ACTA ABP BIOCHIMICA POLONICA

Vol. 59, No 3/2012
417–423
on-line at: www.actabp.pl

Regular Paper

In vitro production of *M.* × *piperita* not containing pulegone and menthofuran

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

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

The essential oils (EOs) and static headspaces (HSs) of *in vitro* plantlets and callus of *Mentha x piperita* were characterized by GC-MS analysis. Leaves were used as explants to induce *in vitro* plant material. The EO yields of the *in vitro* biomass were much lower (0.1% v/w) than those of the parent plants (2% v/w). Many typical mint volatiles were emitted by the *in vitro* production, but the callus and *in vitro* plantelet EOs were characterized by the lack of both pulegone and menthofuran. This was an important difference between *in vitro* and *in vivo* plant material as huge amounts of pulegone and menthofuran may jeopardise the safety of mint essential oil. Regarding the other characteristic volatiles, menthone was present in reduced amounts (2%) in the *in vitro* plantlets and was not detected in the callus, even if it represented the main constituent of the stem and leaf EOs obtained from the cultivated mint (26% leaves; 33% stems). The *M. piperita* callus was characterized by menthol (9%) and menthone (2%), while the *in vitro* plantlet EO showed lower amounts of both these compounds in favour of piperitenone oxide (45%). Therefore, the established callus and *in vitro* plantlets showed peculiar aromatic profiles characterized by the lack of pulegone and menthofuran which have to be monitored in the mint oil for their toxicity.

Key words: *M. x piperita*, *in vitro* plantlets, callus, essential oil, SPME, GC-MS

Received: 18 May, 2012; revised: 17 july, 2012; accepted: 27 August, 2012; available on-line: 03 September, 2012

INTRODUCTION

The genus *Mentha* L. (Lamiaceae) comprises 18 species and 11 named hybrids placed in four sections: *Pulegium, Tubulosae, Eriodontes,* and *Mentha* (Tucker & Naczi, 2007). They are mainly perennial herbs growing wildly in damp or wet zone throughout temperate regions of Eurasia, Australia and South Africa (Dorman *et al*., 2003). Three Mentha species, *M. piperita* (peppermint), *M. arvensis* (cornmint), and *M. spicata* L. (spearmint) are commonly cultivated for essential oil production, especially in China, USA and India (Clark & Menary, 1981; Lawrence, 1997; Srivastava *et al*., 2003). Their essential oils were used extensively in food, cosmetic, and pharmaceutical industries (Bruneton, 1995; Hendriks, 1998; Ansari *et al*., 2000; Duke *et al*., 2002). Peppermint is considered to be a hybrid of *M. aquatica* and *M. spicata* (Murray & Hefendehl, 1972; Tucker & De Baggio, 2000). This species was first cultivated in the Mediterranean basin, but it was commercially grown in England in the late 1700s and then migrated to the United States of America (Clark & Menary, 1979; 1981; Alkire & Simon, 1993). More recently, *M. piperita* has been found to be cold tolerant and thus suitable for Finnish climate conditions (Aflatuni *et al*., 1999; 2000).

Identifying species in the genus *Mentha* is complicated due to extensive hybridization (Tucker & De Baggio, 2000). Furthermore, it may be assumed that the reported variability in peppermint oil is not due to genetic differences, since most of the commercial plantings, at least in North America and Europe, were propagated vegetatively from plants of the Black Mitcham variety, which originated in England. The European Pharmacopoeia reported a standard range of 30–55% menthol as target oil composition. High menthol content (44%) is the main criterion of peppermint oil quality according to ESCOP (1992). In fact, the acceptable commercial quality of peppermint oil is strictly related to the content of menthol, menthone, and menthyl acetate, with little or no pulegone and menthofuran (Burbott & Loomis, 1967; Murray *et al*., 1988).

Therefore, for some species, such as peppermint, a proportion of the oil yield must be sacrificed to ensure the required oil quality (Clark & Menary, 1979; 1981). It is well known that mint of high commercial value can be produced only in certain geographic areas (Clark & Menary, 1981; Lawrence, 1985; Maffei, 1999). Additionally, the yields and the composition of peppermint essential oil are also strongly influenced by yearly weather conditions, harvest date, plant age (Weglarz & Zalecki, 1985, 1987; Murray *et al*., 1988; Kumar *et al*., 2000) as well as fertilization and planting time (Voirin *et al*., 1990; Marotti *et al*., 1994; Misra & Srivastava 2000). Furthermore, the oil composition is also related to leaf position with increasing menthol and decreasing menthone content in the basipetal direction (Maffei *et al*., 1994; Rohloff, 1999; Gershenzon *et al*., 2000).

