Trichomes micromorphology and essential oil variation at different developmental stages of cultivated and wild growing *Mentha pulegium* L. populations from Portugal

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ARTICLE INFO

Article history:

Received 11 April 2012

Received in revised form 11 July 2012

Accepted 31 July 2012

Keywords:

Mentha pulegium L.

Lamiaceae

Trichomes

Essential oil

Histochemistry

GC

GC–MS

ABSTRACT

The indumentum of *Mentha pulegium* L., studied by light and scanning electron microscopy, was characterized by non-glandular and glandular trichomes, which corresponded to the common arrangement described for the Lamiaceae family. Histochemistry revealed the presence of pectins, total lipids, acidic lipids and essential oils in the glandular trichomes secretions. The essential oil yield ranged from 0.3% (w/d.w.) in the vegetative phase to 1.6% at full flowering. Gas chromatography and gas chromatography–mass spectrometry essential oils composition analysis at full flowering revealed mostly quantitative rather than qualitative variations, with pulegone as the major compound (52–82%), followed by isomenthone (2–36%), menthone (0.1–17%), and piperitenone (1–15%). Comparative evaluation of cultivated and wild growing populations showed differences in the relative amounts of the main components of the essential oils isolated from plants harvested at different developmental stages.

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1. Introduction

The genus *Mentha* L. (Lamiaceae), comprising more than 25 species, is responsible for approximately 2000 t of world essential oil, making it the second most important essential oil producing genus, after *Citrus* (Mucciarelli et al., 2001). *Mentha pulegium* L., a member of this genus, commonly known as pennyroyal (*poejo* in Portuguese), is an aromatic perennial herbaceous plant reaching up to 40 cm height (Stengele and Stahl-Biskup, 1993). This species grows wild in humid and damp areas and water banks of central, southern and Western Europe, north Africa and Asia Minor (Chalchat et al., 2000; Tutin et al., 1972). The aerial parts are pubescent bearing glandular trichomes which are responsible for the essential oil secretion. The morphology,

distribution and frequency of these glandular trichomes are distinctive characteristics among the Lamiaceae species (Werker, 2000). In Portugal, the aerial parts of *M. pulegium* and the preparations from it have been traditionally used in Alentejo Region to flavour recipes, as well as for its medicinal properties (Póvoa et al., 2006). In folk medicine it is used as an infusion, preventing different gastric disorders and inflammations of the respiratory tract (Mkaddem et al., 2007; Póvoa et al., 2006). Nevertheless, there are no approved medicinal uses for pennyroyal essential oil (Barceloux, 2008). This plant has also been used as a spice and flavouring agent in different foods (Mkaddem et al., 2007; Monteiro et al., 2007a), despite the fact that the essential oils of *M. pulegium* are generally considered to be rich in pulegone, a toxic compound with potentially lethal hepatotoxic effects (Anderson et al., 1996). Several publications, reports and directives of the UE emphasize the need for a better characterization of botanicals and botanical preparations and for scientific assessment of risks from exposure of consumers to these products. The UE directive 88/388/EEC has stipulated a maximum concentration for this oxygen-containing monoterpene of 100 mg/kg in beverages and 25 mg/kg in foodstuff, with the exception of 250 mg/kg in

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flavoured beverages and 350 mg/kg in mint confectionery (EEC, 1988).

It is known that the chemical composition of plants is influenced by several external factors including growing conditions and climate (Figueiredo et al., 2008). The essential oils from *M. pulegium* have been characterized in different regions of the world, Table 1 (and references therein). Despite these reports, and studies in Portuguese cultivated populations (Lopes et al., 2010; Monteiro et al., 2007b; Teixeira et al., 2012), there is no previous report on the chemical composition of wild growing populations of *M. pulegium* volatile oils from mainland Portugal. One should not forget that, in cultivated mint plants the qualitative oil composition is relatively stable, but in most wild growing mints a great diversity in essential oil constituents has been observed (Mimica-Dukic and Bozin, 2008).

In view of the potential pharmacological, commercial and food industry value of this species, the work described in this paper was carried out to (1) characterize, by light and scanning electron microscopy, the morphology and structure of the indumentum, in particular of the glandular trichomes, which are the main responsible for the essential oils secretion, (2) typify the chemical composition of the essential oil from Portuguese *M. pulegium* wild populations, (3) compare the essential oil composition between cultivated and wild growing populations and (4) evaluate the evolution of the essential oil components throughout the plant developmental stages.

