increasing UV-B irradiation [2], [12], [31], [32] and [33], probably because of the action of other compounds such as flavonoids. Considering the commercial value of peppermint essential oil, we compared the responses to increased UV-B irradiation of plants grown in field (FP) and in a growth

chamber (GCP). We analysed the essential oil composition and the terpenoid gene expression, the latter by using the available peppermint EST sequences [23]. At the same time, we characterized the main flavonoids accumulated after UV-B irradiation. The aim of the present study was to evaluate whether UV-B differentially affects terpenoid gene expression and flavonoid content and whether an interplay between peppermint terpenoid gene expression, essential oil composition and flavonoids does exist.

2. Materials and methods

2.1. Plant material and UV-B irradiation

Peppermint (*Mentha* × *piperita* L. nm *rubescens*) plants grown in field (FP) (Casalgrasso, Cuneo province, Italy) were irradiated a few days before full bloom with UV-B for 1 h in the middle of the day, with a wavelength of 310 nm (Philips TL 40W/12). Tubes were suspended 50 cm above the plants. TL tubes were filtered by using 0.1 mm cellulose diacetate foil which absorbs radiation below 290 nm. UV treatment resulted in a daily UV-B radiation of 7.1 kJ m^{-2} day^{-1} UV_{BE}, weighted according to the generalised plant action spectrum of Caldwell [34]. The five upper leaf pairs were collected 24 h after exposition, whereas peppermint grown in the same conditions without the UV-B treatment were used as a control. Intact leaves were kept in liquid nitrogen and stored at −80 °C before all analyses. UV-B irradiation used in our experiments was corresponding to about 40% of ozone layer depletion.

Rhizomes of peppermint were also transplanted in pots and grown for 6 weeks in controlled environmental chambers (GCP) with 16 h photoperiod (190 μmol s^{-1} m^2, GroLux), 29 °C day/24 °C night temperature cycle and relative humidity of 65 ± 10%. Plants were watered every 2 days and fertilized weekly with standard fertilizers (N:P:K, 20:20:20). Plants in pots (10–15 pots) were then irradiated with UV-B for 1 h in the middle of the day, with a wavelength of 310 nm (Philips TL 40W/12) [30]. Plant sampling was done after 24 h as above.

In order to overcome different developments in FP and GCP conditions, plants were sampled at the same developmental stage and for all the experiments, the first 5 leaf pairs of plants before bloom were always used.

Spectroradiometric analysis was performed using a Photo Research Pritchard (Kollmorgen Co.) Spectroradiometer Model 1980B controlled by a HP-9825 computer [30].

2.2. Extraction and analysis of peppermint essential oil

Peppermint leaves of plants exposed to UV-B irradiation and control plants were ground in liquid nitrogen and then extracted with 5 ml of hexane (Merck) containing 0.04 mg ml^{-1} camphor (Fluka) as internal standard. The extract was loaded on a small glass column containing $MgSO_4$ (Fluka) and the eluent was concentrated under a gentle flow of N_2 to 1 ml and analysed by gas chromatography on a Agilent Technologies 6890 series N gas chromatograph equipped with a capillary (HP5-MS) column (30 m × 0.25 mm; film thickness of 0.25 μm). The injector temperature was 250 °C and the detector temperature 290 °C. Using a constant helium flow of 2.0 ml min^{-1} and a pressure of 6.59 psi, the oven program was as follows: initial temperature 60 °C for 5 min, followed by a ramp of 8 °C min^{-1} to 160 °C and by the second ramp of 40 °C min^{-1} until 290 °C and final time of 10 min. The compounds were identified by gas chromatography–mass spectrometry as previously reported [30]. The column and the program parameters were the same as above. Further identification of compounds was made by comparison of their mass spectra with those stored in NIST 98 and other

custom-made libraries or with pure standards. Essential oil content was calculated on a dry weight basis.

2.3. Total RNA extraction and quantitative RT-PCR

One hundred mg of UV-B irradiated and control frozen leaves were ground by using the Tissue Lyser, Qiagen, Hilden, Germany with two 30-s bursts. For further extraction the method of Chang et al. [35] was used. DNase-treatment was done according to manufacturer's protocol using the Ambion TURBO DNA-*free* reagents (Ambion). RNA sample quality and quantity was checked by using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies) according to manufacturer's instructions. Quantification of RNA was also confirmed spectrophotometrically by NanoDrop ND-1000 (Thermo).

First strand cDNA synthesis was accomplished with 2 µg of total RNA and random primers using the AffinityScript™ Multiple Temperature Reverse Transcriptase (RT) (Stratagene), according to the manufacturer's recommendations. Primers for real-time PCR were designed using the Primer 3 software [36]. The real-time PCR was done on Mx3000P Real-Time PCR System (Stratagene). The reaction was performed with 25 µl of mixture consisting of 12.5 µl of 2× Brilliant SYBR Green QPCR Master Mix (Stratagene), 0.5 µl of cDNA, 100 nM primers (Invitrogen) and 30 mM ROX as a passive reference dye.