Flower oil has much more pulegone and menthofuran than leaf oil and more than 50% of the flower oil may consist of pulegone and menthofuran, with less than half the oil is composed of menthone and menthol (Murray *et al*., 1988). Due to the high commercial value of peppermint EO, several efforts have already been made by plant biotechnological approaches as an alternative promising way to control the EO production and improve peppermint quality (Maffei *et al*., 2007). Callus,

✉e-mail: bertoli@farm.unipi.it

Abbreviations: BA, 6-benzylaminopurine; EOs, essential oils; GC-MS, gas chromatography mass spectroscopy; GC-FID, gas chromatography flame ionization detection; HSs, static headspaces; NAA, naphthaleneacetic acid; 2,4-D, dichlorophenoxyacetic acid; SPME, solid phase microextraction.

cell tissue cultures, biotransformation, immobilization bioreactors have produced considerable amounts of terpenoids, although in each case the choice of donor or parental plants was crucial (Banthorpe, 1996). Some cell lines or suspension cultures of *M. piperita* can synthesize essential oils and numerous efforts have been made to produce essential oil *in vitro*. Culture conditions such as pH, hormone concentration, seeding density, which affect cell growth and essential oil production, have been investigated (Kim *et al*., 1996; Tisserat & Silman, 2000; Maffei *et al*., 2007).

In particular, *M. piperita* shoot cultures are extremely sensitive to artificial light and temperature. In fact, menthofuran biosynthesis is favoured by long periods of relatively low light intensities and warm night temperatures, whereas menthone is accumulated under short light photoperiods and cold nights in peppermint cultures (Spencer *et al*., 1993). *Agrobacterium*-mediated and direct gene transfers into protoplasts of *M. piperita* cv Black Mitcham have been already successfully used to produce stable, transformed peppermint plants with the limonene synthase gene. This regenerated plant material was characterized by high menthone, menthofuran and pulegone content and low menthol level in comparison with the typical Midwest peppermint (Krasnyanski *et al*., 1999). Maximum accumulation of terpenoids has been found in the late exponential phase of the cell culture cycle and is higher in cell suspension than in callus cultures (Banthorpe, 1996). Furthermore, some studies have been undertaken to test the applicability of cell-recycled airlift bioreactors for high-density cultures of *M. piperita* cells (Maffei *et al*., 2007). However, most of these *in vitro* protocols are not yet of commercial relevance. Despite of all these different biotechnological attempts on *M. piperita*, the industrialization of mint cell cultures for the production of their essential oils is still limited by low productivity, low growth rate of cells, and high sensitivity to shearing (Kim *et al*., 1996; Banthorpe, 1996; Diemer *et al*., 1998; Tisserat & Silman, 2000; Bhat *et al*., 2002).

The aim of the present study, as part of a European Project (EC NUTRA-SNACK, 6 FP), was to investigate a selection of adult plants of *M. x piperita* cultivated in Poland as a starting raw material in order to establish callus and *in vitro* plantlets with a standardised volatile profile.

MATERIALS AND METHODS

Plant material. Stolons of *M. x piperita* were received from the National Centre for Plant Genetic Resources at the Plant Breeding and Acclimatization Institute (Radzikow, Poland). Field plant material was obtained in 2009 from the field experiments performed at the Experimental Farm, Pulawy, Poland. Plants were collected during the second vegetative year at the beginning of flowering, air dried and powdered.

Procedure of sterilization and planting. Apical buds of field grown plants were rinsed with tap water prior to surface sterilization with 70% ethanol for 1 min followed by 10% perhydrol (H_2O_2) treatment for 5 min. Then they were rinsed three times with sterile distilled water and placed on half strength LS basal medium (Linsmayer & Skoog, 1965) supplemented with 15 g L^{-1} sucrose and solidified with agar at 6 g L^{-1}, adjusted to pH 5.8, and autoclaved at 121°C and 0.1 MPa for 20 min. The Sterilized material was placed on the medium in Petri dishes and was then were incubated in a growth chamber at 25°C, under a 16 h photoperiod and a light intensity of 140 μE m^{-2} s^{-1}.

After two weeks of growing apical buds were moved to LS medium enriched with 0.2 mg L^{-1} NAA (naphthaleneacetic acid) and 0.2 mg L^{-1} IAA (indole-3-acetic acid). NAA as well as IAA stock solutions were prepared by dissolving the substances in a drop of 1M NaOH and adjust adjusting to a desirable volume with deionized water. The Hormones were filter sterilized and added to the autoclaved LS medium. Young plantlets developed on this medium were propagated *via* cuttings. All plant material was kept in the growth chamber under the same conditions as above.

Callus cultures induction. 30-day old leaves from *in vitro* plants were used as initial explants. They were cut into small pieces and transferred on several LS media variants supplemented with dichlorophenoxyacetic acid (2,4-D) in combination with 6-benzylaminopurine (BA) or isopenthenyladenine (2iP) at the concentration 0.5; 1 and 2 mg L^{-1}. Agar was added to each basal medium and pH was adjusted to 5.8. Callus tissues were grown in Petri dishes, which were incubated in a growth chamber at 25°C and a 16 h photoperiod, provided by cool white fluorescent lamps. The Tissue culture was subcultured every four weeks. For biochemical analysis callus tissues coming from the 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 a 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 Et_2O, dried over anhydrous $MgSO_4$, filtered and the solvent removed by evaporation on a water bath. 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 accomplished by a 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 mL; 10% *n*-hexane solution. Identification of the essential oil constituents was performed, for both columns, by the 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 (C_9–C_{23}).

