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Morphology of secretory structures and essential oil composition in *Mentha cervina* L. from Portugal

Leandra Rodrigues,[1]* Patrícia Monteiro,[1] Orlanda Póvoa,[2] Generosa Teixeira,[3] Margarida Moldão,[1] Ana Cristina Figueiredo[4] and Ana Monteiro[1]

[1] Centro de Botânica Aplicada à Agricultura, Instituto Superior de Agronomia, Technical University of Lisbon, Tapada da Ajuda 1349-017 Lisboa, Portugal
[2] Escola Superior Agrária de Elvas, Rua de Alcamim 19, 7350-903 Elvas, Portugal
[3] Centro de Biologia Ambiental, Botânica Farmacêutica, Faculdade de Farmácia, Universidade de Lisboa, Avenida Prof. Gama Pinto, 1649-003 Lisboa, Portugal
[4] Universidade de Lisboa, Faculdade de Ciências de Lisboa, Departamento de Biologia Vegetal, IBB, Centro de Biotecnologia Vegetal, C2, Piso 1, Campo Grande, 1749-016 Lisboa, Portugal

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ABSTRACT: ***Mentha cervina* L. is an aromatic plant that is traditionally used in the Alentejo region of Portugal to flavour food dishes and for the medicinal properties of the essential oil produced in its glandular trichomes. The morphology and distribution of the secretory structures of 20 populations was studied by light and scanning electron microscopy and revealed a great similarity in the type and distribution of glandular and non-glandular trichomes. In addition, two populations were surveyed at different stages of their life cycles. This showed that both maximum trichome density and maximum filling capacity of the glandular trichomes are attained early on. The GC and GC–MS chemical analyses showed that pulegone (62–80%), isomenthone (3–18%) and limonene (3–7%) are the main components of *M. cervina* essential oils. Cluster analysis of the identified essential oil components revealed a major chemical consistency between the 20 populations evaluated.**

KEY WORDS: *Mentha cervina*; Lamiaceae; essential oils; GC; GC–MS; histochemistry; trichomes; pulegone

Introduction

Mints (*Mentha* spp., family Lamiaceae) are one of the most popular essential oil crops, particularly in the Mediterranean area, where they are widely distributed. Many members of this genus are cultivated for ornamental purposes, but above all for their essential oils, which are secreted by glandular trichomes distributed across the vegetative and reproductive organs. Their oils are valued commercially as additives for food products, cosmetics and pharmaceuticals.[1] Peppermint (*M. piperita* L.) and spearmints (*M. spicata* L. and *M. cardiaca* Baker) are the most widely cultivated species, because peppermint oils are valued for the accumulation of the cyclic monoterpenes menthol and menthone, while spearmint oil is sought for its high carvone content.[2] As such, mints are valuable crops with a substantial importance to the botanical economy.

Mentha cervina L. (commonly known as Hart's pennyroyal) is an aromatic plant that is traditionally used in Portugal to flavour food dishes and for its medicinal properties.[3] Native to the Iberian Peninsula and North Africa, in Portugal it can be found in river banks, damp and wet places (Natural habitat Natura 3130), being representative of the priority habitat Natura 3170 'temporary Mediterranean ponds'.[4] Due to excessive harvesting, overgrazing and habitat destruction, the species has been disappearing from natural settings.[5,6]

The composition of essential oils and the study of the structures responsible for their secretion have been the subject of a great number of studies on mints, which have looked not only at chemical composition[7–9] and seasonal variation[10,11] but also at the effect of different factors on the composition and yield of essential oils.[12–15] However, only two studies concerning the oil composition of *Mentha cervina* were found. In both, one population of *M. cervina* was analysed and pulegone was reported as the major oil component.[16,17] Due to the presence of this toxic compound, essential oils rich in pulegone should not be used in the aromatherapy or food industries.[18] Thus, studies to identify other possible chemotypes are needed, given that the existence of different chemotypes is a common feature in most *Mentha* species and hybrids.[19]

These are the first results of a research project concerning the utilization of unexploited Portuguese aromatic flora,

* Correspondance to: L. Rodrigues, DPPF- Secção de Herbologia, Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisboa, Portugal. E-mail: liarodrigues@isa.utl.pt

