Biological activity of essential oils of *Mentha pulegium* from field-grown and acclimated *in vitro* plants

Amira Benahmed[1,2,*], Boualem Harfi[1] and Abdelmalik Belkhiri[3]

[1]Biotechnology Research Center (C.R.Bt), Nouvelle Ville – Ali Mendjeli 25000, Constantine, Algeria
[2]Department of Biology and Plant Ecology, Faculty of Natural Sciences and Life, Route Ain Elbey 25000, University Constantine 1, Algeria
[3]Laboratory of Pharmacognosy, Faculty of Medicine, University Constantine 3, Nouvelle Ville – Ali Mendjeli 25000, Constantine, Algeria

In this study we discuss *Mentha pulegium* shoot multiplication and the capacity of *in vitro* plants to produce essential oils (EOs). EO was obtained by hydrodistillation from dried leaves and flowering stems of *in vitro* and field-grown plants, and their chemical composition was examined by GC-MS. The differences in yield as well as quantitative and qualitative composition between the oils extracted from *in vitro* and field-grown plants were observed. *M. pulegium* EOs were evaluated for their antioxidant and antimicrobial activities. It was found that antimicrobial activity of EOs extracted from acclimated *in vitro* plants was higher than those from field-grown *M. pulegium* plants. Our aim was to perform phytochemical screening of leaves from a wild-growing plant and compare the results with material produced using biotechnological methods based on tissue culture.

Keywords: Acclimatization, biological activities, essential oils, *in vitro* plants, *Mentha pulegium*.

THE *Mentha* genus includes about 20 species and is considered as one of the largest genera in the family Lamiaceae. *Mentha* species have been used in folk medicine due to their biological properties (antibacterial, antioxidant, anti-inflammatory, stimulant, emmenagogue, anticatarrhal, etc.)[1–4].

Mentha pulegium is a herbaceous plant[5]. The dried aerial flowering parts of *M. pulegium*, locally called 'flioyou', are used in traditional North Africa medicine due to their carminatives and antispasmodic properties in the treatment of flatulence and intestinal colic[6]. The most important biologically active constituents of *M. pulegium* are essential oils (EOs)[7]. Also, other bioactive molecules such as phenolic compounds have been isolated from plant tissue of *M. pulegium*[8].

After the active growth phase and under conditions of deficiency, examination, by some authors, of the metabolic pathways of *M. pulegium*, revealed that the biological importance of many secondary metabolites is not known precisely.

*For correspondence. (e-mail: ami_mouta@yahoo.fr)

EOs have been the subject of several studies[9,10], and different chemotypes have been characterized. Previous reports[9,10] on the composition of *M. pulegium* EO showed that pulegone was the main compound (25–92%).

As known, the biological activity of an EO is linked to its composition, the aim of our study was to evaluate the influence of acclimatization conditions on the chromatographic profile of the EOs of the selected plants.

Mentha pulegium L. plants were collected during flowering in June 2014 for extraction of EOs and juvenile stage in March 2014 for *in vitro* micropropagation, at Beni Hamidene (long. 6.55, lat. 36.5167, elevation 300 m amsl), 16 km northwest of de Constantine, Algeria. Identification of species was based on the botanical characteristics of Quezel and Santa[5].

For the establishment of *in vitro* plants, shoot tips with leaves of *M. pulegium* plants harvested from the same location as defined above, were disinfected with 10% sodium hypochlorite solution containing two drops of Tween 20 for 10 min. This step was followed by treatment with ethanol at 70% for 30 sec, and three passages in sterile distilled water. Then, nodal fragments were aseptically deposited on Murashige and Skoog[11] (MS) agar medium (0.8%) supplemented with sucrose (3%) and different hormonal combinations (gibberellin (GA3) and 6-benzylaminopurine (BAP)). Culture flasks were kept in a culture chamber set at 23°C with a photoperiod 16 h/8 h (light/dark). Subculturing was carried out every 3–4 weeks. The efficiency of cytokinin/gibberellins combinations was determined by recording the stem length and node number.

Shoot culture multiplication was realized on MS agar medium (0.8%) to which 0.5 mg/l BAP and 1 mg/l GA3 were added. All media pH values were fixed at 5.6–5.8. The media were steam-sterilized in an autoclave at 121°C for 20 min. The cultures were grown at 23° ± 2°C with a long photoperiod (16 h light/8 h dark) and 70% humidity level. Subculturing was carried out every 3–4 weeks.

Three-weeks-old plantlets from shoot-tip explants were used for the acclimatization of *in vitro* plants. The plants were removed from the culture flasks and the roots washed in tapwater to remove agar traces. They were then transplanted into pots (7 cm diameter) containing a sterilized mixture of sand and peat (1 : 1 v/v). The pots were acclimated at 23° ± 2°C with a 16 h light/8 h dark photoperiod and 70% humidity level. The plantlets were moistened by spraying with distilled water 3–4 times per day during the first week and then irrigated twice a week. Irrigation was carried out by alternating between distilled water and an irrigation mineral solution (1/10 m/v).

The potted plants were maintained in the growing chamber for a period of 70 days (estimated as the flowering period), under conditions of 23° ± 2°C, photoperiod of 16 h/8h (light/dark) and 70% humidity level. The plantlets underwent abiotic stress (temperature, light) for one week. The aim was to evaluate the influence of various

stress conditions, described below, on the composition, yield and bioactivity of EOs.

The stress parameters of the culture were as follows: Thermic stress: 35° ± 2°C and 16° ± 2°C versus 23° ± 2°C (control); light stress (photoperiod light/dark): 8 h/16 h, continuous dark versus 16 h/8 h (control).