2. Material and methods

2.1. Plant material

In 2009, several field trips were conducted across the geographic range of *M. pulegium*. A total of 14 populations with different geographic origins, representative of the distribution of the species in mainland Portugal, were included in the analysis (Table 2, Fig. 1). To characterize the essential oil (EO) composition and identify possible chemotypes, these 14 populations were collected, during the flowering phase, from natural habitats. Voucher specimens have been deposited in the LISI herbarium (Table 2).

In order to understand the evolution in EO composition and yield along the plant life cycle, and compare cultivated with wild growing conditions, a time-course study was undertaken. In this study, six populations (15 plants per population) were collected from the wild, transported in containers and transplanted to the essay field at Instituto Superior de Agronomia, Lisbon, Portugal. Plants were planted 50 cm apart, in 2 m² plots, and drip irrigated periodically (each 7–10 days). Plots were kept weed free by hand hoeing. Samples from the six populations, in the wild and in the cultivated essay field, were harvested at the vegetative, pre-flowering and full flowering phases.

2.2. Morphological studies

2.2.1. Light microscopy (LM)

Stems, leaves and flowers at the different developmental stages, of 10 individuals for each population, were fixed with 3% glutaraldehyde (Merck, Germany) in a 0.1 M sodium phosphate buffer, pH 7.3, for 4 h at 4 °C, and washed in the same buffer (Ascensão et al., 1999). After dehydration in a graded series of ethanol solutions, hand-cut cross-sections were made and clarified with sodium hypochlorite and washed in distilled water (Evans, 1996). Observations were carried out under a Nikon Eclipse E400 microscope equipped with a Nikon Coolpix MDC lens adapter. Images were obtained with a Nikon Coolpix 995 digital camera. Quantitative

Fig. 1. Map of Portugal with collection sites of *Mentha pulegium* populations analyzed. Symbols, according to the essential oil cluster analysis (Fig. 3). For abbreviations, see Table 2.

characters are the average of, at least, 30 different observations for each population.

2.2.2. Scanning electron microscopy (SEM)

Plant material was fixed as above, critical-point dried in a Polaron BioRad E3500, and coated with gold in a Jeol JFC-1200 (Tokyo, Japan). Observations were carried out at 15 kV, on a Jeol JSM-5220 LV scanning electron microscope (Tokyo, Japan) equipped with an image acquisition system. Measures and counting were obtained by computer-assisted image analysis.

2.3. Histochemical studies

General staining procedures for detecting some of the main chemical groups secreted were carried out using fresh leaves and flowers from 2 populations (Table 1). The histochemical tests included: (1) Sudan III for total lipids (Johansen, 1940); (2) Nile Blue for neutral and acidic lipids (Jensen, 1962); (3) Nadi reagent for essential oils and resin acids (David and Carde, 1964); and (4) Ruthenium Red for pectins (Johansen, 1940). Standard control procedures were carried out simultaneously.

2.4. Essential oil analysis

2.4.1. Isolation procedure

For each sample, aerial parts of 10 individuals per population were collected, grossly pulverized, and 20 g were subjected to hydrodistillation for 1 h in a Clevenger-type apparatus according

Table 1
Data from previous studies on the essential oil composition of *Mentha pulegium* L.

