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Mentha longifolia in vitro cultures as safe source of flavouring ingredients

Alessandra Bertoli[1]✉, Michele Leonardi[1], Justine Krzyzanowska[2], Wieslaw Oleszek[2] and Luisa Pistelli[1]

[1]Department of Pharmaceutical Science – University of Pisa, Pisa, Italy; [2]Department of Biochemistry Institute of Soil Science and Plant Cultivation State Research Institute, Pulawy, Poland

***In vitro* plantlets and callus of *M. longifolia* were established and their volatile constituents characterized by GC-MS analysis of their headspaces (HSs) and essential oils (EOs). Significant quali-quantitative differences were found in the aromatic fingerprints in comparison with the *M. longifolia* parent plants. In fact, limonene and carvone were the main constituents in the EOs of the mother plants, while the aroma of the *in vitro* plant material were especially enriched in oxygenated terpenes. In particular, huge amounts of piperitenone and piperitenone oxide (75%) were found for *in vitro* plantlets, while *trans*-carvone oxide (19%) and *trans*-piperitone epoxide (9%) were found in callus EO. However, the established *in vitro* plant material showed lack of pulegone and menthofurane, thus preserving an important feature observed in the volatile fingerprint of the parent plants. In fact, because of their well-known toxicity significant amounts of pulegone and menthofurane may compromise the safety using of mint essential oil. Therefore the *in vitro M. longifolia* plantlets and callus may be regarded as a potential source of a safe flavouring agent.**

Keywords: *M. longifolia*, *in vitro* plantlets, callus, essential oil, static headspace, GC-MS

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INTRODUCTION

Mentha longifolia L. (Lamiaceae) or wild mint is a perennial herb, extremely variable both morphologically and chemically. It comprises a number of isolated populations extending over the whole of Europe, and from African highlands down to the Cape of Good Hope (Lawrence, 1981). The aerial parts of adult plants are commonly used in folk medicine for the treatment of cold, cough, asthma, and chest inflammations, including pulmonary tuberculosis. It is also used externally to treat wounds and swollen glands (Ikram & Haq, 1980; Evans, 1996; Mimica-Dukic *et al*., 1996; 2003). Mint extracts are commonly used as food flavoring additive and are generally considered safe to use as they provide good defense against oxidative damage and health benefits (Dorma *et al*., 2003). However, a revision of the safety aspects of some mint constituents such as pulegone and menthofurane has been discussed recently (Nair, 2001; JECFA, 2009). *M. longifolia* is known also under synonymous names as *M. spicata var. longifolia* L. or *M. sylvestris*. The majority of *M. longifolia* chemotypes and subspecies contain piperitenone oxide, piperitone oxide, carvone, menthone, and 1,8-cineol as the main constituents, even though major variation in the dominating compounds has been found in wild or cultivated plant material grown in different habitats (Maffei, 1988; Venskutonis *et al*., 1996; Fleisher & Fleisher, 1998; Karousou *et al*., 1998; Baser *et al*., 1999; Abu Al-Futuh *et al*., 2000; Mastelic & Jerkovic, 2002; Jaimand & Rezaee, 2002; Rasooli & Rezaei, 2002; Mathela *et al*., 2005; Oyedeji & Afolayan, 2006; Gulluce *et al*., 2007). A summary of literature data on the essential oil (EO) composition of *M. longifolia* is reported in Table 1.

In the present study, leaves and stems of *M. longifolia* selected from cultivated adult plants (Pulawy, Poland) were used as mother plants to establish *in vitro* plantlets and callus. Both the headspaces (HSs) and the essential oils (EOs) were studied to compare the complete aromatic fingerprint of *in vivo* and *in vitro* biomass. To our knowledge, no studies have been reported on the volatile profile of *in vitro* cultures of *M. longifolia*.

MATERIAL AND METHOD

Plant Material. Seeds of *Mentha longifolia* (catalogue number: 239112) were obtained from the National Centre for Plant Genetic Resources at the Plant Breeding and Acclimatization Institut (Radzikow, Poland). Plants were cultivated in an experimental field of the Institute of Soil Science and Plant Cultivation in Pulawy, Poland. Plants were harvested at the beginning of flowering.