Table 1. Location and details of the populations of *Mentha cervina* studied

Accessions	Collection place	Geo-reference		Type of sample	Voucher number
MC1	Redondo	W7.5678	N38.7128	Cultivated	103/2007-MC
MC2	Mértola	W7.4996	N37.6773	Cultivated	104/2007-MC
MC3	Aljustrel	W8.1040	N37.9373	Cultivated	105/2007-MC
MC7	Santiago do Cacém	W8.6813	N37.8072	Cultivated	106/2007-MC
MC10	Campo Maior	W7.0007	N39.0819	Cultivated	107/2007-MC
MC12	Nisa	W7.6811	N39.6017	Cultivated	108/2007-MC
MC13	Ponte de Sôr	W7.9813	N39.3361	Cultivated	109/2007-MC
MC14	Montargil	W7.9901	N39.3329	Cultivated	110/2007-MC
MC15	Redondo	W7.9680	N38.7928	Cultivated	111/2007-MC
MC16	Reguengos de Monsaraz	W7.4973	N38.4602	Cultivated	112/2007-MC
MC17	Amareleja	W7.2128	N38.1058	Cultivated	113/2007-MC
MC18	Serpa	W7.6001	N37.9466	Cultivated	114/2007-MC
MC19	Portel	W7.7986	N38.2683	Cultivated	115/2007-MC
MC21	Évora	W7.8919	N38.5766	Cultivated	116/2007-MC
MC22	Monforte	W7.4354	N39.0543	Cultivated	117/2007-MC
MC24	Grândola	W8.4880	N38.1336	Cultivated	118/2007-MC
MC29	Elvas	W7.1715	N38.7767	Cultivated	119/2007-MC
MC35	Figueira de Castelo Rodrigo	W6.5335	N40.5203	Wild	527/2005-MC
MC38	Termas de Monfortinho	W6.5416	N39.5812	Wild	526/2005-MC
MC39	Idanha-a-Nova	W7.2113	N39.9245	Wild	556/2005-MC

with a view to expanding new sources of aromatic oils. Here we report the chemical composition of the essential oils of several populations of Portuguese *M. cervina*, as well as the morphology of glandular trichomes—the structures that are responsible for producing the oil.

Experimental

Plant Material

This study was based on 20 populations of *M. cervina* representative of central and southern Portugal. To characterize the chemical composition and identify possible chemotypes, 17 populations were collected from natural habitats and kept under culture in the essay field of Escola Superior Agrária de Elvas (Alentejo). During the flowering phase, samples from aerial parts of the cultivated exemplars were collected. In order to understand the influence of different ecological conditions on the essential oil composition, samples from three populations growing under wild conditions were collected at the same time. Voucher specimens from the 20 populations have been deposited in the LISI herbarium (Table 1).

Morphological Studies

Light microscopy (LM)

Stems, leaves and flowers at different developmental stages, of 10 individuals for each population, were fixed with 3% glutaraldehyde (Merck, Germany) solution in a 0.1 M phosphate buffer, pH 7.3, and post-fixed with 1% osmium tetroxide in the same buffer.[20] 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.[21] 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 characters are the average of at least 30 different observations for each population.

Scanning electron microscopy (SEM)

Plant material was fixed as above, critical-point dried 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 a direct image acquisition system. Measures and counting were obtained by computer-assisted image analysis.

Histochemical Studies

General staining procedures for detecting the main chemical groups secreted were carried out using fresh leaves and flowers from three populations, MC10, MC21 and MC29 (with more contrasting agronomic behaviour). The histochemical tests included: (a) Sudan III for total lipids;[22] (b) Nile blue for neutral and acid lipids;[23] (c) Nadi reagent for essential oils and resin acids;[24] and (4) ruthenium red for polysaccharides with acidic groups.[23] Control procedures were carried out at the same time.

Essential Oil Analysis

Isolation procedure

Full flowering aerial parts of 10 individuals for each population were collected for chemical composition analyses. The samples were grossly pulverized, and 20 g were subjected to hydrodistillation for 1 h in a Clevenger-type apparatus according to the European Pharmacopoeia.[25] The oils were kept at a low temperature until further analysis.

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GC and GC–MS analyses

GC analyses were performed using a Perkin-Elmer 8700 gas chromatograph (Perkin-Elmer, 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, 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). Oven temperature was programmed from 45°C to 175°C at 3°C/min, then at 15°C/min to 300°C, 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. Samples were injected using the split sampling technique, ratio 1:50, with a volume of injection of 0.1 μl 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. The GC–MS unit consisted of a Perkin-Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific) and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin-Elmer). 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; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40–300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices relative to a C_8–C_{18} hydrocarbon standard mixture, and with GC–MS spectra from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercially available standards.