EO from wild and *in vitro* plants was obtained by hydrodistillation (100 g dry matter) with a Clevenger-type apparatus for a period of 3 h. To remove water traces after extraction, anhydrous sodium sulphate (Na_2SO_4) was used. The extracted oils were stored in brown hermetic tubes at 4°C.

For GC-MS analysis, a Hewlett Packard Agilent 6890 plus equipped with a mass-selective detector Hewlett Packard Agilent 5973 with quadruples mass analyser and an HP-5MS-fused silica column (30 m × 0.25 mm × 0.25 µm film thickness) were employed. Parameter of the column were: 60°C holds 8 min to 250°C at 2°C/min and then holds 10 min. Helium (N6) carrier gas was used at a flow rate of 0.5 ml/min. The detector was maintained at 250°C. The volume of sample injection was 0.2 µl with a split ratio of 10 : 1. Mass spectra were recorded in the electron-impact (EI) mode at 70 eV and 1.8 scans/s; *m*/*z* 30–550 was the mass range used; ion source temperature was 230°C while temperature of the connecting parts was 270°C.

Identification of compounds was based on a comparison of their mass spectra with those of reference standards (NIST02.L, NBS/Wiley 7N.1) in mass spectra libraries, and those published in the literature[12], as well as on the comparison of their retention indices (RIs) relative to C8–C28 (*n*-alkanes).

Quantitative analysis was done employing the normalization method, by the electronic integration of the TIC peak areas without the use of correction factors.

EO was tested against five selected pathogenic microbial strains obtained from the American Type Culture Collection (ATCC; supplied by the Pasteur Institute, Algiers, Algeria). These include two strains of Gram-positive bacteria (*Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923)), one strain of the Gram-negative bacteria (*Salmonella enteritidis* (ATCC 13076)), one fungus (*Aspergillus brasiliensis* (ATCC 6404)), and one yeast (*Candida albicans* (ATCC 10231)). The bacterial strains were maintained at 4°C on agar slant.

Microbial cultures were grown in appropriate media, Muller–Hinton agar (MHA) for bacteria and Sabouraud dextrose agar (SDA) for fungi, as previously reported[13]. Three–five well-isolated and perfectly identical colonies from young bacterial and fungal (18 h and 48 h respectively) cultures were removed by loops and placed into tubes containing 5 ml sterile physiological water at 0.9%. After agitation, the measured transmittance values of bacterial and fungal suspension cultures were adjusted between 22% and 32% and 2% and 3% respectively. These values correspond to a concentration of 10^7–10^8 CFU/ml. Petri dishes of 9 cm diameter were first filled with 15 ml of the appropriate agar medium and left to rest until solidification of the agar. Then, 200 µl of each of the microbial suspensions was added aseptically to 50 ml of each of the appropriate liquid culture media. After agitation, 4 ml of the inoculated agar medium was poured as a second layer on the petri dishes. After solidification of the agar, blank sterile disks (S70150A; diameter 6 mm) impregnated with 10 µl of EO were positioned on the surface of MHA- and SDA-inoculated culture media. Petri dishes were kept on the bench for 30 min to allow diffusion of EOs. Incubation was carried out at 37°C after 24 h for bacterial cultures and at 25°C after 48 h for fungal cultures. The growth inhibition zone diameters (mm) were carefully measured with a digital calliper. Each treatment was repeated three times for reproducibility.

DPPH (2,2-diphenyl-β-picrylhydrazyl) was used as a reagent in order to measure free-radical scavenging activity[14]. Butylated hydroxyanisole (BHA) used as the positive control. A twofold serial dilution from stock extract solution (4 µg/ml) was carried out with methanol to obtain test solutions in the range 10,000–156 ppm. Next, 3 ml of DPPH methanol solution (4×10^{-5} M) was added to 1 ml of sample solutions of different concentrations and butylated hydroxyanisole solution separately. All tests were repeated thrice. The reaction mixture was shaken and then incubated for 60 min at room temperature. The amount of DPPH remaining was determined at 517 nm against a blank (methanol with DPPH) using a 96-well micro-plate reader, spectra (Perkin Elmer, EnSpire Multilabel Reader 23001154).

Optical density was recorded and % inhibition was calculated using the formula

$$\% \text{ Inhibition of DPPH activity} = [(A_{\text{cont}} - A_{\text{samp}})/A_{\text{cont}}] \times 100.$$

where A_{cont}: is the optical density of the blank and A_{samp} is the optical density of the sample.

Inhibitory concentration relative to 50% of radicals (IC_{50}) was determined from the equation obtained by plotting the linear curve of inhibition percentage against concentration. DDPH free scavenging ability was considered significant if the IC_{50}-value was low[15].

All measurements were repeated three times for each assay. Results are presented as mean ± SD. Data were analysed (ANOVA, Tukey test) using Windows SPSS, version 20.0. For compared treatments, *P*-values ($P < 0.05$) were regarded as statistically significant.