Seed sterilization and sowing. Seeds were washed in running tap water and sterilized with 70% ethanol for 2 min, then transferred to 10% perhydrol solution with shaking for 20 min, and finally rinsed three times with sterile distilled water. Sterilized seeds were placed for germination on half-strength LS basal medium (Linsmayer & Skoog, 1965) supplemented with 15 g L^{-1} sucrose and solidified with agar 6 g L^{-1}, adjusted to pH 5.8 followed by autoclaving at 121 °C and 0.1 MPa for 20 min. Seeds were sown on the medium in Petri dishes and then were incubated in a growth chamber at 25 °C, under 16 hours light /8 hours dark cycle provided by fluorescent lamps. Sprouting seeds were planted on LS medium enriched

✉e-mail: bertoli@farm.unipi.it

Abbreviations: EO, essential oils; HS, headspace, GC-EI/MS, gas chromatography-electronic impact/mass detector; GC-CI/MS, gas chromatography-chemical ionization/mass detector; LRI, linear retention index; SPME, solid phase microextraction.

Table 1. EO yields collected in different countries.

EO yield	Plant material	Reference	Region
1.63 % ($w/w_{dry\ weight}$)	dry mature plant (no flowers)	Oyedeji & Afolayan, 2006	South Africa
1.09 mL/Kg	fresh plant	Maffei, 1988	Italy
0.93 % (w/w)	fresh aerial parts	Rasooli & Rezaei, 2002	Iran
0.4–0.8 %(w/w)	fresh leaves	Mathela *et al*., 2005	India
2.31 mL/100g	air dried plant	Gulluce *et al*., 1996	Turkey
3.5mL/100g	air dried leaves	Jaimand & Rezaee, 2002	Iran
9.6 mL/100g	air dried flowers	Jaimand & Rezaee, 2002	Iran
0.93 % (w/w)	air dried plant	Mastelic & Jerkovic, 2002	Croatia
0.43 mL/100g	air dried plant	Veskuntonis *et al*., 1996	Lithuania
1.80 mL/100g	air dried plant	Kokkini *et al*., 1988	Greece
1.6 mL/100g	air dried plant	Karousou *et al*., 1998	Greece
0.1–1 % (v/w)	fresh plant	Baser *et al*., 1999	Turkey
2.2±0.5 % v/w 1.6±0.1 % v/w	adult plant stems adult plant leaves	Present study	Poland
0.2±0.02 % v/w 0.4±0.07 % v/w	*in vitro* plantlets callus tissue	Present study	*in vitro* biomass

with 0.2 mg L^{-1} NAA (naphtaleneacetic acid) and 0.2 mg L^{-1} IAA (indole-3-acetic acid). Flasks were kept in the growth chamber under the same conditions. Young *in vitro* plants were used for callus induction. Some of those plants after 30 days of cultivation were harvested, freeze-dried and used for biochemical analysis.

Callus induction. Leaves from 3-week-old *in vitro* plants were used as explants. They were cut into small pieces and transferred on several media variants supplemented with dichlorophenoxyacetic acid (2,4-D) in combination with 6-benzylaminopurine (BA) or isopentenyladenine (2iP) added at the concentration 0.5, 1 and 2 mg L^{-1}. As a control LS basal medium without any hormones was applied sucrose (30 g L^{-1}) and agar (8 g L^{-1}) were added to the media and pH was adjusted to 5.8.

Callus tissues were grown in Petri dishes. For each type of medium at least ten dishes containing explants from five seedlings were used for callus growth. The cultures were maintained in a growth chamber at 25 °C under a 16-h photoperiod, provided by cool white fluorescent lamps. After 30 days the mint callus induction and its characteristic features such as abundance, color, structure, tendency to form roots, shoots and necrosis were observed. Calli were subcultured at four-week intervals. For biochemical analysis calli from 7th passage were used.

Phytochemical analysis. Chemicals. Commercial standards and isolated compounds from aromatic plant species were part of a homemade database of volatiles where each compound was used as reference material after GC-MS grade purity determination (98–99 %). The samples and standard solutions were prepared using *n*-hexane (Carlo Erba, HPLC-grade).