Specifically, PCR conditions were the following: *18S*: initial polymerase activation of 10 min at 95 °C; and 40 cycles of 30 s at 95 °C, 30 s at 52 °C, and 60 s at 72 °C; *Mds*: initial polymerase activation of 10 min at 95 °C; and 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; *Dxs*: initial polymerase activation of 10 min at 95 °C; and 44 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; *Ippi*: initial polymerase activation of 10 min at 95 °C; and 53 cycles of 30 s at 95 °C, 30 s at 57 °C, and 60 s at 72 °C; *Gpps*: initial polymerase activation of 10 min at 95 °C; and 40 cycles of 30 s at 95 °C, 30 s at 59 °C, and 60 s at 72 °C; *Ls*: initial polymerase activation of 10 min at 95 °C; and 45 cycles of 30 s at 95 °C, 30 s at 52 °C, and 60 s at 72 °C; *L3oh*: initial polymerase activation of 10 min at 95 °C; and 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; *Pr*: initial polymerase activation of 10 min at 95 °C; and 47 cycles of 30 s at 95 °C, 30 s at 59 °C, and 60 s at 72 °C; *Mr*: initial polymerase activation of 10 min at 95 °C; and 44 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; *Mfs*: initial polymerase activation of 10 min at 95 °C; and 50 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; *Fpps*: initial polymerase activation of 10 min at 95 °C; and 50 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; *S-Tps*: initial polymerase activation of 10 min at 95 °C; and 40 cycles of 30 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C.

Relative RNA levels were calibrated and normalized with the level of 18S ribosomal mRNA.

Primers used for real-time PCR were as follows: *18S*, accession number NR_022795, forward primer 5′-ATGATAACTCGACGGATCGC-3′, reverse primer 5′-CTTGGATGTGGTAGCCGTTT-3′; *Dxs*, accession number AF019383[37], forward primer 5′-CCACCAGGCTTACCCACACAA-3′, reverse primer 5′-GCCACCGCCATCCCTAAAC-3′; *Mds*, accession number AW255189[23], forward primer 5′-TTGGAGTAGAGCCTCAGTCGG-3′, reverse primer 5′-CAACTTAGGATTGGTGTCGGG-3′; *Ippi*, accession number AW255524[23], forward primer 5′-CTCTTGGGGTGAGAAATGCT-3′, reverse primer 5′-CATCTGAGGGGGCTTTGTA-3′; *Gpps*, accession number BD081617[38], forward primer 5′-ATCTCAGCCGTTCTCCTTCA-3′, reverse primer 5′-CCTTATTGGGATGGATTTCT-3′; *Ls*, accession number AW255536[23], forward primer 5′-CGGTGGTGGAGAAATACTGGGTTT-3′, reverse primer 5′-CCGTAATCAGAGCGTGACTTTGC-3′; *L3oh*, accession number AF124817[39], forward primer 5′-CCCCATCACCACCAACTCCA-3′, reverse primer 5′-GCTCCGCCAGCACCCATAG-3′; *Pr*,

accession number AAQ75423[40], forward primer 5′-ACAGCCTGAAGCAGCCTGAA-3′, reverse primer 5′-CGGCAGAACCATCTCAAGGA-3′; *Mr*, accession number AY288138[26], forward primer 5′-CGCTGTTGCTGTTGCTCACTT-3′, reverse primer 5′-GTTTTGGGATGGAATGGATGTG-3′; *Mfs*, accession number AF346833[41], forward primer 5′-GCAGAACGAGGTGCGAGAAG-3′, reverse primer 5′-TGCGAAAGGTGGATGTAGGC-3′; *Fpps*, accession number AW255100[23], forward primer 5′-GGAGAACCATCCAACTGTAA-3′, reverse primer 5′-GCCTTTACAACCAGCCAAGA-3′; *S-TPS*, accession number AW254922[23], forward primer 5′-CGCAAGAGACAGAAATGTGGAG-3′, reverse primer 5′-GTTGGATAGCGTCGGTGAGAAT-3′. The length of PCR product was from 98 to 255 bp.

PCR conditions were determined by comparing threshold values in dilution series of the RT product, followed by non-template control for each primer pair. Relative expression levels of genes were calculated by using the Pfaffl method [42]. A suitable melt curve analysis was always performed.

2.4. Phenolic compounds analyses

2.4.1. Extraction and total phenol content

Control and UV-B irradiated frozen leaves were ground to a fine powder in liquid nitrogen and then extracted in 5 ml 70% (v/v) ethanol (Merck) in ultrasonic bath for 45 min. Samples were then centrifuged for 10 min at 4200 rpm and the total phenolic content was assessed spectrophotometrically on the resulting supernatants by using the Folin–Ciocalteu phenol reagent (Fluka) method and rutin (Merck) as standard [43].