The present study initially focused on obtaining *in vitro* plants from nodal fragment cultured on MS medium supplemented with different hormonal combinations (BAP and GA3). The methodology used for explant

Table 1. Effect of 6-benzylaminopurine (BAP) and gibberellin (GA3) on induction of shoots and multiplication of *in vitro* plant

Medium	Hormonal balance	Characteristics of *in vitro* plants
Obtaining *in vitro* plants		
M1	0 mg/l BAP, 0 mg/l GA3	Short shoots with serried nodes
M2	0.5 mg/l BAP, 0 mg/l GA3	Medium-sized shoots with serried nodes
M3	0.5 mg/l BAP, 1 mg/l GA3	Medium-sized shoots with well-separated nodes
M4	1 mg/l BAP, 0 mg/l GA3	Long shoots with serried nodes
M5	1 mg/l BAP, 1 mg/l GA3	Long shoots with well-separated nodes
Multiplication of *in vitro* plants		
M6	0 mg/l BAP, 0 mg/l GA3	Short *in vitro* plants malformed
M7	0.5 mg/l BAP, 0 mg/l GA3	Long *in vitro* plants, serried nodes
M8	0.5 mg/l BAP, 1 mg/l GA3	Long *iv vitro* plants, distinct nodes with high multiplication rate
M9	1 mg/l BAP, 0 mg/l GA3	Medium-sized *in vitro* plants, serried nodes
M10	1 mg/l BAP, 1 mg/l GA3	Medium-sized *in vitro* plants, distinct nodes

sterilization was effective in establishing aseptic cultures with a high rate of shoot proliferation. Table 1 shows the results of the effect of plant growth phytohormones on the induction of shoots and multiplication of *in vitro* plants.

Our results show that M5 medium is the best for induction of shoots, while for multiplication, M8 medium is the most suitable. The two media, viz. M5 and M8 were retained for further use.

The action of the phytohormones depends on their concentration at a specific place and time; so it depends on the balances between different phytohormones.

Sun *et al.*[16] reported that cytokinin concentration in the culture medium is critical for shoot organogenesis. The promoter effect of BAP in the induction of multiple shoots has also been reported[17].

Although cytokinins promote the proliferation of axillary shoots, their inhibitory role on shoot elongation is well known[18], which confirms the results obtained in this experiment. For multiplication of *in vitro* plants, hormonal balance maintained for the cytokinin is 0.5 mg/l, a decrease in the concentration of BAP in relation to that of initiation of shoots.

Taiz and Zeiger[19] reported that GA3 inhibits auxin action in meristems and stimulates shoot elongation. The promoter effect of GA3 on shoot elongation in BAP-containing media has been reported in different species[17].

Jordan *et al.*[20] demonstrated the importance of BAP for the multiplication of *Lavandula dentata* and other species of the same genus[21].

Echeverrigaray *et al.*[22] reported a high multiplication rate of *L. dentata* using MS medium supplemented with 2.2 μM (equivalent of 0.5 mg/l) BAP, with an average shoot length of 3.53 cm. Zuzarte *et al.*[18] demonstrated that nodal segments were easier to multiply than axillary buds, and that the largest number of shoots with an average length greater than or equal to 0.5 cm for nodal segment explants was obtained in a medium supplemented with 0.25 mg/l BAP.

Since the shoots rooted spontaneously, no further treatment with the auxins was necessary to promote rooting. This result is consistent with that obtained by Zuzarte *et al.*[18]. This is an advantage for *in vitro* propagation because it allows an important gain of time and avoids the use of auxins.

The *in vitro* rooted plants were successfully acclimatized, with a total survival rate exceeding 95% (Figure 1). Acclimated plants appear normal and show no abnormalities or morphological variations. The protocol of acclimatization used by Arikat *et al.*[23] gave a survival rate of 80%. Paul *et al.*[17] achieved acclimatization with a survival rate between 96% and 100%.

Table 2 shows GC-MS analysis results of *M. pulegium* EOs from naturally grown and *in vitro* plants grown under culture conditions (temperature and light). The *M. pulegium* EO content in wild plants was 0.97% ± 0.06% (v/w). The yield obtained at blooming stage is comparable within the values (0.7–2.3%) reported by other researchers from Algerian wild *M. pulegium* species[24–26].

Oil GC-MS analysis revealed a total of 29 compounds, representing 94.82% (area per cent) of the total oil (Table 1), mostly dominated by oxygenated monoterpenes (87.43%) such as isomenthone (45.76%), pulegone (29.57%) and menthone (6.50%). The predominance of isomenthone over the other constituents places the species in the isomenthone chemotype.

In spite of the common belief that *M. pulegium* EO is always rich in pulegone, exceptions are not rare in the wild. Previous studies have outlined that wild populations of *M. pulegium* plants are marked by the existence of several chemotypes[27,28]. In a large-scale study, Kokkini *et al.*[28] have reported that the pulegone content varies greatly in wild pennyroyal Greek populations, ranging from 0.1% to 90.7% of the total oil[28]. They found that the oils of two populations have traces of pulegone (≤0.1%),