Extraction procedure. Freeze-dried plant samples were hydrodistilled (2 h, 2 L distilled water, flow 2.0 ml/min) by a Clevenger apparatus described in the European Pharmacopoeia V Ed. The essential oils were dissolved in Et2O, dried over anhydrous MgSO4, filtered and the solvent removed by evaporation on a water bath. The essential oil yields are summarised in Table 1. The essential oils were diluted in *n*-hexane (HPLC solvent grade, 10 %) and analysed by GC-FID (injection volume 1 µl, HP-WAX and HP-5 capillary columns) and GC-MS (injection volume 0.1 µl, DB-5 capillary column).

GC-FID analysis. GC-FID analyses were run on an HP-5890 Series II instrument equipped with HP-WAX and HP-DB-5 capillary columns (30 m × 0.25 µm, 0.25 µm film thickness), working with the following temperature program: 60 °C for 10 min, ramp of 5 °C/min up to 220 °C; injector and detector temperatures 250 °C; carrier gas nitrogen (2 ml/min); detector dual FID; split ratio 1 : 30; injection volume of 1 µl; 10 % *n*-hexane solution. Identification of the essential oil constituents was performed for both columns by comparison of their retention times with those of pure authentic samples and by means of their Linear Retention Indices (L.R.I.) relative to a series of *n*- hydrocarbons (C9-C23) on the two columns.

GC-MS analysis. GC/EI-MS analyses were performed on a Varian CP-3800 gas chromatograph equipped with an HP DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures 220 and

Table 2. Calibration parameters of the standard compounds used in the GC-MS quantitative analysis.

Standard compounds	LRI	Representative chemical class	Calibration Curve Equation[a]	R^2	Detection Limit (mg/mL)
2-ctanol	995	hydrocarbon derivatives	$y = 0.4765x + 0.0071$	0.999	0.0054
imonene	1029	monoterpene hydrocarbons	$y = 0.7231x + 0.0154$	0.999	0.0022
enthone	1153	oxygenated monoterpenes	$y = 0.4350x + 0.0893$	0.999	0.0027
β-aryophyllene	1419	sesquiterpene hydrocarbons	$y = 0.5470x + 0.0024$	0.999	0.0063
aryophyllene oxide	1512	oxygenated sesquiterpenes	$y = 0.6454x + 0.0097$	0.999	0.0081

[a] $y = C_{is}/C_s$ and $x = A_{is}/A_s$ where C_S, A_S = concentration and peak area of standard, C_{is} and A_{is} = concentration and peak area of internal standard

Table 3. Some typical *M. longifolia* constituents (relative percentage composition) in the analysed samples compared to literature data.

	Europe								Asia				Africa					
	Poland (present study)				Italy[a]	Crete[b]	Crete[b]	Crete[b]	Lithuania[c]	India[d]	Turkey[e]	Iran[f]	Iran[f]	South Africa[g]	South Africa[g]	South Africa[g]	South Africa[g]	South Africa[h]
	stems	leaves	*in vitro* plantlets	callus tissue	fresh plant	air-dried plant zone A	Air-dried Plant Zone B	air-dried plant zone C	leaves air-dried plant	fresh leaves	plants flowering stage	fresh flower oil	fresh leaf oil	fresh plant	air-dried	oven-dried	sun-dried	fresh plant (no flowers)
menthol	1.9	4.3	2.1	8.9	0.6	0.6	0.1	1.0	t	–	–	–	–					
menthone	4.7	4.2	2.1	–	0.2	2.0	1.3	t	–	–	7.9	0.9	1.1	31.1	47.6	t	38.3	50.9
menthol/ menthone	0.4	1.0	1.0		3.0	0.3	0.1											
pulegone	–	–	–	–	0.1	0.1	–	–	–	–	15.5	4.1	0.0	35.0	18.4	–	20.2	19.3

[a]Maffei, 1988; [b]Karousou *et al.*, 1998; [c]Venskutonis *et al.*, 1996; [d]Mathela *et al.*, 2005; [e]Gulluce *et al.*, 1996; [f]Jaimand & Rezaee, 2002; [g]Asekun *et al.*, 2007; Oyedeji & Afolayan, 2006