Collection country	Plant part	Extraction procedure	Main component(s) (%)	Identification method	Reference
Algeria	Aerial parts	Hydrodistillation	Pulegone 4–87	GC, GC/MS	Beghidja et al. (2007)
Bulgaria	Aerial parts	Water and steam distillation	Pulegone 43–45	GC, GC/MS	Stoyanova et al. (2005)
European countries	Different plant parts	Hydrodistillation	Pulegone 7–85	GC, GC/MS	Stengele and Stahl-Biskup (1993)
Greece	Aerial parts	Hydrodistillation	Pulegone 0.1–91	GC, GC/MS	Kokkini et al. (2004)
India	Aerial parts	Hydrodistillation	Pulegone 65–83	GC, GC/MS, ¹ H NMR, ¹³ C NMR	Agnihotri et al. (2005)
Iran	Aerial parts	Hydrodistillation	Pulegone 38	GC/MS	Aghel et al. (2004)
Iran	Aerial parts	Hydrodistillation	Piperitone 38 Piperitenone 33 Menthone 39	GC, GC/MS	Mahboubi and Haghi (2008)
Iran	Aerial parts	Hydrodistillation	Pulegone 60	GC, GC/MS	Hassanpouraghdam et al. (2011)
Portugal	Aerial parts	Hydrodistillation	Pulegone 65–87	GC, GC/MS	Monteiro et al. (2007b)
Portugal	Aerial parts	Hydrodistillation	Menthone 36 Pulegone 23	GC, GC/MS	Lopes et al. (2010)
Portugal	Aerial parts	Hydrodistillation	Pulegone 35 Piperitenone 27	GC/MS	Teixeira et al. (2012)
Portugal	Aerial parts	Hydrodistillation	Pulegone 80	GC/MS	Mata et al. (2007)
Spain	Aerial parts	Hydrodistillation	Pulegone 41–42	GC, GC/MS	Reis-Vasco et al. (1999)
Tunisia	Aerial parts	Hexane extract	Pulegone 42	GC, GC/MS	Maroto-Diaz et al. (2007)
Tunisia	Aerial parts	Hydrodistillation	Pulegone 44	GC, GC/MS	Mkaddem et al. (2007)
Turkey	Aerial parts	Hydrodistillation	Pulegone 72	GC, GC/MS	Hajlaoui et al. (2010)
Uruguay	Leaves	Hydrodistillation	Pulegone 73	GC-FID, GC/MS	Sarikurku et al. (2012)
Yugoslavia	Aerial parts	Steam Distillation	Menthone 31	GC, GC/MS	Lorenzo et al. (2002)
					Chalchat et al. (2000)

to the European Pharmacopoeia (Council of Europe, 2007). The oils were kept at 4 °C until further analysis.

2.4.2. Gas chromatography (GC)

GC analysis were performed using a PerkinElmer 8700 gas chromatograph (PerkinElmer, Shelton, CT, USA) equipped with two FIDs, a data-handling system, and a vaporizing injector port in which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Agilent Technologies, Santa Clara, CA, USA); and a DB-17HT fused-silica column (30 m × 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc.). Oven temperature was programmed, 45–175 °C, at 3 °C min⁻¹, subsequently at 15 °C min⁻¹ up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures were 280 °C and 290 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm s⁻¹. Samples were injected using the split sampling technique, ratio 1:50, with a volume of injection of 0.1 µl of a pentane-oil solution. The percentage composition of the oils was computed by the normalization method from the GC peak areas, which were calculated as mean values of two injections of each oil sample, without using response factors.

Table 2
Data on collection site and sample type of *Mentha pulegium* wild populations studied.

Populations	Sample type ^a	Specific sample collection sites					
		Localization	Altitude (m)	Latitude	Longitude	Hidrografic basin	Voucher
Mp2	W/C/DS	Ouguela, Campo Maior	207	39° 4' 54.96" N	7° 0' 4.33" W	Guadiana	1059/2010
Mp32	W/C/DS	Valência de Alcântara	313	39° 28' 1.17" N	7° 12' 24.16" W	Tejo	1060/2010
Mp33	W/C/DS	Alburquerque	234	39° 11' 0.69" N	7° 1' 59.03" W	Guadiana	1061/2010
Mp34	W	Montalvão, Nisa	116	39° 39' 50.86" N	7° 32' 19.27" W	Tejo	1062/2010
Mp36	W	Bagaúte, Peso da Régua	50	41° 9' 0.41" N	7° 45' 2.24" W	Douro	1063/2010
Mp40	W	Gomes Aires, Almodôvar	200	37° 30' 58.11" N	8° 11' 5.17" W	–	1064/2010
Mp41	W/C/DS	La Codosera	298	39° 16' 48.08" N	6° 52' 20.89" W	Guadiana	1065/2010
Mp54	W/C/DS	Segura, Idanha-a-Nova	235	39° 49' 11.06" N	6° 58' 52.99" W	Tejo	1066/2010
Mp55	W/C/DS	Salvaterra do Extremo, Idanha-a-Nova	253	39° 53' 37.50" N	6° 54' 18.38" W	Tejo	1067/2010
Mp59	W	Monfortinho, Idanha-a-Nova	255	39° 59' 9.96" N	6° 52' 50.23" W	Tejo	069/2010
Mp60	W	Entradas, Castro Verde	154	37° 44' 36.51" N	7° 58' 44.60" W	Guadiana	1070/2010
Mp62	W	Póvoa, Miranda do Douro	750	41° 34' 22.71" N	6° 19' 17.53" W	Douro	1071/2010
Mp63	W	Escarigo, Figueira de Castelo Rodrigo	560	40° 50' 34.73" N	6° 49' 33.62" W	Douro	1072/2010
Mp64	W	Vilar seco, Miranda do Douro	725	41° 31' 25.48" N	6° 24' 5.56" W	Douro	1073/2010

^a Sample type: W, wild; C, cultivated; DS, developmental stage.