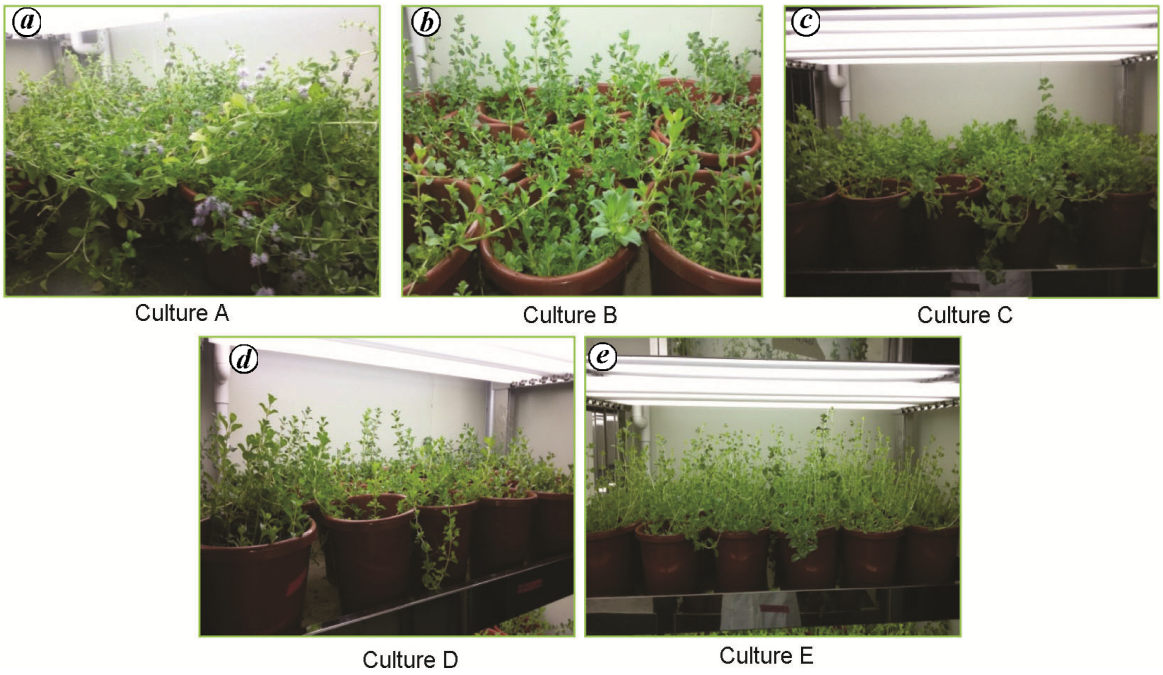

Figure 1. Acclimatized plants in different conditions. Culture A – 23°C, 16 h, light/8 h, dark, Culture B – 16°C, 16 h, light/8 h dark, Culture C – 35°C, 16 h, light/8 h dark and Culture D – 23°C, 16 h, dark/8 h, light, Culture E – 23°C, total darkness.

while the amount of piperitone exceeds 80%. In a study of an Austrian *M. pulegium* population, 70% piperitone was reported, whereas pulegone was not present in the oil[29].

Acclimatization of *M. pulegium* (culture A) under conditions of 23°C temperature and photoperiod cycle 16 h light/8 h dark showed yield and composition of EOs to be similar to those of wild plants (Table 2), with the chromatographic profile marked by a predominance of isomenthone and pulegone (58.52% and 25.26% respectively).

However, comparative evaluation of EOs produced from wild growing and *in vitro* plants that have undergone heat or light stress (cultures B–E) showed significant differences, especially in the yield and for one of the cultures (culture C) in the composition (Table 2). A rise in the acclimatization temperature to 35°C (culture C) or its lowering to 16°C (culture B), while maintaining a photoperiod cycle of 16 h light/8 h dark, leads to significant increase in the yield of EOs with respective values of 1.84% and 1.16%, compared to control (0.94%, culture A). Similarly, a reduction in the light exposure time (culture D) or in case of total darkness (culture E), while maintaining a temperature of 23°C, leads to significant increase in the yield of EOs with respective values of 1.37% and 1.65%, compared to control (0.94%, culture A). The highest yield (1.84 ± 0.01 ml/100 g dry wt) of EO was recorded for culture C, which was subjected to a temperature stress of 35°C. In addition to its high yield, culture C provided an EO composition quite different compared to those of other *in vitro* cultures (cultures A, B, D and E) and that of field-grown plants. Chromatographic profile of EO of culture C was dominated by pulegone (49.18%) and *l*-menthone (26.40%), while isomenthone was not detected. Similarly, the levels of piperitenone, 8-hydroxy-delta-4(5)-*p*-menthen-3-one and (1*RS*, 4*SR*)-8-hydroxy-*p*-menthane-3-one were higher in culture C compared to cultures A, B, D and E.

The biosynthesis of *p*-menthane in mint species, has been characterized by Lawrence[30]. In *M. pulegium*, limonene is hydroxylated at the C3 position to trans-isopiperitenol and the pennyroyal-type species produces monoterpenes bearing an oxygen function at C3, such as pulegone – a highly hepatotoxic compound[31]. Piperitenone is considered to be an essential intermediate in the biosynthesis of C3-oxygenated *p*-menthane monoterpenes in mint species[31]. Since menthone is the precursor of menthol, isomenthone is the precursor of isomenthol and pulegone is the precursor of menthone and isomenthone in the biosynthesis of monoterpenes in the genus *Mentha*. Results may indicate possible variations in the biosynthetic pathways of the studied populations.

Our results indicate a possible conversion of pulegone to menthone instead of isomenthone in culture C maintained at 35°C and photoperiod cycle of 16 h light/8 h dark. The absence of isomenthone in culture at high temperature suggests that the reductive enzymes catalysing the conversion of pulegone to isomenthone are not optimally operative in the plantlets. This observation is consistent with other studies, where micropropagated

Table 2. Yield and composition of essential oils from naturally grown and *in vitro* plants