GC-MS analysis. GC/EIMS analyses were performed by a Varian CP-3800 gas chromatograph equipped with a 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 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. The identifica-

Table 1. Calibration parameters of the standard compounds used for the GC-MS quantitative analysis.

Standard compounds	LRI	Representative chemical class	Calibration Curve Equation[a]	R^2	Detection Limit (mg/mL)
2-octanol	995	Hydrocarbon derivatives	$y = 0.4765x + 0.0071$	0.999	0.0054
limonene	1029	Monoterpene hydrocarbons	$y = 0.7231x + 0.0154$	0.999	0.0022
menthone	1153	Oxygenated monoterpenes	$y = 0.4350x + 0.0893$	0.999	0.0027
β-caryophyllene	1419	Sesquiterpene hydrocarbons	$y = 0.5470x + 0.0024$	0.999	0.0063
caryophyllene 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

tion of the constituents was based on the comparison of the retention times with those of authentic samples, comparing their linear retention indices (LRI) relative to a series of *n*-hydrocarbons (C_9–C_{30}). A computer matching of mass spectra by two commercial databases (NIST 2000; ADAMS) as well as a homemade library, built up from pure substances or known oils, were used to perform identification of the volatile constituents. Moreover, the molecular weights of the all identified substances were confirmed by GC/CIMS, using MeOH as CI ionizing gas.

HS-SPME-GC-MS analysis. 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. piperita*. Each aliquot was inserted separately into a 50 mL glass conic 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 (5 min), the fiber was withdrawn into the needle and transferred to the injection port of the GC and GC-MS system, operating in the same conditions described for the essential oils, apart from the splitless injection mode and the injector temperature (250ºC).

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. The calibration curves of the analytes were performed by using standards, which have chemical similarity with the compounds of interest in the volatile oils (Table 1). The correspondent regression lines (five points) of each standard were obtained with chromatographic injections of solutions obtained by mixing different accurate volumes of the standard stock solution and an accurate volume of an 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 1). 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 the volatile fraction, 100 μL of the internal standard solution (1 mg/mL) and *n*-hexane to 1 mL (three measurements for the same sample**)**. All results of the quali-quantitative GC-MS analysis are in Tables 2–3.

RESULTS AND DISCUSSION

M. x piperita was collected during the second vegetative year (2009) when plants were very well established. Plants were harvested at the early flowering stage and dried in an open air in the shade. The leaves and stems were studied separately in order to evaluate the volatile constituents in different plant organs.

M. piperita calli were easily developed from the leaves of *in vitro* grown seedlings. The addition of 2 mg L^{-1} 2iP and 0.5 mg L^{-1} 2,4-D gave satisfying results. Callus induction was observed after 15 days. The callus obtained on this medium was light green and friable. Furthermore, small necroses on the explants surfaces were observed, but no roots or shoots were formed. The established tissues were successively subcultured and hydrodistilled by the same procedure used for the leaves and stems of the adult plants. The EO yields obtained from the open-field grown adult plants were found to be 1.8% v/w and 1.5% for stems and leaves, respectively. Therefore, this Polish mint selection showed an EO production similar to other European no-Mediterranean plant samples (Aflatuni *et al.*, 2000; Stojanova *et al.*, 2000). However, the EO yields dropped to 0.1% v/w in the *in vitro* plantlets and callus established from the selected Polish *M. x piperita*.

Regarding the EO composition, menthone, menthol, menthyl acetate, carvone, piperitone, 1,8-cineole, and pulegone were the major compounds in the *M. x piperita* adult plants. The hydrocarbon monoterpenes, α-pinene and sabinene (8 and 7%, respectively), were found especially in the stem EO (Table 2). Carvone and piperitone were present in similar amounts (3%) both in the stem and leaf EOs, while the other two characteristic constituents of mint spp., menthofuran and piperitenone, were not detected (Table 2). The HS-SPME-GC-MS analysis confirmed the absence of these two constituents in the aroma emitted spontaneously from the cultivated plant samples (Table 3).

Among the sesquiterpenes, β-caryophyllene and germacrene D were predominant both in stem and leaf EOs, but significant quali-quantitative differences were registered especially in the total sesquiterpene composition (3%, stems; 18% leaves; Table 2). A larger variety of sesquiterpenes was found in the leaf than in stem EO. This fact was confirmed also by HS-SPME-GC-MS analysis (Table 3). Many studies demonstrated that environmental conditions and geographical origins influenced greatly the EO production in *M. piperita* (Clark & Menary, 1981; Aflatuni, 1999; Maffei, 1999). Further-