2.4.3. Gas chromatography–mass spectrometry (GC–MS)

The GC–MS unit consisted of on PerkinElmer Autosystem XL gas chromatograph (PerkinElmer, Shelton, Connecticut, USA), equipped with DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc., Agilent Technologies, Santa Clara, CA, USA), and interfaced with a PerkinElmer Turbomass mass spectrometer (software version 4.1, PerkinElmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280 °C; ion trap temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm s⁻¹; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 µA; scan range, 40–300 µm; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to a C₉–C₁₇ hydrocarbon standard mixture, and with GC–MS spectra from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesised components and commercial available standards.

2.4.4. Data analysis

The percentage composition of the isolated essential oils was used to determine the relationship between the different samples

by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) (Rohlf, 2000). For cluster analysis, correlation coefficient was selected as a measure of similarity among all accessions, and the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (2000) and classified as very high (0.9–1), high (0.7–0.89), moderate (0.4–0.69), low (0.2–0.39) and very low (<0.2).

3. Results and discussion

3.1. Morphological studies

The indumentum of *M. pulegium* includes non-glandular and glandular trichomes scattered all over the vegetative and reproductive organs. The non-glandular trichomes are of three different types: (i) unicellular, with a warty surface, a swollen basal epidermal cell and acute apices (Fig. 2A), which is seen on stems and sepals and on both leaf surfaces, but more abundant on the adaxial surface; (ii) short multicellular, 2–4 cells, uniseriate, warty surface, supported by a cellular pedestal formed by two to five epidermal cells arranged around the base and acute apices, sparse on adaxial leaf surface but common on sepals inner and outer faces (Fig. 2A); (iii) long multicellular, up to 8 cells, with bigger cell dimensions, thin, uniseriate, acute apices, warty surface, always leaned toward the apex and supported by a cellular pedestal formed by two to five epidermal cells, only seen on the petal apex outer face (Fig. 2B). The glandular trichomes belong to two morphologically different types, peltate and capitate, which are considered as the common glandular trichome arrangement in the Lamiaceae family (Werker et al., 1993) and were as well described in *Mentha spicata*, *M. spicata* × *suaveolens* (Martins, 2002), *M. cervina* (Rodrigues et al., 2008) and other Lamiaceae species (Ascensão et al., 1999; Corsi and Bottega, 1999; Rodrigues et al., 2006). In *M. pulegium* peltate trichomes are seen all over both leaf surfaces, dominant on the abaxial surface, on the stem, on the inner and outer surfaces of sepals, and on the outer face apex of petals. They have a short stalk and a smooth large head, with a variable number of secretory cells arranged in one or two circles. Our results show that in *M. pulegium* 12 cells are probably the most common number in a peltate trichome of mature leaves, usually 4 in the inner circle and 4–8 in the outer (Fig. 2G). That number can reach up to 16 cells in the petals peltate trichomes, usually 4 in the inner circle and 8–12 in the outer. According to Turner et al. (2000) and Martins (2002), the number of cells forming the head disc in peltate trichomes depends on the development stage as well as of the plant species, a mature leaf peltate trichome exhibited 10 disc cells in *M. pulegium*, 8 in *M. piperita* (Turner et al., 2000), 12 in *M. spicata* and *M. spicata* × *suaveolens* (Martins, 2002), and 16 in *Prostanthera ovalifolia* (Gersbach, 2002). Upon maturation, these glandular trichomes are sunken in epidermal depressions and the cuticle of the cells of the secretory head lifts, forming a subcuticular space that encloses secretions. The head dimensions of peltate trichomes are variable, but bigger on the reproductive structures: diameter up to 109 μm ($\pm 9 \mu\text{m}$) on the corolla, compared to 88 μm ($\pm 10 \mu\text{m}$) on the adaxial leaf surface and 92 μm ($\pm 9 \mu\text{m}$) on the abaxial leaf surface. Morphologically well developed peltate glandular trichomes, were also observed on cotyledons (Fig. 2C), as in other Lamiaceae, such as *Salvia officinalis* and *Ocimum basilicum* (Croteau et al., 1981; Werker et al., 1993). The peltate trichomes are the only kind of glandular trichomes seen on reproductive structures, occurring along the lower side of the connective tissue, between the two anther lobes (Fig. 2D). This report on the presence of peltate trichomes between the two anther lobes is noteworthy but was also reported

for other Lamiaceae species (Ascensão et al., 1995; Rodrigues et al., 2008).