Compounds	RI	Wild plants (%)	*In vitro* plants (%[a]; temperature/light: dark)[b]				
			A (23°C/16 : 8 h)	*B* (16°C/16 : 8 h)	*C* (35°C/16 : 8 h)	*D* (23°/8 : 16 h)	*E* (23°C/0 : 24 h)
Yield (ml/100 g dry matter)	–	0.97 ± 0.06	0.94 ± 0.01	1.16 ± 0.06	1.84 ± 0.01	1.37 ± 0.01	1.65 ± 0.01
α-Pinene	931	0.19	0.05	0,29	0.11	0.22	0.20
Sabinene	971	0.06	0.12	0.17	0.02	0.36	0.15
β-Pinene	974	0.18	0.06	0.19	0.09	0.17	0.16
Octan-3-one	986	0.25	0.11	0.14	0.15	0.13	0.13
Octan-3-ol	998	2.30	1.20	1,07	1.07	0.92	0.94
o-cymene	1023	nd	nd	tr	tr	0.09	0.06
Limonene	1027	0.18	2.69	1.76	0.79	3.38	1.47
1,8-Cineole	1029	0.08	0.07	0.06	0.12	0.07	0.08
Octan-3-ol, acetate	1126	0.86	nd	nd	0.08	0.51	0.51
Limonene oxide, *cis*	1132	nd	0.10	nd	nd	nd	0.04
Limonene oxide, *trans*	1137	nd	0.08	nd	0.09	nd	nd
L-Menthone	1155	6.50	1.11	0.73	26,40	1.02	1.52
Isomenthone	1174	45.76	58.52	47.74	nd	51.98	74.25
Isopulegone	1179	0.80	0.58	0.63	0.98	0.65	0.44
Neomenthol	1184	nd	nd	0.11	nd	0.14	nd
(+)-Carveol, *trans*	1226	nd	0.12	nd	0.24	nd	tr
Carveol, *cis*	1230	nd	nd	0.09	nd	0.14	nd
Pulegone	1248	29.57	25.26	40.88	49.18	32.72	13.96
(1*RS*, 4*SR*)-8-Hydroxy-*p*-menthane-3-one	1252	0.11	0.03	nd	0,61	nd	nd
Piperitone	1257	1.88	1.44	1.50	0.44	1.98	2.69
5-(1′,1′-Dimethylethyl) bicycle [3.1.0]hexan-2-one	1285	0.51	0.53	0.23	nd	0.29	nd
8-Hydroxy-delta-4(5)-*p*-menthen-3-one	1291	1.32	1.11	0.52	4.21	0.56	0.34
Carane, *trans*	1308	0.56	0,11	0,18	nd	nd	0.20
Piperitenone	1343	2.86	nd	1.62	3.48	2.62	0.80
Piperitenone oxide	1365	0.06	0.26	nd	nd	0.06	nd
Mint furanone 1	1495	0.14	nd	nd	0.27	nd	nd
Mint furanone 2	1527	nd	0.10	nd	0.05	0.07	nd
Caryophyllene oxide	1580	0.22	0.14	0.07	0.06	0.08	0.08
Humulene-1,2-epoxide	1607	0.43	0.49	0.27	0.15	0.29	0.30
Monoterpènes		0.69	3.11	2.47	1.37	4.29	2.12
Monoterpenesoxygenes		87.43	87.35	93.1	80.57	91.03	93.7
Sesquiterpenes		0.65	0.63	0.34	0.21	0.37	0.38

nd, Not detected; Tr, Traces (<0.05%); RI, Retention index. [a]Normalized peak area abundances without correction factors. [b]Culture conditions.

shoots accumulate pulegone as the main constituent instead of menthone and menthol[32]. Alternatively, a reversible reaction such as conversion of isomenthone or menthone to pulegone is also possible.

Plant development factors and environmental conditions have been shown to significantly influence the performance and composition of peppermint oil, with implications for the commercial production of this product[32]; however, the specific steps of monoterpene metabolism influenced by these changes (growth factors and environment) are not known.

Clark and Menary[33] reported that long photoperiod (16 h light), high night temperature (20°C) and high light density improve the yield of EOs. Also, these are determining factors of EO composition.

Studies on the biosynthesis, metabolism and accumulation of monoterpenes reveal the influence of endogenous factors (genetics and stage of development), as well as environmental factors (temperature and luminance)[32]. It can be supposed that the origin of the reported compositional changes in our study is not due to genetic differences, as *in vitro* cultures were vegetatively propagated from a single stock of mother plants.

The EO samples from field-grown and *in vitro* plants were examined for their antioxidant and antimicrobial activities. The antioxidant assay revealed that all samples of *M. pulegium* EO and BHA act as a free-radical scavenger by reducing DPPH (purple colour) to 2,2-diphenyl-1-picryl hydrazine (colourless; Table 3). The IC_{50} values ranged from 11.99 to 51.97 mg/ml. According to the categories defined by Scherer and Godoy[34], all the *M. pulegium* EOs tested in this study revealed poor antioxidant activity (antioxidant activity index (AAI) values ranged from 0.005 to 0.001). Previous studies have reported wide variation in the antioxidant activity of *M. pulegium* EO. For example, EO of *M. pulegium*

Table 3. Results of 2,2-diphenyl-β-picrylhydrazyl radical scavenging assay obtained with essential oils