Table 2. Mass percent composition[a] of *M. piperita* essential oils (*in vivo/in vitro*)

		adult plants			*in vitro*				
		stems	leaves		*in vitro* plantlets			callus tissue	
Components	***LRI***		***RSD***		***RSD***		***RSD***		***RSD***
a-pinene	939	8.0[a]	0.78	0.7[a]	0.01	3.1[a]	0.19	9.6[a]	0.54
camphene	954					0.3	0.01	2.6	0.12
sabinene	975	6.7	0.49	0.7	0.01	2.7	0.08	2.9	0.10
b-pinene	979	0.7	0.01	1.0	0.01	6.3	0.20	11.9	0.72
myrcene	991	0.8	0.01	0.7		6.2	0.33	1.1	0.01
2-octanol	995	0.5	0.01	0.2				2.7	0.04
iso-sylvestrene[c]	1009							1.0	0.01
a-terpinene	1017							1.4	0.03
p-cymene	1025					1.6	0.03	1.5	0.18
limonene	1029	1.2	0.06	1.3	0.01	10.2	0.17	6.6	0.47
1,8-cineole	1033	5.1	0.19	4.4	0.02	3.6	0.09	10.2	0.41
(Z)-*b*-ocimene	1037	1.0	0.02	1.3	0.02			1.7	0.04
(E)-*b*-ocimene	1050	0.9	0.01	0.8	0.01			1.5	0.01
pentyl isobutanoate	1056					0.2	0.01		
isopentyl isobutanoate[c]	1058					0.4	0.04		
g-terpinene	1060	1.0	0.01					1.2	0.01
cis-sabinene hydrate	1069							5.7	0.23
camphelinone[§]	1070							5.8	0.09
linalool	1097	3.3	0.01					5.9	0.06
allo-ocimene	1132	0.6	0.01	t[b]		2.4	0.03		
neo-isopulegol[§]	1141			t					
isopulegol	1150			2.1	0.02	1.5	0.02		
menthone	1153	26.2	0.51	32.9	0.24	2.1	0.01		
iso-menthone	1163	3.4	0.01	8.1	0.03				
neo-menthol	1166	6.0	0.01	t		2.5	0.02		
menthol	1172	6.7	0.26	11.8	0.04	2.1	0.02	8.9	0.49
4-terpineol	1177	3.4	0.17						
iso-menthol	1183	3.3	0.01						
n-decanal	1202	0.4	0.06						
isopulegone	1208			2.2	0.01				
cis-hexenyl isovalerate	1235			0.2	0.01				
pulegone	1237	3.3	0.02	2.2	0.01				
carvone	1243	3.4	0.01	2.5	0.01	2.1	0.01	6.2	0.01
piperitone	1253	3.8	0.02	3.4	0.03				
menthyl acetate	1295	3.4	0.01	4.8	0.10	2.5	0.02	7.5	0.36
piperitenone	1315								
piperitenone oxide	1369	3.3	0.01			45.3	0.80		
b-bourbonene	1388	0.4	0.01	2.2	0.01	0.2	0.01		
b-elemene	1391	0.4	0.02	t		0.5	0.01		
b-caryophyllene	1419	0.8	0.02	3.3	0.08	0.4	0.02		
trans-muurola-4(14),5-diene	1450			t					
a-humulene	1455			t					
(E)-*b*-farnesene	1457			2.1	0.01				
germacrene D	1485	1.1	0.05	4.0	0.21	2.5	0.03	2.9	0.36
bicyclogermacrene	1500			2.2	0.02	1.1	0.04	0.4	0.01
a-bulnesene	1510			2.3	0.03				
zonarene[c]	1530			t					
globulol	1585			2.2	0.03				
Total		**99.3**		**99.6**		**99.8**		**99.2**	

[a]mass percentage composition was calculated as mg/100mg; [b]t = traces (% < 0.1); [c]tentative identification

more, physiological features such as leaf/stem ratio and herb biomass are positively correlated with oil content, whereas plant height generally influences it negatively (Sharma & Tyagi, 1991). It is important to point out that the acceptable commercial quality of peppermint oil is strictly related to the content of menthone, menthol, and menthyl acetate in addition to traces or lack of pulegone and menthofuran (Burbott & Loomis, 1967; Lawrence, 1985). Regarding peppermint oils and the Pharmacopoeia requirements such as those of the British Pharmacopoeia (1968), menthol must exceed 45% and menthyl acetate must range from 4 to 9%. However, some European and extra-European peppermint oils may contain less than 45% of menthol (Maffei *et al.*, 1994). It is well known that large variations in the menthol content are possible in some regional productions. However, these

Table 3. SPME-GC-MS analysis of adult plants and *in vitro M. piperita* plant material.