It is within the peltate trichomes that most of the essential oil is believed to be synthesized (Turner et al., 2000). The material secreted by the glandular head cells passes through the apical cell walls and accumulates within a large space formed by the detachment of the cuticle together with the pectin layer of the secretory cell walls. The secretory products remain in this space, giving a spherical shape to each mature peltate trichome. A similar pattern of subcuticular space formation was described in oil glands of other Lamiaceae species (Turner et al., 2000; Werker et al., 1993). Measurements of the glandular secretory head cells and of the subcuticular fillings show that the maximum diameter of the secretory head cells is achieved during an earlier stage of development, and that the increase in total diameter of the peltate glandular trichome is due to further secretion during leaf growth and dependent of the organ in which it is present. Because the accumulation of the secreted material continues during the growth of the organs that bear them, they are considered long-term trichomes (Werker et al., 1993; Werker, 2000 and references there in).

Capitate trichomes are also widespread in Lamiaceae. The types of capitate trichomes found differ in stalk length and head shape and include: (i) capitate type I, with one stalk cell 10 μm ($\pm 0.1 \mu\text{m}$) in length, and a round/oval secretory head cell, with a smooth surface (Fig. 2E), 27 μm ($\pm 2.2 \mu\text{m}$) in length and 21 μm ($\pm 1.7 \mu\text{m}$) in diameter at the head, uniformly distributed on both leaf surfaces, calyx and stems; (ii) capitate type II, with a lower conical stalk cell, 28 μm ($\pm 6 \mu\text{m}$) in length and 1 to 2 elongated neck cells, 12 μm ($\pm 0.7 \mu\text{m}$) and a round secretory head cell, with a smooth surface (Fig. 2F), and 13 μm ($\pm 0.1 \mu\text{m}$) in diameter at the head, only on the adaxial petal surface. The capitate trichomes found correspond to the capitate types I and II described by Werker et al. (1985). In capitate glandular trichomes much less material is accumulated in the cell lumen and no rupture of the cuticle was observed.

On fully expanded mature leaves of *M. pulegium*, the capitate glandular trichomes were densely distributed while the peltate were scattered among them. Similar results were found also in *M. pulegium* by Karray-Bouraoui et al. (2009) and in both *M. spicata* and *M. spicata* × *suaveolens* by Martins (2002). The densities observed were: (i) 2.7 and 3.9 peltate trichomes/ mm^2 on the adaxial and abaxial leaf surface respectively, (ii) 10 and 13 capitate trichomes/ mm^2 on the adaxial and abaxial leaf surface, respectively. These results showed a larger distribution on the abaxial surface, a common feature for several other Lamiaceae species (Ascensão et al., 1995; Rodrigues et al., 2006, 2008; Turner et al., 2000; Werker et al., 1993). Nevertheless, the densities found in *M. pulegium* were the lowest values compared to other studies in *M. pulegium* (Karray-Bouraoui et al., 2009) and other mints (Turner et al., 2000).

3.2. Histochemical studies

Data from histochemical tests revealed that the secreted material composition was similar in both leaves and flowers and had a complex nature, containing lipophilic as well as hydrophilic components. The presence of these compounds, although independent of the organ and of its developmental stage, was dependent on the trichome type (Table 3). The chemical composition of trichomes secretions seems to be dependent on the type of trichome and be independent of the organ and its development. Secretions from peltate hairs stained positive with Ruthenium Red (pectins), Sudan III (total lipids), Nile Blue (acidic lipids) and Nadi reagent (essential oils), while secretions from capitate hairs only stained positive with Ruthenium Red, Sudan III and Nadi reagent.