Essential oils	% Inhibition (ppm)							IC_{50} (mg/ml)
	156.25	312.5	625	1250	2500	5000	10000	
Wild plant	0.86 ± 1.45	0.44 ± 1.06	0.33 ± 0.44	–1.08 ± 1.34	4.56 ± 0.56	12.57 ± 2.95	22.88 ± 6.86	22.93 ± 6.58
Culture A	–2.53 ± 1.04	–2.29 ± 0.60	–1.85 ± 2.09	–0.48 ± 1.08	0.84 ± 2.61	5.42 ± 1.44	14.60 ± 2.14	30.88 ± 7.54
Culture B	3.31 ± 1.14	0.87 ± 1.45	1.68 ± 0.99	5.82 ± 1.19	7.79 ± 1.72	15.88 ± 0.60	23.34 ± 6.09	29.28 ± 18.14
Culture C	–0.20 ± 0.18	1.36 ± 0.80	2.37 ± 0.74	6.18 ± 0.39	11.91 ± 2.34	24.55 ± 1.32	41.84 ± 2.60	11.99 ± 0.43
Culture D	–1.61 ± 3.10	–0.52 ± 0.64	–2.34 ± 3.04	1.64 ± 2.48	4.20 ± 1.10	7.72 ± 2.00	22.18 ± 1.05	21.52 ± 0.51
Culture E	3.86 ± 1.21	4.55 ± 1.28	2.83 ± 0.92	4.40 ± 2.94	6.35 ± 2.06	8.60 ± 3.74	14.84 ± 2.73	51.97 ± 32.73
Butylated hydroxyanisole[b]	90.54 ± 0.10	91.06 ± 0.21	91.47 ± 0.18	92.10 ± 0.00	92.51 ± 0.52	–	–	0.006 ± 0.13

Table 4. Diameter of zones of inhibition (mm)

Multiple (cross) effects between EO and strains (post hoc test)		Diameter of inhibition zones (mm)	Classification into homogeneous groups of EO activity	Classification into homogeneous groups of susceptibility of strains
EOs	Strains			
Wild plant	*Staphylococcus aureus*	20.33 ± 5.69	38.53 ± 26.76A	19.22 ± 2.80c
	Bacillus subtilis	14.67 ± 1.53		
	Salmonella enteritidis	17.67 ± 2.08		
	Aspergillus brasiliensis	70.00 ± 0.00		
	Candida albicans	70.00 ± 0.00		
Culture A	*Staphylococcus aureus*	20.00 ± 1.73	37.40 ± 22.01A	
	Bacillus subtilis	22.67 ± 1.15		16.83 ± 3.93c
	Salmonella enteritidis	20.33 ± 4.04		
	Aspergillus brasiliensis	53.33 ± 14.47		
	Candida albicans	70.00 ± 0.00		
Culture B	*Staphylococcus aureus*	20.00 ± 1.73	29.60 ± 21.45B	
	Bacillus subtilis	17.67 ± 2.52		
	Salmonella enteritidis	13.67 ± 3.51		17.28 ± 5.09c
	Aspergillus brasiliensis	70.00 ± 0.00		
	Candida albicans	26.67 ± 2.31		
Culture C	*Staphylococcus aureus*	17.33 ± 1.53	30.53 ± 20.87B	
	Bacillus subtilis	15.67 ± 0.58		
	Salmonella enteritidis	25.00 ± 4.36		
	Aspergillus brasiliensis	70.00 ± 0.00		67.22 ± 8.09a
	Candida albicans	24.67 ± 1.15		
Culture D	*Staphylococcus aureus*	18.00 ± 1.73	27.07 ± 23.03B	
	Bacillus subtilis	11.33 ± 2.08		
	Salmonella enteritidis	13.33 ± 1.15		
	Aspergillus brasiliensis	70.00 ± 0.00		
	Candida albicans	22.67 ± 11.55		38.00 ± 24.17b
Culture E	*Staphylococcus aureus*	19.00 ± 2.65	27.13 ± 22.59B	
	Bacillus subtilis	19.00 ± 1.73		
	Salmonella enteritidis	13.67 ± 0.58		
	Aspergillus brasiliensis	70.00 ± 0.00		
	Candida albicans	14.00 ± 8.72		

Values are expressed as mean ± standard deviation. 70: total inhibition. Means with different letters are significantly different ($P < 0.05$). Lowercase letters: classification into homogeneous groups of strains. Upper case letters: classification in homogeneous groups of EOs.

showed high antioxidant activity (AAI = 1.6 in Hajlaoui *et al.*[35]). The contrary was also true (AAI = 0.003 in Kamkar *et al.*[36]; AAI = 0.01 in Teixeira *et al.*[37]).

Temperature and light effects on EO composition showed no significant impact on antioxidant activity. The lowest IC_{50} value (11.99 ± 0.43 µg/ml) obtained by EO

produced from *in vitro* cultures subjected to temperature stress of 35°C has been classified as poor antioxidant activity[34].

It is reported that antioxidant activity may be partially due to phenols. These are organic compounds that contain a hydroxyl group bound directly to the aromatic ring. The H-atom of the hydroxyl group can trap DPPH radicals, thus preventing other compounds from getting oxidized[38]. EO had statistically the lowest activity, may be in part due to the presence of phenols.

Table 4 presents results of susceptibility essays of pathogenic microorganisms to EOs. Among the microorganisms tested, *Aspergillus brasiliensis* was the most sensitive to all EOs of *Mentha pulegium*, with mean diameter of inhibition being 67.22 ± 8.09 mm. *Candida albicans* strain was more sensitive to EOs from naturally grown plants and *in vitro* plants acclimated under conditions of 23°C and 16 h light photoperiod (culture A). All the bacteria tested showed moderate sensitivity to *M. pulegium* EOs.

EOs of wild *M. pulegium* and acclimated plants (culture A) have maximum antimicrobial activity compared to those of other acclimated plants (cultures B–E).

The studied *M. pulegium* EOs are effective against the tested strains, showing that Gram-positive and Gram-negative bacteria have the same sensitivity to EOs. However, Mahboubi and Haghi[39] reported strong antimicrobial activity of *M. pulegium* EO against *Staphylococcus aureus*, *Staphylococcus epidermis* and *B. cereus*. Whereas the least susceptible were Gram-negative bacteria especially *Escherichia coli*. According to previous studies, the low sensitivity of Gram-negative bacteria to the action of EO is explained by the presence of a second membrane which has hydrophilic chains of polysaccharides (LPS) acting as barriers to EO having a hydrophobic character. In the present study, it is suggested that the volatile compounds (small amount) that enter through this membrane are able to kill the bacteria.