2.4.2. LC–ESI-MS/MS analysis of phenolic extracts

Extracts were analysed using an Agilent ChipCube LC/MS interface (Agilent Technologies, Inc., Palo Alto, CA, USA). The system includes a binary capillary-flow pump system (Agilent Technologies, Inc.): a capillary Agilent 1200 Series pump for loading of sample and an Agilent 1200 Series nano-pump system for LC separation. Mobile phases were degassed with a vacuum degasser (Agilent Technologies, Inc.), while samples were injected via an autosampler. HPLC-Chip Zorbax 300SB-C18 (45 mm × 75 μm 300A) with an HPLC-ChipCube-MS interface (G4340A) (Agilent Technologies, Inc.) has been used for chromatographic separation of compounds. The eluents were: (A) ultra pure water/methanol (Merck) 97:3 + 0.1% formic acid (Merck) (pH 3.00) and (B) methanol/ultra pure water 97:3 + 0.1% formic acid. The same A mobile phase has been used for both capillary and nano-pumps. Gradient for LC separation was 0% B at time 0.00 min, 60% B at 10.00 min, 85% B at 13.00 min, 100% B at 15.00 min and 0% B at 18.00, with a flow rate of 0.70 μl min^{-1} and injection volume of 0.05 μl. Separated compounds were identified by using a 6300 Series Ion Trap Mass Spectrometer equipped with a ChipESI source (Bruker Daltonik GmbH, Bremen, Germany). Analyses were carried in negative mode, capillary entrance was −1.9 kV relative to needle and −500 V relative to endcap. Spray was stabilized with a nitrogen flow at 4.00 l min^{-1} and 325 °C to evaporate solvents in the spray chamber. In the MS^3 experiments, Ultra Scan mode, Target Mass of 600 *m/z*, Trap Drive Level at 60% (Skimmer −40.0 V, Capillary exit −127.0 V, Oct 1 DC −12.00 V, Oct 2 DC −1.74 V, Trap Drive 48.5), Scan Average 5, threshold 500,000, ICC 70,000 200.00 ms, fragmentation amplitude 1.0 V were used and a range of 50–800 *m/z* was acquired. Spectra data were processed and analysed through DataAnalysis for 6300 Series Ion Trap LC/MS 4.0 software (Bruker Daltonik GmbH, Bremen, Germany).

2.5. Statistical analysis

The overall data sets are expressed as mean values of at least three biological replicates. Three technical replicates were run for each biological replicate. Metric bars indicate standard error. ANOVA and following Tukey–Kramer's HSD test were used to determine significant differences among treatments using the SYSTAT 10 software.

3. Results

3.1. Differential expression of genes related to terpenoid biogenesis

Industrial plants such as peppermint show drastic metabolic changes in response to environmental conditions, including light quality and quantity [28], [29] and [30]. One of the open questions related to terpenoid metabolism is whether light differentially modulates the expression of genes involved in terpenoid metabolism [11]. To better understand terpenoid modulation by UV-B light, we exposed peppermint FP and GCP to supplemental UV-B irradiations and evaluated some of the main genes involved in the essential oil biogenesis (Fig. 1).