240 °C, respectively; oven temperature programmed from 60 °C to 240 °C at 3 °C/min; carrier gas helium at 1 ml/min; injection volume 0.1 µl (10% *n*-hexane solution); split ratio 1:30. Identification of the constituents was based on a comparison of the retention times with those of authentic samples, comparing their LRI with those of a series of *n*-hydrocarbons (C9-C30). Computer matching of the mass spectra by two commercial data bases (NIST 2000, ADAMS) as well as a home-made library built up from pure substances or known oils were used to perform identification of the volatile constituents. Moreover, the molecular weights of the identified substances were confirmed by GC/CIMS, using MeOH as CI ionizing gas.

HS-SPME-GC-MS. The HS-SPME analyses were performed with Supelco SPME devices, coated with two different kinds of fibers (PDMS, PDMS-Carboxen, 100 µm) in order to sample the static headspace of a fixed portion of the freeze-dried plant material (stems, leaves, *in vitro* plantlets, callus) of *M. longifolia*. Each aliquot was inserted separately into a 50-ml conic glass flask and allowed to equilibrate for 30 min. After the equilibration time, each fiber was exposed to the sample headspace for 5 min at room temperature, and when the sampling was finished the fiber was withdrawn into the needle and transferred to the injection port of the GC and GC-MS system, operating in the conditions described for the essential oils, apart from the splitless injection mode and the injector temperature (250 °C).

Quantitative analysis. Quantification of the essential oils was conducted using an internal standard (*is*, *n*-undecane) added to the volatile oil under the conditions of the GCMS analysis used for standard mixtures. Calibration curves of the analytes were performed by using standards which have chemical similarity with the compounds of interest in the volatile oils (Table 2). The correspondent regression lines (five points) of each standard in Table 2 were obtained with chromatographic injections of solutions obtained by mixing accurate volumes of the standard stock solution and an accurate volume of internal standard solution at 10 mg/ml (*n*-hexane as solvent). The limits of detection of the standard target compounds are given in mg/mL (Table 2). The quali-quantitative GC-MS results are given as a mass percentage composition (mg/100 mg) of each volatile sample which was determined by the injection of a solution (0.1 µL) obtained by mixing 10 µL of volatile fraction, 100 µL of internal standard solution (1 mg/mL) and *n*-hexane to 1 mL (three measurements for sample).

The quali-quantitative results are shown in Tables 3–5.

RESULTS AND DISCUSSION

The volatile constituents emitted from field and *in vitro* biomass of *M. longifolia* were extracted both by hydrodistillation to obtain the essential oil (EO) and by solid phase microextraction (SPME) to sample the spontaneous aroma. The EO yields were 2.2% v/w for stems and 1.5% for leaves collected from adult plants of *M. ongifolia*.

The EO yields of *in vitro* plants and callus were much lower (0.2 and 0.4% v/w, respectively) (Table 1). However, these yields from *in vitro* biomass were similar or higher than those obtained from air-dried or fresh wild *M. longifolia* reported in the literature (Table 1). The EO composition was similar for the stems and leaves of field-grown adult plants, even if significant quantitative

Table 4. Mass percent composition[a] of *M. longifolia* essential oils obtained by hydrodistillation from *in vivo* and *in vitro* plant material.