		adult plants		*in vitro*					
		stems	leaves	*in vitro* plantlets				callus tissue	
	LRI	PDMS[a]	CARB[a]	PDMS	CARB	PDMS	CARB	PDMS	CARB
Components				Relative percentage composition (%)[b]					
a-pinene	939	0.7		1.7	0.3	1.6	0.3	0.2	0.2
camphene	954	1.0	0.7	t[c]		t			
sabinene	975			1.2	0.4	1.5	0.3	0.5	0.5
b-pinene	979	2.2	1.5	2.3	0.9	3.7	0.8	0.1	0.1
myrcene	991	2.2	1.0	1.1	0.4	3.4	0.6	2.2	2.4
3-octanol	995		1.1	0.3	0.1	0.8		0.3	0.4
3-carene	1003					t		1.5	1.8
iso-sylvestrene [d]	1009					0.2			
a-terpinene	1017					0.3	0.1	3.0	1.5
p-cymene	1025			t	t	0.1		0.3	0.4
limonene	1029	8.2	6.2	0.8	0.3	5.8	1.4	0.3	0.4
1,8-cineole	1033	17.4	13.0	16.2	6.3	20.8	5.7	0.6	0.4
(Z)-*b*-ocimene	1037	5.2	3.9	0.1	0.1			0.6	0.8
2-heptyl acetate	1043							10.6	1.5
(E)-*b*-ocimene	1050	0.5	1.1	0.1	t			4.6	4.9
dihydro tagetone [d]	1053							1.2	1.2
pentyl isobutanoate	1056					0.9	0.2		
isopentyl butanoate [d]	1058					0.4	0.1		
g-terpinene	1060			0.1	t			t	t
cis-sabinene hydrate	1070					0.4	0.1	0.3	0.6
2,5-dimethyl styrene [d]	1099					0.1	0.1		
linalool	1097	0.2	0.3	0.3	0.2	0.3	0.1	3.9	5.4
n-nonanal	1101					0.3	0.1		
1,3,8-*para*-menthatriene [d]	1110					0.1	t		
allo-ocimene	1120	1.1	0.8			0.4	0.1	0.5	0.5
isopulegol	1150	0.2	1.1	0.1	0.1				
menthone	1153	36.1	39.1	51.9	47.0	t	t	0.6	1.1
iso-menthone	1163	6.6	5.9	9.3	7.6	0.1	0.1	1.8	2.8
neo-menthol	1166	0.9	0.5	0.4	0.9	0.1	0.3	2.3	2.4
menthol	1172	6.0	4.6	6.4	9.3	0.1	0.1	0.1	0.1
g-terpineol	1177							3.4	5.2
iso-menthol	1183	0.9	0.5						
n-decanal	1189	0.3	0.4			0.7	0.1	1.7	2.4
verbenone	1192	0.3	0.3			0.1	1.4	3.2	3.9
isopulegone	1193			t	t	0.1	0.7		
pulegone	1237				0.2				
carvone	1243	2.2	2.1	t	0.1	0.7	0.6	1.1	1.9
2-(Z)-hexenyl isovalerate	1245	0.2	1.1	t	0.1			0.3	0.1
2-(E)-hexenyl isovalerate	1247							2.2	3.7
piperitone	1253			0.5	1.1	0.7	0.4	2.9	2.4
cis-piperitone epoxide	1254							0.3	0.1
trans-piperitone epoxide	1256							2.2	3.7
cis-carvone oxide	1263							0.6	0.8
trans-carvone oxide	1276							8.7	11.8
menthyl acetate	1295	1.9	2.7	1.8	4.8	1.1	1.5	23.1	24.9
piperitenone	1343								
piperitenone oxide	1369	0.2	1.8	0.1	1.0	43.7	66.8	2.7	2.3
b-bourbonene	1388	0.2	0.3	0.1	0.4	0.3	0.3	t	1.2
b-elemene	1391	0.1	1.1	0.2	1.7	0.2	0.7	0.6	1.2
b-caryophyllene	1419	1.1	2.1	1.2	4.4	1.9	3.5	0.7	0.6
phenyl ethyl butanoate [d]	1441						0.2		
cis-muurola 3,5-diene	1450			t	0.2		0.1		
trans-muurola 3,5-diene	1454			0.1	0.3				
a-humulene	1455					0.1	0.8	0.5	0.1
cis-muurola-4(14),5-diene	1467			0.1	0.5		0.3	1.2	
germacrene D	1485	0.6	1.6	1.0	6.0	0.8	2.5	2.0	1.2
bicyclogermacrene	1500		0.4		0.3		0.6	0.3	0.2
spathulenol	1578					t	0.3		
caryophyllene oxide	1583				0.9	0.1	1.9		
Total		**96.5**	**95.2**	**97.6**	**95.9**	**91.9**	**93.4**	**94.1**	**98.1**

[a]PDMS polydimethylsiloxane fiber; CARB carboxen fiber. [b]Percentage average calculated as relative percentage composition (%) on DB-5 column without correction factors. [c]t = traces (%<0.1). [d]tentative identification

areas are generally capable of producing EOs with acceptable menthol concentration, even if some variability within the same area is possible due to the unevenness in plant maturity. However, although the menthol content is often below the required level, menthyl acetate concentration is generally satisfied (Shah & Gupta, 1989; Chalchat *et al*., 1997).

In the present study, menthol and menthyl acetate were both present in the leaf and stem EOs hydrodistilled from the adult plants cultivated in Poland. These EOs were richer in menthol than in menthyl acetate, even if menthol (7–12%, stems; 3–5%, leaves) was much lower than the quality parameters fixed by ESCOP (ESCOP, 1992). The established *M. x piperita* callus produced menthol (9%) in comparable amounts with its mother plant (12% leaves, 7% stems), while menthyl acetate was enhanced (8%) significantly in comparison with the stem and leaf EOs (3% and 5%, respectively). On the other hand, the *in vitro* plantlets showed much lower levels of menthol and menthyl acetate (2%) than callus. With respect to biosynthetic processes in mint spp., menthones derive from the reduction of menthylacetate, which is routinely found in measurable quantities in the peppermint oil (Croteau & Hooper, 1978). In the present study, the EOs obtained from adult plants cultivated in Poland showed a menthol-menthone ratio of 0.26 for stems and 0.36 for leaves. Lower values were determined in the correspondent static headspaces (0.16 stems; 0.12 leaves), but these results confirmed that menthone contributed more than menthol to the spontaneous aroma of the cultivated adult plants. In fact, menthone was predominant both in the stem (26%) and leaf (33%) EOs, while menthol showed lower levels (7 and 12% stems and leaves, respectively; Table 2) Pulegone was detected only in the leaf and stem EOs of adult plants (3–2%, respectively), while it was not observed in the EOs of the *in vitro* plant material. However, the composition of monoterpene alcohols and ketones showed interesting quali-quantitative differences especially in the *in vitro* biomass established from the different plant organs.