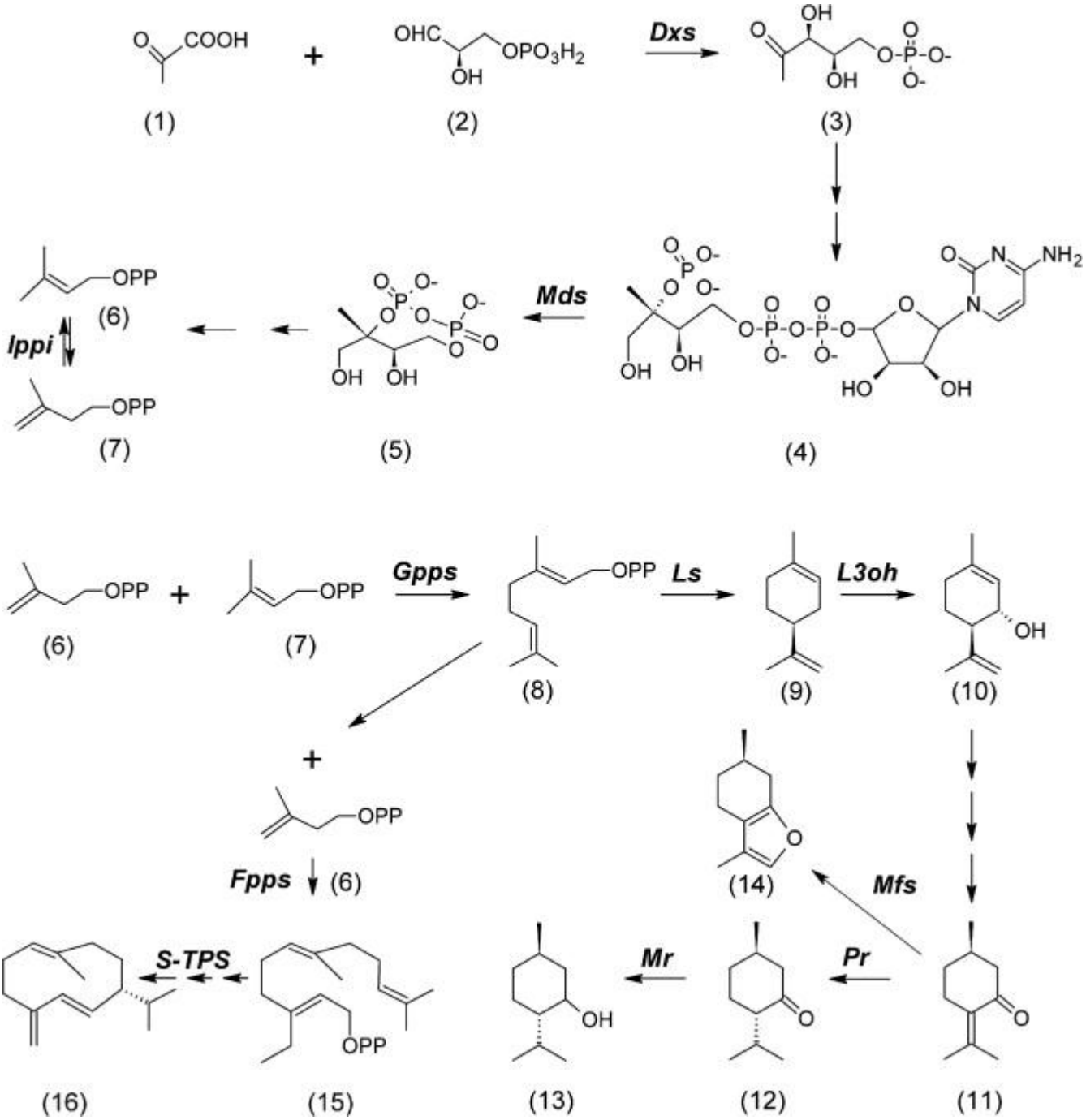

Fig. 1. Outline of monoterpene metabolism in peppermint. Scheme of peppermint genes and molecules studied in this work. *Dxs*, 1-deoxy-d-xylulose-5-phosphate synthase; *Mds*, 2-C-methyl-d-erythritol-2,4-cyclodiphosphate synthase; *Ippi*, isopentenyl diphosphate isomerase; *Gpps*, geranyl diphosphate synthase; *Ls*, (−)-limonene synthase; *L3oh*, (−)-limonene-3-hydroxylase; *Pr*, (+)-pulegone reductase; *Mr*, (−)-menthone reductase; *Mfs*, (+)-menthofuran synthase; *Fpps*, farnesyl diphosphate synthase; *S-TPS*, putative sesquiterpene synthase. (1), pyruvate; (2), d-glyceraldehyde 3-phosphate; (3), 1-deoxy-d-xylulose 5 phosphate; (4), 4-(cytidine 5′-diphospho)-2-C-methyl-d-erithritol; (5), 2-C-methyl-d-erythritol 2,4-cyclodiphosphate; (6), isopentenyl diphosphate; (7), dimethylallyl diphosphate; (8), geranyl diphosphate; (9), (−)-limonene; (10), (−)-*trans*-isopiperitenol; (11), (+)-pulegone; (12), (−)-menthone; (13), (−)-menthol; (14), (+)-menthofuran; (15), farnesyl diphosphate; (16), germacrene-D.

In peppermint GCP, UV-B irradiation induced up-regulation (fold change >2, with respect to control plants) of five genes: *Dxs*, *Ippi*, *Gpps*, *Mr* and *Fpps*, while the expression of *L3oh* and *S-TPS* was down-regulated (fold change <0.5, with respect to control plants). The expression of *Mds*, *Ls*, *Pr*, and *Mfs* did not show significant variations (Fig. 2).

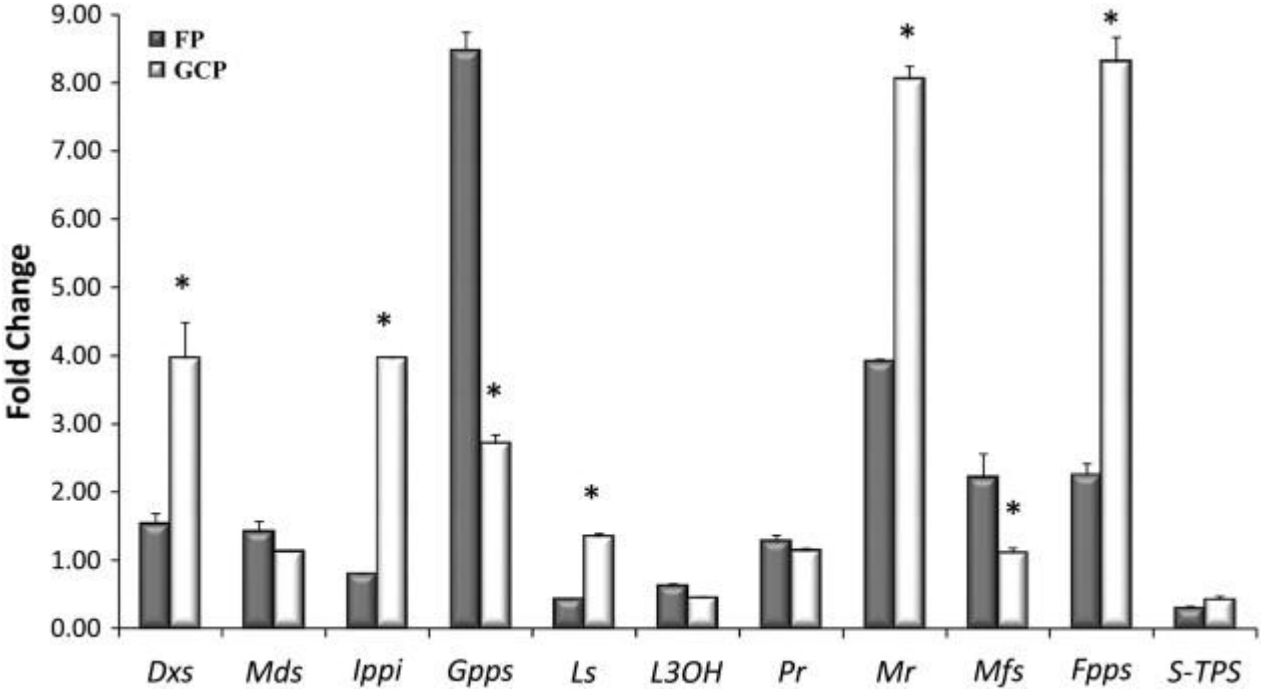

Fig. 2. Effect of UV-B irradiation on peppermint gene expression. Peppermint plants growing in field (FP) and in the growth chamber (GCP) are exposed to supplemental UV-B irradiation. Gene expression is given as fold change, by considering the increase/decrease of UV-B irradiated plant gene expression with respect to not irradiated control plants. For gene legends see Fig. 1. Bars indicate standard error (calculated over at least three replicates), asterisks indicate significant differences ($P < 0.05$) between FP and GCP.