		Stems		Leaves		*in vitro* plantlets		callus tissue	
Components	LRI	(%)[a]	RSD[b]	(%)	RSD	(%)	RSD	(%)	RSD
2-(E)-hexenal	805	0.6	0.05	0.3	0.01	0.1	0.01		
α-thujene	930	0.4	0.06	0.7	0.02			0.1	0.00
α-pinene	939	4.9	0.19	1.2	0.01	0.3	0.04	0.1	0.01
camphene	954	0.8	0.07	0.8	0.01				
sabinene	975	2.3	0.08	1.1	0.02	0.7	0.03	2.1	0.11
b-pinene	979	6.8	0.16	1.7	0.01	1.3	0.11	1.2	0.10
myrcene	991	3.6	0.17	1.6	0.02	1.4	0.01	1.0	0.10
2-octanol	995	0.4	0.03	0.4	0.01	0.1	0.00	0.1	0.00
3-carene	1002	0.2	0.01						
iso-sylvestrene	1009	t[c]		0.8	0.01				
α-terpinene	1017	0.9	0.09	0.7	0.01	0.4	0.01		
p-cymene	1025	0.4	0.04	0.7	0.00				
limonene	1029	15.3	0.57	5.8	0.29	1.3	0.01	0.7	0.10
1,8-cineole	1033	8.2	0.15	5.4	0.02	2.3	0.02	3.4	0.16
(Z)-*b*-ocimene	1037	9.1	0.18	3.7	0.13			10.1	0.27
(E)-*b*-ocimene	1050	8.5	0.13	3.8	0.11			3.0	0.21
dihydro tagetone	1053			4.1	0.01				
g-terpinene	1060	0.3		0.8	0.01	0.2	0.01		
n-octanol	1068	t							
cis-sabinene hydrate	1069			4.1	0.05			2.1	0.11
camphelinone	1070			4.1	0.09			t	
linalool	1097	t		4.1	0.02	0.9	0.13	4.4	0.20
n-nonanal	1101			0.3	0.00				
1,3,8-*p*-menthatriene	1110			0.7	0.01				
1,3,8-*cis*-menthatriene	1120			0.7	0.01				
allo-ocimene	1132							1.2	0.10
menthone	1153	4.7	0.11	4.2	0.01				
iso-menthone	1163	1.5		4.1	0.02				
neo-menthol	1166			4.0	0.02				
menthol	1172	1.9		4.3	0.12				
4-terpineol	1177			4.1	0.11				
3-(Z)-hexenyl butanoate	1185			0.3	0.01				
α-terpineol	1189	1.2	0.12	4.2	0.13	1.1	0.01		
methyl salicylate	1192			t					
cis-dihydro carvone	1193			4.1	0.12				
n-decanal	1202	0.3	0.02						
verbenone	1205	1.2	0.03						
isopulegone	1208	1.2	0.05						
cis-hexenyl isovalerate	1235	1.3	0.10	4.3	0.21				
pulegone	1237								
carvone	1243	15.1	0.10	7.9	0.21				
piperitone	1253	2.2	0.01	4.8	0.04	3.3	0.12		
cis-piperitone epoxide	1254							2.4	0.15
trans-piperitone epoxide	1256							8.0	0.22

differences were observed. The main components were limonene and carvone both in stems (15.3 and 15.1%, respectively) and leaves (5.8 and 7.9%, respectively). The hydrocarbon monoterpenes such as α-and β-pinene, 1,8-cineole, as well as *Z*- and *E*-ocimene were much more abundant in stems than in the leaf EOs (Table 4). On the other hand, the oxigenated *p*-menthene compounds, which are other typical constituents of mint spp., showed higher percentages in the leaf than in the stem EOs. The menthol/menthone ratio in the stem (0.4) and in leaf EOs (0.1) of the parent plants were similar to those reported in the literature for air-dried Cretan *M. longifolia* plants (0.3–0.1), but it was much lower than found in Italian fresh samples (3.0) (Maffei, 1988; Karousou *et al.*, 1998). Previous studies on wild extra-European *M. longifolia*, such as Asian and Australian samples, showed a menthol/menthone ratio in favour of menthone, as those plants did not produce menthol. On the other hand, wild *M. longifolia* samples from Turkey, Iran, and South Africa, had a large amount of pulegone instead of menthone or menthol. In fact, many mint species have been reported to have an efficient conversion of piperitenone to pulegone (Table 3). Pulegone (monoterpene monocyclic ketone) has been detected in several EOs of mint spp. and it is considered the precursor of another typical mint ketone, menthone, the monocyclic alcohol menthol, and the bicyclic epoxydic monoterpene menthofuran (Fig. 1). Many studies have shown that piperitenone, piperitone, and pulegone generally co-exist in the