The presence of total lipids in secretions of the two types of trichomes and acidic lipids only on peltate trichomes had also been

Fig. 2. (A–G) SEM micrographs showing distribution and types of *Mentha pulegium* trichomes. (A) Abaxial leaf surface exhibiting unicellular (1) and short multicellular (2) non-glandular trichomes with a regular distribution. Also visible are the peltate (3) and the capitate type I (4) glandular trichomes sunken in epidermal depressions. (B) Petal apex outer face showing large multicellular non-glandular trichomes, leaned toward the apex. (C) Cotyledons exhibiting morphologically well developed peltate and capitate glandular trichomes. (D) Stamens showing peltate trichomes between the two anther lobes. (E) Capitate type I glandular trichome with one stalk cell and an oval secretory head cell. (F) Capitate type II glandular trichome with a lower conical stalk cell, exhibiting 1–2 elongated neck cells. (G) Peltate glandular trichomes with twelve secretory cells, arranged in two circles (bar = 20 μ m).

mentioned in previous studies with *Tymbra capitata* (Rodrigues et al., 2006). The secretions from both trichomes types showed a blue colour with the Nadi reagent, assuming a predominance of essential oils. Similar results also have been verified by Huang et al. (2008) in *Lavandula pinnata* and also the presence of pectins in the cell walls by Ruthenium Red is referenced in *Plectranthus ornatus* (Ascensão et al., 1999) and *Satureja subspicata* (Marin et al., 2010).

3.3. Essential oil composition

The essential oil yield, in the 14 wild populations of *M. pulegium*, collected at full flowering ranged from 0.7% to 1.6% (w/d.w.)

(Table 4). The average essential oil yield (1.1%) achieved at the flowering phase is in accordance with some reported oil yields at full flowering for wild *M. pulegium* plants (1.2%, Hassanpouraghdam et al., 2011), *M. arvensis*, *M. piperita*, *M. spicata* and *M. longifolia* (1.7%, 1.2%, 1.2%, 1.0%, respectively, Hussain et al., 2010) but there have been studies reporting twice the yield in *M. pulegium* (3.8%, Kokkini et al., 2004; 3.9%, Cook et al., 2007). Several studies suggest that oil yield is associated with climatic factors; higher temperatures, summer water deficit and higher summer sunshine are factors that seem to favour the overall oil yield (Kokkini et al., 2004; Voirin et al., 1990). Although Portugal has higher temperatures and higher summer sunshine, the typical habitat of this species is not

Table 3

Histochemistry of the secretions of the glandular trichomes present on the vegetative and reproductive organs of *Mentha pulegium*.

Histochemical test	Type of compounds/reaction colour	Peltate trichomes	Capitate trichomes	
			Type I	Type II
Sudan III	Total lipids/Red	+	+	+
Nile Blue	Neutral lipids/Pink	–	–	–
	Acidic lipids/Blue	+	–	–
Nadi	Essential oils/Blue	+	+	+
	Acidic resins/Red	–	–	–
Ruthenium Red	Pectins/Red	+	+	+

characterized by summer water deficit, which may explain the low yields found.

Thirty-nine components were identified in the EOs isolated from the *M. pulegium* populations studied, ranging from 92 to 99% of the total oil composition. The identified oil components are listed in Table 4 in order of their elution on the DB-1 column, arranged according to the four types of essential oils obtained by agglomerative cluster analysis, with the lowest and the highest percentages found for each component in each volatile oil type.

Mostly quantitative rather than qualitative variation was observed in all the essential oils analyzed. Oxygen-containing monoterpenes (83–98%) were dominant in all oils, Table 4. Pulegone was the major compound in all of the populations (52–82%) at full flowering, followed by isomenthone (2–36%), menthone (0.1–17%), and piperitone (1–15%). Despite some variability among the evaluated populations, cluster analysis (Fig. 3), confirmed a high chemical correlation among all accessions ($S_{\text{corr}} \geq 0.9\%$).

Table 4

Minimum and maximum percentage range of components identified in the essential oil, isolated from the aerial parts of 14 *Mentha pulegium* wild populations collected at full-flowering phase. For samples grouped on each of the clusters I–II and subclusters a–c, see Fig. 3.