A different pattern of gene expression was found when additional UV-B irradiation was given to FP. After UV-B irradiation, FP showed an up-regulation of four genes: *Gpps*, *Mr*, *Mfs* and *Fpps*. Down-regulation was found for *Ippi*, *Ls*, *L3oh* and *S-TPS* (Fig. 2).

A direct comparison between FP and in GCP reveals a similar gene modulation for *Mds*, *Pr* and *S-TPS*. *Dxs*, *Ippi*, *Mr*, and *Fpps* were significantly more up-regulated in GCP, whereas *Gpps* was significantly more up-regulated in FP (Fig. 2). An opposite trend was found for *Ippi* and *Ls* gene expression, with a down-regulation in FP and up-regulation in GCP (Fig. 2). *L3oh* and *S-TPS* were always down-regulated by UV-B irradiation in both FP and GCP (Fig. 2).

3.2. Chemical composition of peppermint essential oil

The peppermint essential oil composition is characterized by the presence of several monoterpenes and some sesquiterpenes [30] and [44]. In order to evaluate whether terpenoid gene expression correlates with qualitative and quantitative variations in the essential oil chemical composition, we extracted and analysed by GC–MS the volatile fraction of UV-B irradiated peppermint FP and GCP.

GCP showed a total increased content of essential oil with respect to FP, both in control and in UV-B irradiated plants. This was found to depend mainly on the increased contents of (−)-menthone,

(+)-menthofuran and (+)-pulegone in GCP (Table 1). After UV-B irradiation, GCP showed an almost significant increased content of 1,8-cineole, and a significant increase of linalool, (+)-menthofuran, (+)-pulegone, (*E*)-β-caryophyllene and germacrene-D; whereas an almost significant decreased content was found for piperitone and a significant decrease was observed for (−)-menthol and menthyl acetate (Table 1). All other identified compounds were not significantly changed. The effect of UV-B irradiation on peppermint FP was a significant increase in linalool and (+)-pulegone, whereas the only compound showing a significant reduction was menthyl acetate (Table 1).

Table 1. Chemical oil composition of peppermint plants growing in growth chamber (GCP) and in field (FP) after irradiation with UV-B. Data are expressed as μg g^{-1} dry weight. *P* values from the ANOVA between controls and UV-B irradiated plants are indicated.

Compound	GCP			FP		
	Control	**UV-B**	***P***	**Control**	**UV-B**	***P***
(−)-Limonene	7.38	7.6	0.69	6.51	7.44	0.57
1,8-Cineole	1203.19	1460.41	0.06	1406.96	1417.1	0.94
E-(β)-Ocimene	119.73	125.70	0.84	12.9	13.28	0.33
Sabinene hydrate	469.75	514.69	0.31	477.17	513.88	0.21
Linalool	70.06	114.73	0.04	62.4	82.95	0.03
(−)-Menthone	8510.40	7226.96	0.31	6167.04	6832.18	0.13
(+)-Menthofuran	4016.97	5169.42	<0.01	1204.61	1280.83	0.38
(−)-Menthol	2012.88	1376.04	0.02	2848.64	2727.15	0.12
(+)-Cis-Isopulegone	162.92	144.00	0.46	28.27	23.57	0.38
(+)-Pulegone	2441.34	3285.88	0.04	142.76	201.4	0.03
Piperitone	96.41	58.89	0.06	137.17	153.35	0.11
Menthyl acetate	11.64	7.17	0.03	3.34	1.28	0.01
Piperitenone	21.32	20.43	0.34	26.34	31.37	0.25
(*E*)-β-Caryophyllene	279.07	378.02	0.05	465.44	469.67	0.88
Germacrene D	404.90	573.42	<0.01	483.1	503.47	0.42
Total	19853.02	20497.08	0.11	13501.65	14286.64	0.38

A direct comparison between FP and GCP UV-B irradiated plants shows significant differences. In particular, GCP showed a significant increase of 1,8-cineole, linalool, (+)-menthofuran, (*E*)-β-caryophyllene and germacrene-D, with respect to FP. The latter, showed a significant increase of (−)-menthone, (−)-menthol, piperitone and piperitenone, with respect to GCP (Table 1).

3.3. Chemical composition of peppermint phenolic compounds

In peppermint, the total phenol content was always significantly increased by UV-B irradiation, with FP showing significantly higher amounts with respect to GCP (Fig. 3A).