cis-carvone oxide	1263							2.3	0.11
trans-carvone oxide	1276							18.9	0.41
menthyl acetate	1295							2.6	0.07
piperitenone	1315					30.4	0.54		
piperitenone oxide	1369					44.8	0.10	3.3	0.16
b-bourbonene	1388	0.5	0.01	0.2	0.01				
b-elemene	1391	0.2	0.01	0.2	0.01			t	
b-caryophyllene	1419	2.8	0.04	0.6	0.02			t	
phenyl ethyl butanoate	1444			0.1	0.00			2.1	0.06
trans-muurola-4(14),5-diene	1450	t		0.1	0.01				
α-humulene	1455	0.2	0.01	0.1	0.01			1.1	0.05
(E)-*b*-farnesene	1457	t							
cis-muurola-4(14),5-diene	1467	t		0.1	0.00				
germacrene D	1485	2.0	0.02	0.5	0.03			4.2	0.17
bicyclogermacrene	1500	0.3	0.01	0.2	0.01			2.2	0.08
germacrene A	1501			0.1	0.01				
d-cadinene	1523			0.1	0.01				
spathulenol	1578			0.5	0.01				
caryophyllene oxide	1583			0.5	0.01				
globulol	1585	0.5	0.02						
epi-α-cubenol	1640			0.5	0.03				
α-cadinol	1654			0.5	0.02				
Total		99.8	0.27	99.5	0.17	99.8	0.16	98.1	0.49

[a]Content of compound in mg/100 mg of the essential oil; [b]RSD relative standard deviation (triplicate analysis; $p \leq 0.05$); [c]t = traces (% < 0.1).

mint essential oils (Karousou *et al*., 1998; Abu Al-Futuh *et al*., 2000), but some chemotypes of *M. longifolia* have been reported to contain piperitenone oxide (1,2-epoxy-*p*-menth-4-(8)-en-3-one) as the major constituent (Maffei, 1988; Venskutonis, 1996). In the present work, piperitone was at very low levels both in the stem (2.2%) and in leaf EOs (4.8%), while pulegone, menthofuran, piperitenone, and piperitenone oxide were not detected. Regarding sesquiterpenes, β-caryophyllene (2.8%) and germacrene D (2.0%) were the most abundant volatiles in stem EOs, while the same compounds dropped under 1% in the leaves of *M. longifolia* parent plants. Previous studies on *M. longifolia* EO reported that germacrene D is a typical sequiterpene of different varieties of this species (Maffei, 1988; Evans, 1996; Rasooli & Rezaei, 2002). SPME-GC-MS analyses confirmed these results for the EO extracted from adult plants (Table 5). Both stems and leaves of Polish *M. longifolia* were used to establish *in vitro* plant material and the induction of callus tissue was noticed on all tested media (outside the control variant). The *M. longifolia* callus induction on the medium enriched with isopenthenyladenine and dichlorophenoxyacetic acid generally gave poor or very poor callus growth. A slightly better effect was observed when 6-benzylaminopurine was used as cytokinin, but the callus tissues obtained were also poor. Small necroses on explant surfaces were observed, and roots or shoots were not formed. The best effects were observed on media containing 1.0 mg L^{-1} BA plus 0.5 mg L^{-1} 2,4-D. The callus obtained on this medium was creamy, fragile which is important in the case of suspension cultures. Moreover this tissue was characterized by good biomass production. The GC-MS analysis of *in vitro* plantlets and callus of *M. longifolia* showed a large variety of the typical volatile constituents already found in their parent plant material. Furthermore, most of these volatile constituents were also emitted spontaneously by *in vitro* biomass as confirmed by the SPME-GC-MS analysis carried out directly on this material (Table 5). However, significant quali-quantitative differences were observed between the *in vivo* and *in vitro* biomass. The ratio of oxides/ketones was found to be an important parameter to indicate the efficiency of the conversion from piperitenone to piperitenone oxide in the *in vivo* plants. Piperitone and piperitenone were not detected in the callus EO, while their corrispondent oxides, *cis*- (2.4%) and *trans*- (8.0%) piperitone epoxide as well as piperitenone oxide (3.3%) were present (Tables 3–5).