In fact, menthone and menthol were detected in the same amounts (2%) in the EOs of *in vitro* plantlets. On the other hand, menthone was not detected in the callus EOs that was rich especially in menthol (9%), 1,8-cineole (10%), methyl acetate (7%), and carvone (6%). The production of these typical mint oxygenated monoterpenes was enhanced in the callus tissues in comparison with the correspondent mother plants (Table 2). Otherwise, the biosynthesis of the hydrocarbon sequiterpenes was drastically reduced in the *in vitro* plant material of *M. x piperita* and only germacrene D was produced in significant amounts (2.5 and 2.9% *in vitro* plants, callus respectively).

The *in vitro* plantlets oil showed a completely different composition in comparison with the callus because it was characterized by especially huge amounts of the piperitenone oxide (45.3%, Table 2). As reported in the literature, the mint plants with low menthofuran content generally produce measurable amounts of *trans*-piperitone oxide and piperitenone oxide. These epoxyketones are reported infrequently in peppermint, and the total content very rarely exceeds a few percent in the distilled essential oil (Croteau, 1991; Wise & Croteau, 1999).

The EO of *in vitro* plantlets was also richer in limonene (10%) than callus. This compound is considered the precursor of pulegone, carvone, and piperitenone (Wise & Croteau, 1999; McConkey *et al*., 2000; Turner & Croteau, 2004). In the EOs of *in vitro* plantlets, pulegone was not detected and carvone (2%) was lower in comparison with the other samples. Therefore, limonene may be preferentially transformed to piperitenone, which is then oxidized to piperitenone oxide especially in the *in vitro* plantlets. Piperitenone oxide was also found in the HSs of *M. x piperita* callus and mother plants, but at much lower levels (2–3% and 0.1–2%, respectively; Table 3).

The commercial importance of peppermint mint depends on the percentage of menthol and menthone as well as the presence of some undesirable compounds such as pulegone and menthofuran (EMEA, 2004). In the present study, menthone and menthol characterised the EOs of the adult plants cultivated in Poland and *in vitro* plantlets. The callus tissue yielded an essential oil especially enriched in menthol and not containing menthone, menthofuran and pulegone. In the present study, the lack of menthofuran characterized both the *in vivo* and *in vitro* EO production.

This common feature was confirmed in all the analysed samples by the HSs analyses (Table 3).

It is worth to notice that pulegone, the precursor of menthofuran, was detected in the stem and leaf EOs of open-field plants (3 and 2%, respectively), but neither in the EOs and HSs of the correspondent *in vitro* plant material (Tables 2–3). As reported in the literature, pulegone and menthofuran are closely related to mint safety (Murray *et al*., 1988). The hepatoxicity of pulegone is believed to result from its metabolism to menthofuran (Thomassen *et al*., 1990). The Working Party on Herbal Medicinal Products of the EMEA Agency released a draft position paper on the use of herbal medicines containing pulegone and menthofuran (EMEA/HMPWP, 2004). More recently, the Scientific Committee on Food of EFSA was asked to provide scientific advice to the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the EU Member States. Pulegone and menthofuran were evaluated too and, accordingly, cannot be used as flavouring substances in the EU market (JECFA, 2009).

In conclusion, *M. x piperita* callus and *vitro* plantlets were established by sustainable biotechnological protocols using selected adult plants cultivated in Poland, which were already previously characterized by reduced amounts of pulegone as well as the lack of menthofuran. The essential oil obtained from the established callus did not contain pulegone or menthofuran. It was enriched in the typical mint volatiles such as 1,8-cineole, menthol, menthyl acetate, carvone, which were found also in the analysed parent plants. Although further studies will be needed to improve the EOs yields from the *in vitro* biomass, the established callus may be regarded as a potential source of a mint-type essential oil not containing pulegone or menthofuran.

Acknowledgement

The work has been financially supported by the European Community by the NUTRA-SNACK, 6FP project.

REFERENCES

Alkire BH, Simon JE (1993) Water management for midwestern peppermint (*Mentha* x *piperita* L.) growing in highly organic soils, Indiana, USA. *Acta Hort* **344**: 544–556.

Aflatuni A (1999) The comparative study of mint species grown in Northern Finland. In *Mint Research in Finland*, Salo R ed. Symposium of Mint Research, Jokioinen, Agricultural Research Centre of Finland, Serie A **66**: 74–81.