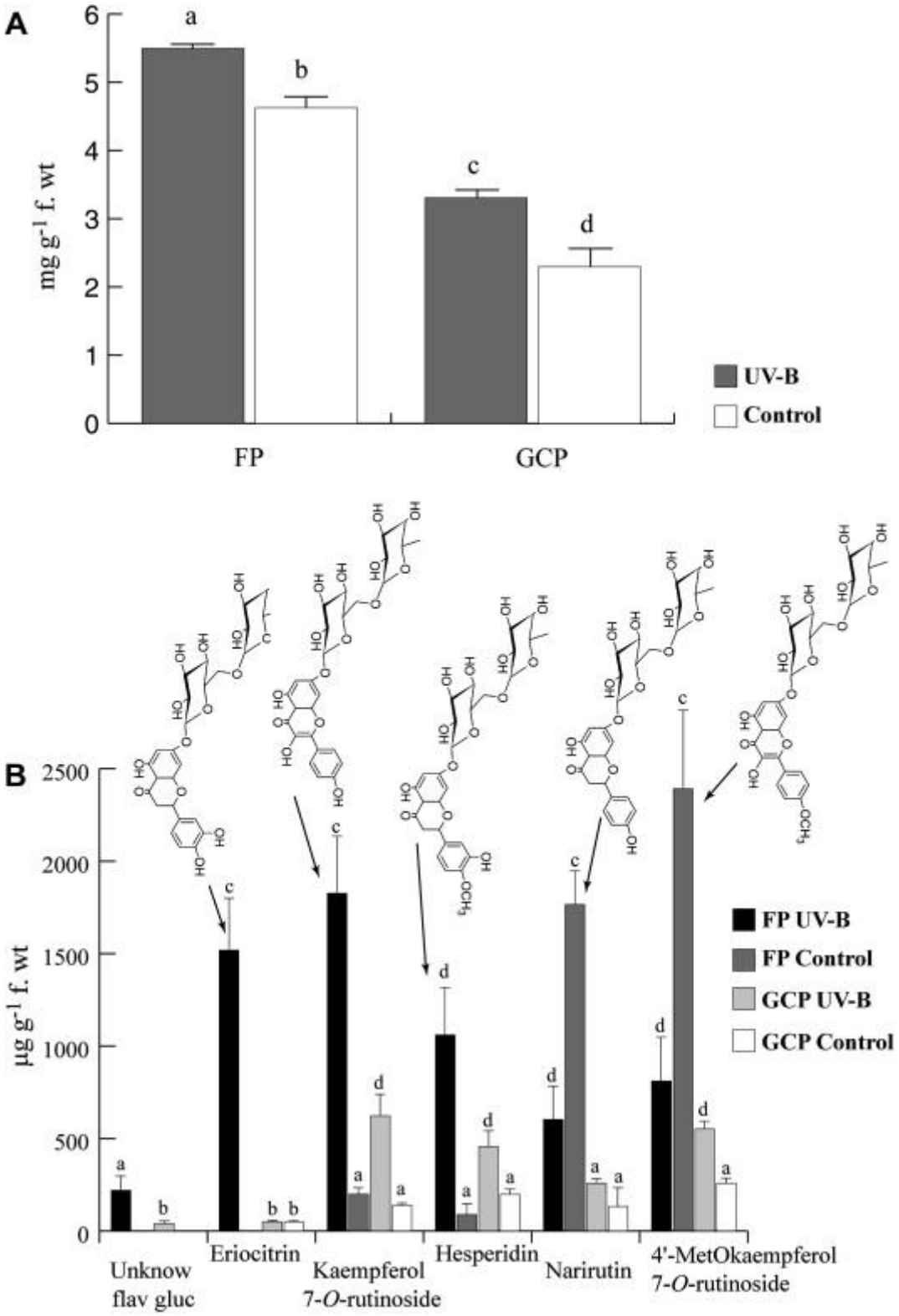

Fig. 3. Effect of UV-B irradiation on peppermint phenolic compounds. (A) Total phenol content of peppermint plant cultivated in field (FP) and in the growth chamber (GCP) in controls and in UV-B irradiated plants. (B) Content of some identified and unidentified flavonoids in controls and UV-B irradiated FP and GCP, along with structure formulae. Bars indicate standard error (calculated over at least three replicates), different letters indicate significant differences ($P < 0.05$).

In general, the qualitative composition of peppermint phenolic compounds was not altered by UV-B irradiation, with the sole exception for an unknown flavonoid glycoside (*m/z*: 637 $[M-H]^-$; MS^2

[637]: 351, 285, 193; MS^3 [351]: 307, 289, 193) that was only present in UV-B irradiated FP and GCP. In FP, the content of eriocitrin (*m*/*z*: 595 $[M-H]^-$; MS^2 [595]: 287; MS^3 [287]: 151, 107), kaempferol 7-*O*-rutinoside (*m*/*z*: 593 $[M-H]^-$; MS^2 [593]: 447, 285; MS^3 [285]: 243, 151, 105) and hesperidin (*m*/*z*: 609 $[M-H]^-$; MS^2 [609]: 301; MS^3 [301]: 286, 283, 257, 125) was always increased by UV-B irradiation, with respect to control; whereas the content of narirutin (*m*/*z*: 577 $[M-H]^-$; MS^2 [577]: 431, 269; MS^3 [269]: 255, 241, 151, 117) and 4′-methoxykaempferol 7-*O*-rutinoside (*m*/*z*: 607 $[M-H]^-$, 461; MS^2 [607]: 284, 299; MS^3 [299]: 284) was reduced by UV-B irradiation, with respect to control. In GCP, the content of eriocitrin and narirutin was not affected by UV-B irradiation, which increased the content of kaempferol 7-*O*-rutinoside, hesperidin and 4′-methoxykaempferol 7-*O*-rutinoside, with respect to control (Fig. 3B). In all analyses, the flavonoid content was always significantly higher in FP with respect to GCP, in agreement with the total phenol data. Interestingly, most of the flavonoids increased by UV-B irradiation showed absorbance peaks in the UV-B range (e.g., eriocitrin, 285 and 326 nm; hesperidin, 285 and 327 nm).