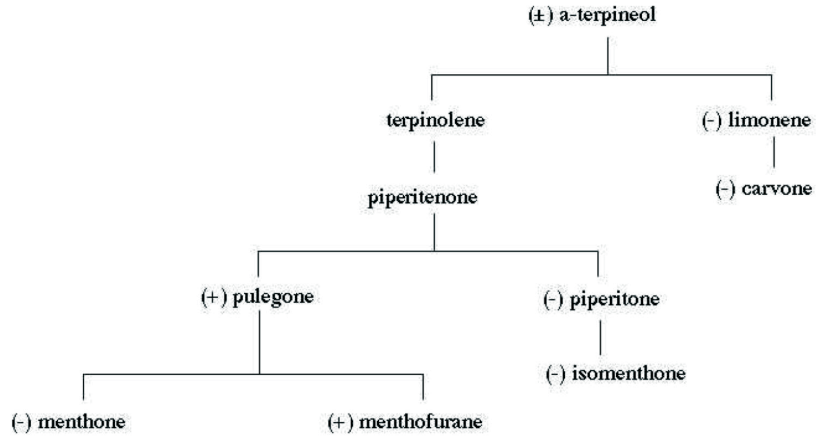

Figure 1. General biosynthetic scheme of cyclic monoterpene ketones reported in mint species (Maffei, 1999).

Furthermore, the huge amounts of carvone which were detected in the *M. longifolia* EO of adult plants (15.1 and 7.9% in stems and leaves, respectively) were not pro-

Table 5. Relative percentage composition of *M. longifolia* aroma obtained from the *in vivo* and *in vitro* plant material by static HS-SPME-GC-MS.

		stems		leaves		*in vitro* plantlets		callus	
	LRI	PDMS[b]	CARB[b]	PDMS	CARB	PDMS	CARB	PDMS	CARB
Components		%	%	%	%	%	%	%	%
2-(E)-hexenal				t[c]	t				
α-thujene	930			0.1	0.1	t			
α-pinene	939	0.9		1.5	1.0	0.3	0.1	t	0.1
camphene	954	0.1		0.1	0.1	t			
sabinene	975	1.1		1.4	1.1	0.3	0.2		
b-pinene	979	3.8	0.8	4.0	4.1	0.8	t	0.1	0.5
myrcene	991	2.8	0.8	3.9	4.0	0.9	0.1		
3-octanol	995	0.0	0.1	0.4	0.4	t			
3-carene	1003			0.1	0.1	t			
iso-sylvestrene	1009	t	t						
α-terpinene	1017	t	t	t		0.2	0.1		
p-cymene	1025	0.1		0.1	0.1	t			
limonene	1029	12.7	14.7	15.1	21.2	0.8	2.6	0.3	0.5
1,8-cineole	1033	20.6	9.8	23.1	11.2	1.7	0.4	2.3	1.9
(Z)-*b*-ocimene	1037	9.6	3.3	11.9	12.9			6.3	2.1
2-heptyl acetate	1043								
(E)-*b*-ocimene	1050	1.3	0.1	1.0	2.4	0.3	0.1	2.7	2.0
dihydro tagetone	1053			t					
g-terpinene	1060	0.3		0.2	0.1	0.1	t		
cis-sabinene hydrate	1070	0.2	t			t	t	1.2	2.5
camphenilone	1072			0.2	0.3	t	t		
2,5-dimethyl styrene	1099								
linalool	1097	0.4	t	0.3	0.5	0.3	0.1	3.5	3.9
n-nonanal	1101			0.1	0.1				
1,3,8-*p*-menthatriene	1110			0.1				0.9	1.1
allo-ocimene	1120			2.1	1.7	0.4	t	0.3	1.2
isopulegol	1150								
menthone	1153	15.3	31.5	13.8	17.9	0.1	t	0.8	1.1
iso-menthone	1163	2.5	4.3	2.3	1.0	0.2	0.1	2.2	0.9
neo-menthol	1166	0.1	0.1	0.2	0.1	0.2	t	2.7	3.8
(+)-menthol	1172	1.5	2.9	2.0	1.0	0.1	t	1.1	2.3
g-terpineol	1177							5.4	8.2
iso-menthol	1183							4.9	2.1
n-decanal	1189	0.1	0.1	0.2	t	0.8	2.1	2.3	4.2
verbenone	1192	0.2	0.1	0.2	0.1			3.8	2.1
isopulegone	1193	0.2	t	0.8	0.7				
pulegone	1237								t
carvone	1243	13.4	5.8	7.5	8.1			3.6	3.0
2-(Z)-hexenyl isovalerate	1245	1.1	0.6	0.1	0.3			2.2	3.1
2-(E)-hexenyl isovalerate	1247							0.8	0.8
piperitone	1253	2.1	1.4	1.7	2.2	35.7	24.6	4.2	5.6
cis-piperitone epoxide	1254							1.7	2.0
trans-piperitone epoxide	1256							7.7	5.5
cis-carvone oxide	1263							1.1	0.5