Aflatuni A, Heikkinen K, Tomperi P, Jolanen J, Laine K (2000) Variation in the extract composition of mints of different origin cultivated in Finland. *J Essent Oil Res* **12**: 462–466.

Ansari MA, Vasudevan P, Tandon M, Razdan RK (2000**)** Larvicidal and mosquito repellent action of peppermint (*Mentha piperita*) oil. *Bioresource Technol* **71**: 267–271.

Banthorpe DV (1996) *Mentha* species (mints): *in vitro* culture and production of lower terpenoids and pigments. In *Biotechnology in Agriculture and Forestry*, Bajaj PS ed, vol. 37, pp 202–225. Medicinal and Aromatic Plants IX. Springer-Verlag, Berlin.

Bhat S, Maheshwari P, Kumar S, Kumar A. (2002). Mentha species: *in vitro* regeneration and genetic transformation. *Mol Biol Today* **3**: 11–23.

Bruneton J (1995) *Pharmacognosy, Phytochemistry, Medicinal Plants,* pp 405–466. Lavoisier Publ. Londres, New York, Paris.

Burbott AJ, Loomis WD (1967) Effects of light and temperature on the monoterpenes of peppermint. *Plant Physiol* **42**: 20–28.

EMEA/HMPWP/52/04, Draft Position Paper ON the use of herbal medicinal products containing pulegone and menthofuran. EMEA 2004, pp 1–4.

European Pharmacopeia (1997) 3rd edn. Strasbourg, France: Council of Europe, 1298–1300.

Chalchat JC, Garry RP, Michet A (1997) Variation of chemical composition of essential oil of *Mentha piperita* L. during the growing time. *J Essent Oil Res* **9**: 463–465.

Clark RJ, Menary RC (1979) The importance of harvest data and plant density on the yield and quality of Tasmanian peppermint oil. *J Amer Soc Hort Sci* **104**: 702–706.

Clark RJ, Menary RC (1981). Variations in composition of peppermint oi1 in relation to production areas. *Econ Bot* **35**: 59–69.

Croteau R, Hooper LC (1978) Metabolism of monoterpenes. Acetylation of (-)-menthol by a soluble enzyme preparation from peppermint (*Mentha piperita*) leaves. *Plant Physiol* **61**: 737–742.

Croteau R (1991) Metabolism of monoterpenes in mint (*Mentha*) species. *Pl Med* Suppl **57**: 10–14.

Diemer F, Jullien F, Faure O, Moja S, Colson M, Matthys-Rochon E, Caissard JC (1998) High efficiency transformation of peppermint (*Mentha x piperita* L.) with *Agrobacterium tumefaciens*. *P1 Sci* **136**: 101–108.

Dorman HJD, Koşar M, Kahlos K, Holm Y, Hiltunen R (2003) Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *J Agric Food Chem* **51**: 4563–4569.

Duke JA, Bogenschultz-Godwin MJ, duCellier J, Duke PAK (2002) In *Handbook of Medicinal Herbs*, 2nd edn, pp 22. CRC, Press, Boca Raton, FL,.

ESCOP (1992) Proposal for a European monograph on the medicinal use of Menthae Piperitae aetheroleum peppermint oil. In *Proposal for European Monograph on the Medicinal Use*. Vol 3.

European Pharmacopeias (1994) 2nd edn *European Treaty Series No 50*, 405–457. Maissonnueve SA, France.

Gershenzon J, McConkey ME, Croteau RB (2000) Regulation of monoterpene accumulation in leaves of peppermint. *Pl Physiol* **122**: 205–213.

Hefendehl FW, Underhill EW, von Rudloff E (1967) The biosynthesis of the oxygenated monoterpenes in mint. *Phytochemistry* **6**: 823–835.

JECFA, 55th meeting (2009) Scientific Opinion of the Panel on Food Contact Material, Enzymes, Flavourings & Processing Aids on a request from the Commission on Flavouring Group Evaluation.

Kim T, Kim TY, Bae GW, Lee HJ, Chae YA, Chung I.S. (1996) Improved production of essential oils by two-phase culture of *Mentha piperita* cells. *Plant Tissue Cult Lett* **13**: 189–192.

Hendriks H (1998) Pharmaceutical aspects of some *Mentha* herbs and their essential oils. *Perfum Flavor* **23**: 15–23.

Krasnyanski S, May RA, Loskutov A, Ball TM, Sink KC (1999) Transformation of the limonane synthase gene into peppermint (*Mentha piperita* L.) and preliminary studies on the essential oil profiles of single transgenic plants. *Theoretical and Applied Genetics* **99**: 676–682.

Kumar S, Bahl JR, Bansal RP, Kukreja AK, Garg SN, Naqvi AA, Luthra R, Sharma S (2000) Profits of Indian menthol mint *Mentha arvensis* cultivars at different stages of crop growth in northern plains. *J Med Aro Pl Sci* **22**: 774–786.

Lawrence BM (1985) A review of the world production of essential oils (1984). *Perfumer & Flavorist* **10**: 1–16.

Lawrence BM (1997) Progress in Essential Oils: Peppermint Oil. *Perfumer & Flavorist* **22**: 57–66.