4. Discussion

Plants react to environmental stresses by modulating the expression of genes involved in both primary and secondary metabolism. Industrial plants producing essential oils, such as peppermint, respond to changing environmental conditions through alteration of quality and quantity of the essential oil components [27] and [45]. Light quality was found to modulate peppermint essential oil composition [28], [29] and [30] and different mint species and chemotypes show different responses to enhanced UV-B irradiation [12], [29], [32] and [46]. Furthermore, plants grown in controlled climatic conditions may show different responses to UV-B irradiation with respect to plants grown in natural conditions [1] and [47].

The results of this work show that UV-B irradiation causes the modulation of expression of some peppermint genes involved in essential oil biogenesis and the increase of some phenolic compounds, with particular reference to the flavonoids eriocitrin, hesperidin and kaempferol 7-*O*-rutinoside. The interplay between terpenoid and flavonoid production in response to UV-B is evidenced by the higher content of essential oils in GCP (which show a lower total phenol content) and the reduced amount of terpenoids in FP (that show increased content of phenolic compounds). Most genes directly involved in the biosynthesis of *p*-menthane monoterpenes in peppermint are transcriptionally regulated in a coordinated fashion [48] and it seems likely that the expression of these genes is controlled by a common transcription factor [25]. The effect of increased UV-B irradiation on the expression of genes involved in early steps of terpenoid biosynthesis was observed in GCP for the mevalonate-independent (MEP)-pathway gene *Dxs*, the product of which is considered to catalyze one of the rate-limiting steps of the pathway [49] and [50]. There are clear indications that precursor (IPP and DMAPP) supply is a limiting factor in the biosynthesis of monoterpenes from the plastidial deoxyxylulose phosphate pathway [25] and [51]. Recent investigations have shown that overexpression of *Dxs* improves the production of various types of isoprenoids [25] and [52]. In rice, UV irradiation induced the expression level of *Dxs* up to ninefold compared with that of unirradiated controls [53], suggesting a possible relationship between UV-B light and activation of isoprenoid pathway.

Another gene that was always up-regulated by UV-B irradiation is *Gpps*, the product of which yields the building block for monoterpene biosynthesis, geranyl diphosphate [54]. Overexpression of *Gpps* would be expected to result in increased production of GPP and, consequently, of monoterpene end products [25]. However, in FP and GCP UV-B did not change significantly the total amount of essential oil with respect to control plants, whereas the increased expression of this gene might correlate to the increased (+)-menthofuran production in GCP. The regulatory features

of *Gpps* have not yet been described, but overexpression of the mint gene is now being investigated [20].

The monoterpene giving the economic value to peppermint essential oil is (−)-menthol, and the gene involved in (−)-menthol production is *Mr* [26]. *Mr* expression was always up-regulated by UV-B irradiation, no matter the growing condition. However, the (−)-menthol content was found to be not significantly different from control plants, in all growing conditions.

Despite this common up-regulation, the expression trend of other genes involved in monoterpene and sesquiterpene biosynthesis was clearly different between FP and GCP.

The synthesis of (−)-limonene, in providing the first committed intermediate of the pathway, represents a possible rate-limiting step of monoterpene production in peppermint. However, the expression of *Ls* (e.g. down-regulation in FP) was not affecting significantly the (−)-limonene content of the essential oil. Also in transgenic peppermint overexpressing *Ls* and *L3oh*, expression under the regulation of the 35S viral promoter was insufficient to significantly increase production of essential oil biosynthesis and, thus, no influence was observed on oil composition or yield of the transformed plants [55].

One of the major differences between FP and GCP is the modulation of plastidial vs. cytosolic genes. FP responded to UV-B by inducing a higher expression of the plastidial *Gpps*, whereas GCP responded to UV-B irradiation by a major expression of the cytosolic *Fpps*. While *Gpps* activity could be correlated to monoterpene production, as observed in GCP for (+)-menthofuran, *Fpps* up-regulation might be related to other metabolic pathways. In fact, the product of *Fpps*, farnesyl diphosphate, is located at a key position in the mevalonate (MVA) pathway leading to the synthesis of essential isoprenoids, such as sterols and brassinosteroids, cytokinins, ubiquinone, dolichols, and prenylated proteins [56].

In GCP, the UV-B irradiation prompted a higher content of (+)-menthofuran, a competitive inhibitor of *Pr* [20]. In transgenic lines with increased expression levels of *Mfs* and higher (+)-menthofuran amounts in the essential oil, (+)-pulegone amounts were higher than in controls, which led to the hypothesis that the metabolic fate of (+)-pulegone is controlled by a (+)-menthofuran-mediated transcriptional down-regulation of *Pr* levels [57]. In UV-B irradiated GCP, high contents of (+)-menthofuran always negatively correlate with (+)-pulegone amounts, indicating that UV-B induced (+)-menthofuran content might affect (+)-pulegone content, thus affecting (−)-menthol production [19]. However, expression of *Mfs* was not significantly altered by UV-B, which possibly implies either a posttranscriptional activation of Mfs enzyme activity or catabolic conversion of other monoterpenes such as (+)-pulegone [41].