Data Analysis

Quantitative results of the morphological characters did not distinguish between populations growing in either the same or different locations, therefore the results are mean values of the data. The percentage compositions of the essential oil samples were used to determine the relationship between the different samples of *M. cervina* by cluster analysis with the NTSYS-pc software (version 2.02, Exeter Software).[26] Correlation coefficient was selected as a measure of similarity among the 20 populations, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition. The degree of correlation was evaluated as: very high, if correlation was in the range 0.9–1.0; high, 0.7–0.89; moderate, 0.4–0.69; low, 0.2–0.39; and very low, if <0.2.[27]

Results

Morphological Studies

Mentha cervina leaves are linear-oblanceolate, attenuate at the base, and entirely or obscurely toothed. Bracts are like the leaves but wider. Epidermal cells are polygonal in shape, more or less isodiametric, with sinuous anticlinal walls and dotted cell walls. On both surfaces epidermal cells are similar, although abaxial cells are smaller and more variable in shape. Diacytic stomata are present on both surfaces, albeit more abundant on the lower surface, and are evenly distributed, with no particular orientation. The leaves are dorsiventral with the midrib abaxially prominent. Leaf cross-sections revealed a mesophyll composed of one to two layers of palisade parenchyma cells and four to five layers of spongy parenchyma cells. Midribs showed a vascular bundle surrounded by three to six collenchyma layers. Calcium oxalate crystals (raphyds and sphere crystals) are observed in both mesophyll and epidermal cells. The stem cross-section is circular and maintains its form throughout the life cycle.

M. cervina indumentums include non-glandular and glandular trichomes scattered over the vegetative and reproductive organs. The non-glandular trichomes are of three different types: (a) unicellular, with a warty surface, a swollen basal epidermal cell and acute apices (Figure 1A, E), which is seen on stems and sepals and on both leaf surfaces but is more abundant on the adaxial surface; (b) small multicellular, two to four cells, uniseriate, warty surface, with a swollen basal cell and acute apices (Figure 1B), sparse on adaxial leaf surface but common on sepals inner and outer faces; (c) large multicellular, up to 8 cells long, thin, uniseriate, acute apices, warty surface, leaned toward the apex and supported by a cellular pedestal formed by two to five epidermal cells arranged around the base, only seen on the petal outer face apex (Figure 1C).

The glandular trichomes are of two different types: peltate and capitate. The peltate type is seen all over both leaf surfaces, being predominant on the abaxial leaf surface, on the stem, on the inner and outer surfaces of sepals and on the outer face apex of petals. They comprise a short stalk and a large, smooth head, with a variable number of secretory cells (eight to 12 in the leaves and up to 20 in the petals) arranged in one or two circles (Figure 1G). Upon maturation they are sunken in epidermal depressions and the cuticle of the cells of the secretory head lifts, forming a subcuticular space to enclose secretions. The head dimensions of peltate hairs are variable, but bigger on the reproductive structures: diameter up to 132 μm (±6μm) on the corolla, compared to 89 μm (±8 μm) on the adaxial leaf surface and 98 μm (±8 μm) on the abaxial one.

The two capitate trichomes found differ from each other in the shape of the head and the length of the stalk head: (a) capitate type I, with one stalk cell 10 ± 0.1 μm in length, and a round/oval secretory head cell, with a smooth surface (Figure 1F), 32 ± 4 μm in length and 22 ± 2 μm in diameter at the head, uniformly distributed on both leaf surfaces, calyx and stems; (b) capitate type II, with a lower conical stalk cell, 26 ± 6 μm in length, and one or two elongated neck cells, 13 ± 0.7 μm, and a