Linsmayer EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol Plantarum* **18**: 100–127.

Linsmaier EM,d Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. *Plant Physiol* **21**: 487–492.

Maffei M (1999) Sustainable methods for a sustainable production of peppermint (*Menta × piperita* L.) Essential Oil. *J Essent Oil Res* **11**: 267–282.

Maffei M, Bertea CM, Mucciarelli M. (2007). Anatomy, physiology, biosynthesis, molecular biology, tissue culture, and biotechnology of mint essential oil production. In *Mint: The genus Mentha*, Lawrence BM ed, pp 41–85. CRC Press, Boca Raton, Florida.

Maffei M, Mucciarelli M, Scannerini S (1994) Are leaf area index (LAI) and flowering related to oil productivity in peppermint? *Flavour Frag J* **9**: 119–124.

Marotti M, Piccaglia R, Giovanelli E, Deans SG, Eaglesham E (1994) Effects of planting time and mineral fertilization on peppermint (*Mentha piperita* L.) essential oil composition and its biological activity. *Flavour Frag J* **9**: 125–129.

McConkey ME, Gershenzon J, Croteau RB (2000) Developmental regulation of monoterpene biosynthesis in the glandular trichomes of peppermint. *Plant Physiol* **122**: 215–223.

Misra A, Srivastava NK (2000) Influence of water stress on Japanese mint. *J Herbs Spices Med Plants* **7**: 51–58.

Murray MJ, Hefendehl FW (1972) Changes in monoterpene composition of *Mentha aquatica* produced by gene substitution from *M. arvensis*. *Phytochemistry* **11**: 2469–2474.

Murray MJ, Marble P, Lincoln D, Hefendehl FW (1988) Peppermint oil quality differences and the reason for them. In *Flavor and Fragrances: A World Perspective*, Lawrence BM, Mookherjee BD, Willis BJ, eds, pp 189–210. Elsevier Press, Amsterdam.

Rohloff J (1999) Monoterpene composition of essential oil from peppermint (*Mentha x piperita* L.) with regard to leaf position using solid-phase microextraction and gas chromatography/mass spectrometry analysis. *J Agric Food Chem* **47**: 3782–3786.

Shah SC, Gupta LK (1989) Response of mentha species to different harvesting intervals. *Prog Hort* **21**: 148–150.

Sharma S, Tyagi BR (1991) Character correlation, path coefficient and heritability analyses of essential oil and quality components in corn mint. *J Genet* **45**: 257–262.

Spencer A, Hamill JD, Rhodes MJC (1993) *In vitro* biosynthesis of monoterpenes by *Agrobacterium* transformed shoots cultures of two *Menta* species. *Phytochemistry* **32**: 911–919.

Srivastava NK, Misra A, Sharma S (2003) Variation among commercial cultivars of Japanese mint (*Mentha arvensis* L.) in the morphological and metabolite characters associated with essential oil yield. *J Hortic Sci Biot Technol* **78**: 154–160.

Stojanova A, Paraskevova P, Anastassov Ch, (2000) A comparative investigation on the essential oil composition of two Bulgarian cultivar of *Mentha piperita* L. *J Essent Oil Res* **12**: 438–440.

Thomassen D, Slattery JT, Nelson SD (1990) Menthofuran-dependent and independent aspects of pulegone hepatotoxicity: roles of glutathione. *J Pharmacol Exp Ther* **253**: 567–572.

Tisserat B, Silman R (2000) Interactions of culture vessels, media volume, culture *density, and carbon dioxide levels on lettuce and spearmint shoot growth in vitro*. *Plant Cell Rep* **19**: 464–471.

Tucker AO, De Baggio T (2000) In *The big book of herbs*, pp 688. Interweave Press, Loveland, CO.

Tucker AO, Naczi RFC (2007) *Mentha*: an overview of its classification and relationships. In *Mint. The Genus Mentha. Medicinal and Aromatic Plants — Industrial Profiles* (1–39). Lawrence BM red. CRC Press, Boca Raton, Florida, Taylor & Francis Group.

Turner GW, Croteau R (2004) Organization of monoterpene biosynthesis in mentha. immunocytochemical localizations of geranyl diphosphate synthase, limonene-6-hydroxylase, isopiperitenol dehydrogenase, and pulegone reductase. *Plant Physiol* **136**: 4215–4227.

Voirin B, Brun N, Bayet C (1990) Effects of daylength on the monoterpene composition of leaves of *Mentha × piperita*. *Phytochemistry* **29**: 749–755.

Weglarz Z, Zalecki R (1985) Investigations of dependence of the crop season of peppermint (*Mentha piperita* L.) herb upon the crop itself and the quality of the raw material. *Herba Pol* **31**: 175–180.

Weglarz Z, Zalecki R (1987). Effect of plantation age, herb harvest time and time of rhizome digging on the value of rhizome seedlings (*Mentha piperita* L.). *Herba Pol* **33**: 43–48.

Wise ML, Croteau R (1999) Monoterpene biosynthesis. In *Comprehensive natural products chemistry: isoprenoids*. Cane ed, pp 9715. Elsevier, Oxford.