M. pulegium EO antimicrobial activity is attributed to its high pulegone content[40]. The antimicrobial activity of EOs is directly related to the nature of their major constituents and their functional groups. Also, the possible synergistic interactions between the different constituents.

Generally, it is accepted that oxygenated monoterpenes are more reactive than hydrocarbon monoterpenes[41]. The chemical composition of EOs determines their antimicrobial activity[37]. Often, their main constituents were responsible of their biological activities[40]. It has been reported that the antibacterial activity of *M. pulegium* could be attributed to its major compounds[35–39]. However, it has also been reported that minor compounds play an important role. It is difficult to determine which compounds or mixtures are responsible for the activity[40]. According to Mahboubi and Haghi[39], the synergistic effects between the different constituents (major and minor) may explain the antimicrobial activity of mint EOs.

Despite the importance of the species as a medicinal and aromatic culture with industrial potential, research on *M. pulegium* is still limited. So far, there are no published reports in Algeria regarding the effect of *in vitro* culture and acclimatization conditions on accumulation of EOs, as well as on biological activity of EOs *in vitro* plantlets.

In conclusion, our work shows that the micropropagation of *M. pulegium* by the *in vitro* proliferation of nodal fragment is a reliable method for rapid multiplication, allowing the production of the same secondary metabolites as those found in the wild plants.

Successful experimental models to increase yields or manipulate chemical profiles suggest that future commercial exploitation is possible. *In vitro* plant cultures can be used for large-scale reproduction of EO-producing plants, which is important for the conservation of natural genetic resources.

1. Daferera, D. J., Ziogas, B. N. and Polissiou, M. G., The effectiveness of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *Michiganensis*. *Crop Prot.*, 2003, **22**, 39–44.
2. Cowan, M. M., Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, 1999, **12**, 564–582.
3. Iscan, G., Kirimer, N., Kurkcuoglu, M., Baser, K. H. C. and Demirci, F., Antimicrobial screening of *Mentha piperita* essential oils. *J. Agric. Food Chem.*, 2002, **50**(14), 3943–3946.
4. Moreno, L., Bello, R., Primo-Yufera, E. and Esplugues, J., Pharmacological properties of the methanol extract from *Mentha suaveolens* Ehrh. *Phytother. Res*., 2002, **16**, 10–13.
5. Quezel, P. and Santa, S., Nouvelle flore d'Algérie et des régions désertiques méridionales, 1963, vol. 2, pp. 785–786.
6. Newall, C. A., Anderson, L. A. and Phillipson, J. D., *Herbal Medicine. A Guide for Health-Care Professionals*, The Pharmaceutical Press, London, UK, 1996.
7. Lorenzo, D., Paz, D., Dellacassa, E., Davies, P., Vila, R. and Canigueral, S., Essential oils of *Mentha pulegium* and *Mentha rotundifolia* from Uruguay. *Bras. Arch. Biol. Technol*., 2002, **45**(4), 519–524.
8. Strycharz, S. and Shetty, K., Peroxidase activity and phenolic content in elite clonal lines of *Mentha pulegium* in response to polymeric dye R-478 and *Agrobacterium rhizogenes*. *Proc. Biochem*. 2002, **37**, 805–812.
9. Lawrence, B. M., Progress in essential oils. *Perfum. Flavor.*; 1998, **23**, 63–68.
10. Pino, J. A., Rosado, A. and Fuentes, V., Chemical composition of the essential oil of *Mentha pulegium* L. from Cuba. *J. Essent. Oil Res*., 1996, **8**, 295–296.
11. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant Physiol.*, 1962, **15**(3), 473–497.
12. Adams, R. P., *Identification of Essential Oil Components by Gas Chromatography Mass Spectrometry*, Allured Publishing Corporation, Carol Stream Illinois, 2007, 4th edn.
13. Gochev, V., Dobreva, A., Girova, T. and Stoyanova, A., Antimicrobial activity of essential oil from *Rosa Alba*. *Biotechnol. Biotechnol. Equip*., 2010, 24 (Supp1. 1), pp. 512–515.