Components	RI	Cluster I		Cluster II					
		Min	Max	a		b		c	
				Min	Max	Min	Max	Min	Max
3-Methyl cyclohexanone	914	t	t	t	t	t	t	t	t
α -Thujene	924	t	t	t	t	t	t	t	t
α -Pinene	930	t	0.3	0.5	0.7	0.3	0.8	0.4	0.7
Camphene	938	t	t	t	t	t	t	t	t
Sabinene	958	t	0.1	0.2	0.2	t	0.3	0.1	0.2
1-Octen-3-ol	961	t	t	t	t	t	t	t	t
β -Pinene	963	0.3	0.4	0.4	0.5	0.2	0.6	0.3	0.5
3-Octanol	974	1.0	1.4	1.5	1.8	1.1	1.8	1.2	2.1
β -Myrcene	975	t	t	t	t	t	t	t	t
<i>p</i> -Cymene	1003	t	t	t	t	t	t	t	t
1,8-Cineole	1005	t	t	0.3	0.3	t	0.4	t	0.2
Limonene	1009	t	t	0.3	0.4	0.2	0.5	0.3	1.2
γ -Terpinene	1035	t	t	t	t	t	t	t	0.2
Linalool	1074	t	t	t	t	t	0.1	t	0.1
3-Octanol acetate	1086	0.1	0.2	t	0.1	t	0.1	t	t
<i>trans</i> -Verbenol	1114	t	t	t	t	t	t	t	t
Menthone	1120	1.5	4.2	0.9	1.1	0.1	4.0	6.5	17.0
Isomenthone	1126	28.6	36.0	17.7	22.7	8.1	22.7	1.9	10.8
Menthofuran	1134	t	t	t	t	t	t	t	t
<i>cis</i> -Isopulegone	1134	0.7	0.7	0.7	0.8	0.8	1.2	0.8	0.9
Menthol	1148	t	t	t	t	t	t	t	t
Terpinen-4-ol	1148	t	t	t	t	t	0.1	t	t
α -Terpineol	1159	t	0.2	t	0.2	t	0.2	t	0.2
Myrtenol	1168	t	t	t	t	t	0.1	t	0.1
Pulegone	1210	52.0	55.5	60.3	61.4	61.4	81.8	57.0	69.8
Piperitone epoxide	1210	t	t	t	t	t	t	t	t
Piperitone	1211	1.0	3.1	1.1	2.2	t	1.1	0.5	2.2
Menthyl acetate	1278	t	t	t	t	t	t	t	t
Isomenthyl acetate	1288	t	t	t	t	t	t	t	t
Piperitenone	1289	3.0	6.6	6.8	13.1	0.6	6.8	5.0	14.9
Nepetalactone	1291	t	t	t	t	t	t	t	t
Piperitenone oxide	1315	t	t	t	t	t	t	t	0.3
β -Bourbonene	1379	t	t	t	1.1	t	5.7	t	1.1
β -Caryophyllene	1414	t	t	t	t	t	0.1	t	0.1
α -Humulene	1447	t	t	t	t	t	t	t	t
2-Methoxy-6-methylacetophenone ^a	1447	t	t	t	t	t	t	t	t
β -Caryophyllene oxide	1561	t	t	t	t	t	t	t	t
Humulene epoxide	1580	t	t	t	t	t	t	t	t
2-Methyl jasmonate	1634	t	t	t	t	t	t	t	t
% Identification		97.4	99.5	98.0	99.3	92.1	98.9	95.0	99.0
Grouped components									
Monoterpene hydrocarbons		0.5	0.6	1.5	1.7	0.7	2.2	1.1	2.6
Oxygen-containing monoterpenes		95.4	97.7	94.4	95.2	82.5	96.9	90.4	95.7
Sesquiterpene hydrocarbons		t	t	t	1.1	t	5.7	t	1.2
Oxygen-containing sesquiterpenes		t	t	t	t	t	t	t	t
Others ^b		1.2	1.5	1.5	1.9	1.1	1.9	1.2	2.1
Oil yield (w/d.w.)		0.7	1.2	1.1	1.1	0.9	1.6	0.7	1.1

RI, retention index relative to C₉–C₁₇ *n*-alkanes on the DB-1 column; t, traces (<0.05%).

^a Identification based on mass spectra only.

^b Components that do not fit on the classification of terpenes or phenylpropanoids and which are mainly non aromatic alcohols, ketones and alkenes.

Fig. 3. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the *Mentha pulegium* samples examined, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA). For abbreviations, see Table 2.

even though defining two clusters, and 3 sub-clusters, in total. Although some clusters overlapped, others were clearly separated and grouped populations according to their geographical collection site (Fig. 1). The main differences between the two clusters were the pulegone ($\leq 55\%$) and isomenthone ($>28\%$) relative amounts in cluster I. Menthone relative amount ($>6\%$) separated sub-cluster IIc, and isomenthone relative amount ($>17\%$) sub-cluster IIa, from the other EO in sub-cluster IIb.