duced in the callus EO in favour of *cis*-(2.3%) and *trans*-(18.9%) carvone oxides. Although the *M. longifolia* callus did not show limonene and carvone as main constituents like their mother plants, they were characterized by the highest oxidative status of carvone and piperitone. In addition, the scarce amount of limonene (0.7%) and the lack of terpinolene, which are the precursors of piperitenone and carvone, could be justified by the complete transformation of these two latter compounds into their corrispondent oxides (Fig. 1). Regarding the *in vitro* plantlets, they showed the specific terpenes of *M. longifolia* generally reported in the literature.

Piperitenone (30.4%) and piperitenone oxide (44.8%) were the main constituents in their EOs (Table 4). However, as mentioned above, neither these compounds was not detected in the Polish *M. longifolia* mother plants. Nevertheless, the established *in vitro* plantlets could be considered similar to a chemotype of *M. longifolia* rich in piperitenone oxide (75%). In fact, a chemotype of *M. longifolia* Hudson (L.) particularly rich in piperitenone oxide (77%) was selected from populations growing wild in Piedmont Valley (Italy) (Maffei, 1988) and Lithuania (Venskutonis, 1996).

trans-carvone oxide	1276							16.1	14.3
menthyl acetate	1295	0.4	1.7	0.5	0.3			2.4	3.9
iso-ascaridol	1303							1.5	0.9
piperitenone	1343					0.8	1.0	1.8	0.9
piperitenone oxide	1369			t		49.8	48.9	2.7	3.0
b-bourbonene	1388	0.5	0.5	0.3	0.2			0.6	0.5
b-elemene	1391	0.1	0.3	0.1	0.1	0.7	0.2	0.8	0.9
b-caryophyllene	1419	1.5	2.5	1.3	2.1	2.1	4.4	1.0	1.1
trans-muurola 3,5-diene	1454	t	0.1						
α-humulene	1455	0.1	0.2	t		0.2	0.5	0.5	0.6
cis-muurola-4(14),5-diene	1467	0.2	t	0.1	0.2			0.6	0.9
germacrene D	1485	0.5	1.7	0.8	0.7	1.1	3.0	0.4	5.3
bicyclogermacrene	1500	0.2	3.7	t		1.0	8.5	2.4	4.0
caryophyllene oxide	1583	0.1	2.3	t					
Total		94.0	89.3	97.6	96.4	98.9	97.0	93.1	97.3

[a]PDMS and Carboxen fibers, 100 mm; [b]RSD relative standard deviation (triplicate analysis; $p \leq 0.05$); [c]t = traces (% < 0.1).

In our work, the *in vitro* plantles of *M. longifolia* showed an aromatic fingerprint characterized by huge amounts of piperitone and piperitenone oxide (75%) with a clear reduction in other typical *p*-menth-compounds detected in their parent plants (Tables 3–4). Furthermore, the *in vitro* callus and plantlets did not show pulegone and its major metabolite menthofuran. The present study is the first report on the aromatic profiling of *M. longifolia* as *in vivo* and *in vitro* plant material. Both types of *in vitro* plant material, callus and plantlets, were characterized by a lack of pulegone and methofurane. It is important to point out that the EFSA Scientific Committee on Food was recently asked to provide scientific advice on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the EU Member States. According to EU Flavourings List, pulegone and menthofuran cannot be used as flavouring substances (Moorthy, 1991; Tisserand & Balacs, 1995; Nair, 2001; JECFA, 2009). Therefore, the *in vitro* plantlets and callus of *M. longifolia* which did not contain pulegone and menthofurane preserved an important safety feature of their parent plants and they may be qualified as alternative flavouring ingredients.

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