14. Brand, W., Cuvelier, W. and Berset, M. E., Use of a free radical method to evaluate antioxydant activity. *Lebensm. Wiss. Technol.*, 1995, **28**, 25–30.
15. Malika, B. and Laib, I., Chemical composition and antioxidant activity of the essential oil of dry flowers of *Lavandula officinalis*. *Ind. Eng. J*., 2011, **6**, 46–54.
16. Sun, Y. *et al.*, Adventitious bud regeneration from leaf explants of *Platanus occidentalis* L. and genetic stability assessment. *Acta Physiol. Plant*, 2009, **31**, 33–41.
17. Paul, A., Thapa, G., Basu, A., Mazumdar, P., Kalita, M. C. and Sahoo, L., Rapid plant regeneration, analysis of genetic fidelity and essential aromatic oil content of micropropagated plants of Patchouli, *Pogostemon cablin* (Blanco) Benth. – an industrially important aromatic plant. *Ind. Crops Prod*., 2010, **32**, 366–374.
18. Zuzarte, M. R., Dinis, A. M., Cavaleiro, C., Salgueiro, L. R. and Canhoto, J. M., Trichomes, essential oils and *in vitro* propagation of *Lavandula pedunculata* (Lamiaceae). *Ind. Crops Prod.*, 2010, **32**, 580–587.
19. Taiz, L. and Zeiger, E., *Plant Physiology*, Sinaur Associates, Sunderland., MA, USA, 1998, 2nd edn.
20. Jordan, A. M., Calvo, M. C. and Segura, J., Micropropagation of adult *Lavendula dendata* plants. *J. Hortic. Sci. Biotechnol*., 1999, **73**, 93–96.
21. Calvo, M. C. and Segura, J., *In vitro* propagation of lavender. *Hortic. Sci.*, 1989, **24**, 375–376.
22. Echeverrigaray, S., Basso, R. and Andrade, L. B., Micropropagation of *Lavendula dentata* from axillary buds of field-grown adult plants. *Biol. Plant*., 2005, **49**, 439–442.
23. Arikat, N. A., Jawad, F. M., Karam, N. S. and Shibli, R. A., Micropropagation and accumulation of essential oils in wild sage (*Sativa fructicosa* Mill.). *Sci. Hortic*., 2004, **100**, 193–202.
24. Abdelli, M., Moghrani, H., Aboun, A. and Maachi, R., Algerian *Mentha pulegium* L. leaves essential oil: chemical composition, antimicrobial, insecticidal and antioxidant activities. *Ind. Crops Prod*., 2016, **94**, 197–205.
25. Ouakouak, H., Chohra, M. and Denane, M., Chemical composition, antioxidant activities of the essential oil of *Mentha pulegium* L., south East of Algeria, *Int. Lett. Nat. Sci.*, 2015, **39**, 49–55.
26. Benomari, F. Z. *et al.*, Essential oils from Algerian species of *Mentha* as new bio-controlagents against phytopathogen strain. *Environ. Sci. Pollut. Res*., 2018, **25**, 29889–29900.
27. Lawrence, B. M., A study of monoterpene interrelations in the genus *Mentha* with special reference to the origin of pulegone and menthofuran. Ph D thesis, Rijksuniversiteit Groningen, The Netherlands, 1978.
28. Kokkini, S., Handilou, E., Karousou, R. and Lanaras, T., Variations of pulegone content in pennyroyal (*Mentha pulegium* L.) plants growing wild in Greece. *J. Essent. Oil Res*., 2002, **14**, 224–227.
29. Zwaving, J. H. and Smith, D., Composition of the essential oil of Austrian *Mentha pulegium*. *Phytochemistry*, 1971, **10**, 1951–1953.
30. Lawrence, B. M., MINT the genus *Mentha*. In *Medicinal and Aromatic Plants – Industrial Profiles*, Taylor and Francis Group, 2007.
31. Kjonaas, R. and Croteau, R., Demonstration that limonene is the first cyclic intermediate in the biosynthesis of oxygenated *p*-menthane monoterpenes in *Mentha piperita* and other *Mentha* species. *Arch. Biochem. Biophys*., 1983, **220**, 79–89.
32. Burbott, A. J. and Loomis, W. D., Effects of light and temperature on the monoterpenes of peppermint. *Plant Physiol*., 1967, **42**, 20–28.
33. Clark, R. J. and Menary, R. C., Environmental effects on peppermint (*Mentha piperita* L.). I. Effect of day length, photon flux density, night temperature and day temperature on the yield and composition of peppermint oil. *Aust. J. Plant Physiol*., 1980, **7**, 685–692.
34. Scherer, R. and Godoy, H. T., Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chem*., 2009, **112**, 654–658.
35. Hajlaoui, H., Trabelsi, N., Noumi, E., Snoussi, M., Fallah, H., Ksouri, R. and Bakhrouf, A., Biological activities of the essential oils and methanol extract of two cultivated mint species (*Mentha longifolia* and *Mentha pulegium*) used in the Tunisianfolkloric medicine. *World J. Microbiol. Biotechnol*., 2009, **25**, 2227–2238.
36. Kamkar, A., Javan, A. J., Asadi, F. and Kamalinejad, M., The antioxidative effect of Iranian *Mentha pulegium* extracts and essential oil in sunflower oil. *Food Chem. Toxicol*., 2010, **48**(7), 1796–1800.
37. Teixeira, B. *et al.*, European pennyroyal (*Mentha pulegium*) from Portugal: chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil. *Ind. Crops Prod*., 2012, **36**, 81–87.
38. Nguyen, T., Sherratt, P. J. and Pickett, C. B., Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu. Rev. Pharmacol. Toxicol*., 2003, **43**, 233–260.
39. Mahboubi, M. and Haghi, G., Antimicrobial activity and chemical composition of *Mentha pulegium* L. essential oil. *J. Ethnopharmacol*., 2008, **119**, 325–327.
40. Ait-Ouazzou, A. *et al.*, Evaluation of the chemical composition and antimicrobialactivity of *Mentha pulegium*, *Juniperus phoenicea* and *Cyperus longus* essential oils from Morocco. *Food Res. Int*., 2012, **45**, 313–319.
41. Carson, C. F. and Riley, T. V., Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. *J. Appl. Bacteriol*., 1995, **78**, 264–269.

ACKNOWLEDGEMENTS. This study was supported by the Directorate General for Scientific Research and Technological Development, Algeria. We thank the staff *In Citro* Culture and Microbiology Laboratories, Biotechnology Research Center (Constantine, Algeria) and of Physico-Chemical Analysis Research Center, Algeria for performing chromatography analysis of the samples.

Received 30 November 2017; revised accepted 21 March 2019

doi: 10.18520/cs/v116/i11/1897-1904