After UV-B irradiation, GCP also show slightly increased contents of some sesquiterpenes (*E*-β-caryophyllene and germacrene-D), which correlates with *Fpps* up-regulation.

The lack of correlation between gene expression and essential oil production might depend on different rates of terpenoid emissions from the secretory structures. In control, healthy plants the monoterpene volatilization from glandular trichomes occurs at a very low rate [48] and [58]; however, UV-B exposed plants show a significant reduction of cuticular waxes [59] and [60], which constitute the covering of the glandular trichomes [13] and [61]. Thus, we cannot exclude that a significant amount of terpenoids could be lost due to the rupture of glandular trichomes. However, it is not too surprising to observe a poor correlation between gene expression and metabolite accumulation due to the possible numerous posttranscriptional reactions involved.

Our data on comparative essential oil production between FP and GCP are in agreement with previous results, showing that in field plants there is a not significant effect of enhanced UV-B irradiation [12] and [32], while in controlled climatic conditions essential oil composition can be modified [9] and [29]. The latter consideration is particularly important when industrial aromatic plants, such as peppermint, are cultivated in greenhouses [9]. However the results obtained were quite contrasting. On the one hand, we could assess a direct correlation between gene expression and terpene synthesis, as in the case of (+)-menthofuran and *Dxs*, *Ippi* and *Gpps* up-regulation in GCP, but such a correlation was not found for *Mfs*, the gene that codes for the enzyme responsible for (+)-menthofuran biosynthesis. In GCP, (−)-menthol and the sesquiterpenes were not increased, even though the expression of *Mr* and *Fpps* was almost 8-fold with respect to control. The same was found for *Gpps* and terpene synthesis in FP. Hence we focused on other UV-B responsive molecules such as flavonoids.

The accumulation of phenolic compounds is a carefully controlled process with both the levels of phenolics and the composition of the phenolic pool varying considerably in relation to environmental conditions, including UV-B irradiation [6], [62], [63], [64] and [65]. Peppermint leaves contain several flavonoids [14], [15], [16], [17], [66], [67], [68] and [69], and some of these compounds are known to be involved in protection from or elicited by UV radiation [70]. Peppermint phenolic content was increased by UV-B irradiation in both FP and GCP. FP, which are adapted to natural UV-B irradiation showed higher contents of phenolic compounds with respect to either control and UV-B irradiated GCP. The presence of eriocitrin, hesperidin and narirutin has been widely demonstrated in peppermint [14], [15], [17] and [68]. UV-B irradiation of FP and GCP prompts the accumulation of flavonoids absorbing in the UV-B waverange, suggesting a possible shielding activity of these compounds. Light regimes and UV-B radiation have been also found to significantly increase the specific concentrations of flavonoids and other phenolic compounds in other Lamiaceae such as rosemary and basil [64] and [71]. In FP, the observed reduction of narirutin and 4′-methoxykaempferol 7-*O*-rutinoside after UV-B irradiation suggests a possible metabolic transformation of these molecules into other related compounds. In fact, the increase of kaempferol 7-*O*-rutinoside correlates with the decrease of 4′-methoxykaempferol 7-*O*-rutinoside, while the decrease of narirutin is correlated to the increase of the related compounds eriocitrin and hesperidin. The latter being formed by the methoxylation of eriocitrin [72].

The higher flavonoid content observed in peppermint FP probably act as a UV-blocking system in leaf epidermis, thereby decreasing the UV radiation reaching the mesophyll [73] and [74]. We can argue that terpenoid metabolism is less sensitive to UV-B radiation in FP because less UV-B is reaching the mesophyll due to the accumulation of flavonoids. The support to this hypothesis is the lower concentration of essential oils in FP compared to CGP, whereas the opposite is observed for the flavonoids.

In conclusion, the results of this work show that UV-B can induce increased contents of flavonoids and the differential expression of peppermint genes involved in essential oil biogenesis. UV-B irradiated GCP show a different profiling of gene modulation when compared to FP, suggesting an evident adaptation to natural UV-B radiation in the latter, as shown by the increased flavonoid content. Flavonoids are the classical UV-B-regulated compounds in plants, and their biosynthesis and regulation have been very thoroughly explored [10].

Further studies with dose-dependent UV-B irradiations are under way to determine the possible effects on peppermint glandular trichomes and on photoreceptors involved in the signal transduction pathway leading to terpenoid/phenylpropanoid regulation by UV-B irradiation. Although putative UV-B photoreceptors seem to be restricted to the Brassicacae [75], promoter regions that are

essential for the UV-B-driven transcriptional activation have been identified in other plants [10] and [76].

5. Abbreviations

Dxs, 1-deoxy-d-xylulose-5-phosphate synthase; *Mds*, 2-C-methyl-d-erythritol-2,4-cyclodiphosphate synthase; *Ippi*, isopentenyl diphosphate isomerase; *Gpps*, geranyl diphosphate synthase; *Ls*, (−)-limonene synthase; *L3oh*, (−)-limonene-3-hydroxylase; *Pr*, (+)-pulegone reductase; *Mr*, (−)-menthone reductase; *Mfs*, (+)-menthofuran synthase; *Fpps*, farnesyl diphosphate synthase; *S-TPS*, putative sesquiterpene synthase; FP, peppermint plants cultivated in field; GCP, peppermint plants cultivated in the growth chamber.

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