A literature assessment (Table 1) showed that in pennyroyal pulegone-type essential oils, pulegone usually ranges from 60 to 90%. In Portugal, a recent study on one *M. pulegium* population described menthone (36%) and pulegone (23%) as the main essential oil components (Teixeira et al., 2012). In the present study, all the essential oils belong to the pulegone chemotype, supporting the results of Monteiro et al. (2007b) and Lopes et al. (2010), on nineteen cultivated Portuguese populations collected during the flowering phase; Reis-Vasco et al. (1999) in one population collected in Sintra, and Mata et al. (2007), in one sample bought in a local market in Alentejo Region. *M. pulegium* studied oils showed a different behaviour from the oils of most of the other mints, since the existence of different chemotypes is a common feature in *Mentha* species and hybrids (Kokkini and Vokou, 1989).

In plant developmental terms, the essential oil yield had a different behaviour according to the growing conditions. For wild growing populations, the essential oil yield increased from the vegetative stage (mean value 0.5% w/d.w.) until full flowering, June and July (mean value 1.1% w/d.w.). Opposite behaviour was observed in the cultivated ones (1.9% w/d.w. at the vegetative stage for 1.1% w/d.w. at the flowering stage). In general, wild growing populations showed a lower oil yield compared with the cultivated ones. The analysis of the main EO constituents revealed that pulegone remained the major constituent, along the life cycle of the plant, for both growing conditions, although the behaviour of the main components was slightly different (Fig. 4).

In the EO isolated from cultivated populations, pulegone relative amount increased from the vegetative until the full-flowering phase and then it started to decrease towards the end of the cycle.

In the EO isolated from wild growing populations, the relative amount in pulegone increased until the vegetative phase and then decreased anticipating the pre-flowering phase. Towards the full flowering the pulegone relative amount suffered a new increase. These changes were followed by changes in the isomenthone and menthone relative amounts, whenever the pulegone decreased, the isomenthone and menthone tended to increase. In the EO isolated from cultivated populations, menthone and isomenthone reached their maximum at the vegetative phase and decreased towards the end of the cycle (Fig. 4). These variations may be due to the influence of the developmental stage and environmental conditions on the regulation of the biosynthesis of essential oil, since it is

Fig. 4. Time-course study of the main components of the *Mentha pulegium* essential oils isolated in wild (open symbols) and cultivated (closed symbols) growing conditions at different developmental stages. Given the extended period of flowering, two sampling points, one month apart, were considered at this developmental stage. Flowering corresponding to 50% of the plants blooming and Full-flowering when 100% of the plants where blooming. The values are the mean values from 6 populations.

known that the biosynthesis of the essential oils is affected by physiological variations (i.e. organ and leaf position), environmental conditions (i.e. harvest date and planting time), geographic variations and genetic factors and evolution (Figueiredo et al., 2008). It can be hypothesized that wild growing plants are subject to more stress variables (overgrazing, human disruption, water deficit), and that under these conditions they prioritize the metabolism, which results in the reduction of pulegone, favouring isomenthone and menthone. In *Mentha x piperita* leaves, pulegone is reduced by a NADPH-pulegone reductase to yield (–)-menthone and (+)-isomenthone, in an approximately 10:1 ratio (Davis et al., 2005). In this study menthone was also clearly dominant, but in a higher ratio, except for the wild growing populations in the pre-flowering phase. The change observed in the present study, between the relative amounts of menthone and isomenthone through the life cycle may reflect some degree of substrate specificity or other environmental and/or physiological condition.

4. Conclusions

M. pulegium aerial parts showed different types of glandular and non glandular trichomes similar to those previously described for Lamiaceae. Histochemistry studies revealed the presence of pectins in the cell walls and total lipids, acidic lipids and essential oils in the secretions of the peltate and capitate glandular trichomes. The attained essential oil yield for this species was in accordance with those reported in previous studies. *M. pulegium* populations studied showed a pulegone chemotype essential oil, although the menthone and isomenthone relative amounts could further differentiate these oils. The evaluation of the effect of the developmental stage and growing conditions on essential oil composition showed mostly quantitative rather than qualitative variations, supporting the view that both factors together can influence the regulation of the biosynthesis of essential oils. Our results also showed that cultivation only seems to affect the essential oil yield, increasing its content, not affecting the essential oil composition that seems to be more stable and uniform. These are features that turn this species into interesting products for cultivation and commercialization.

Acknowledgements

Leandra Rodrigues is grateful to the Foundation for Science and Technology (FCT) for the PhD grant SFRH/BD/38143/2008. This study was partially funded by the Portuguese Ministry of Agriculture and Rural Fisheries Development, Agro Program, Project n° 522.

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