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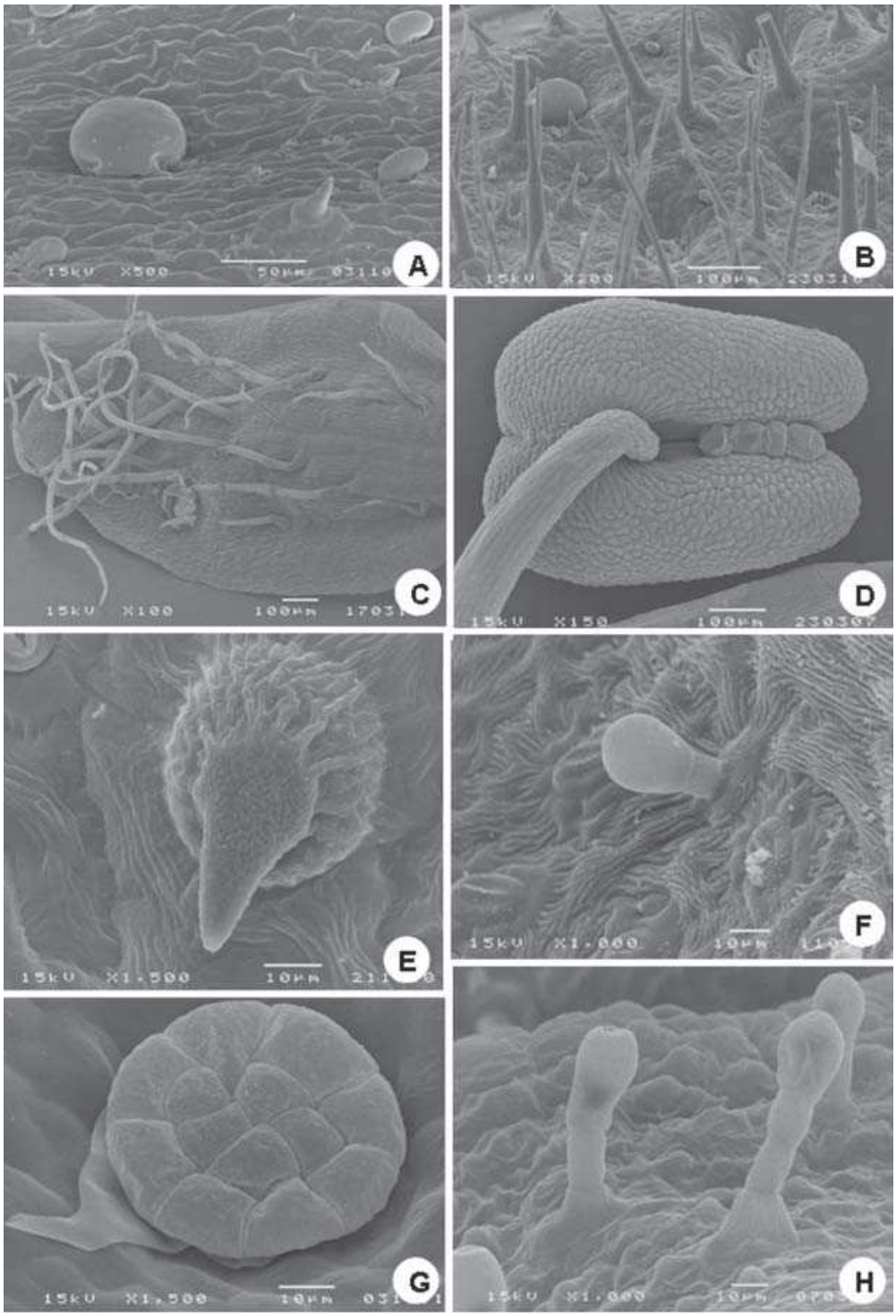

Figure 1. SEM micrographs showing distribution and types of trichromes of *Mentha cervina.* (A) Abaxial leaf surface with glandular and non-glandular trichomes. (B) Adaxial leaf surface exhibiting small multicellular non-glandular trichomes, with two to four cells, with a regular distribution. (C) Petal outer face, showing large multicellular non-glandular trichomes, up to eight cells long, leaning towards the apex and supported by a cellular pedestal formed by two to five epidermal cells arranged around the base. (D) Stamens showing peltate trichomes between the two anther lobes. (E) Unicellular non-glandular trichome, showing a warty surface, a swollen basal epidermal cell and acute apex. (F) Capitate type I glandular trichome with one stalk cell and an oval secretory head cell, with a smooth surface. (G) Peltate glandular trichome with 13 secretory cells, arranged in two circles. (H) Capitate type II glandular trichome with a lower conical stalk cell, exhibiting one to two elongated neck cells

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Table 2. Histochemistry of glandular trichomes on vegetative and reproductive organs of *Mentha cervina*

Histochemical test	Type of compounds/colour reaction	Peltate trichomes secretion	Capitate trichomes cells	
			Type I	Type II
Sudan III	Total lipids/red	+ +	+	+
Nile blue	Neutral lipids/pink	–	–	–
	Acid lipids/blue	+	–	–
Nadi	Essential oils/blue	+ + +	+	+ +
	Acidic resins/red	–	–	–
Ruthenium red	Acidic polysaccharides/red	+ +	+ +	+ +

–, negative; +, slightly positive; + +, positive; + + +, strongly positive.

round secretory head cell, with a smooth surface (Figure 1H), 33 ± 2 μm in length and 23 ± 0.1 μm in diameter at the head, only on the adaxial petal surface.

The only trichomes on the stamens or carpels were peltate trichomes, which occurred along the lower side of the connective tissue between the two anther lobes (Figure 1D), and small multicellular non-glandular trichomes that were found in the style.

Morphologically well-developed glandular trichomes were already observed on cotyledons. The peltate trichome densities in fully expanded mature leaves at full flowering were about two peltate glands/mm^2 on the adaxial leaf surface and 5.25 glands/mm^2 on the adaxial leaf surface, with twice as many glands produced as on the adaxial leaf surface. The same could not be seen for capitate trichomes, which were about 10 trichomes/mm^2 on each leaf surface.

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 hydrophilic and lipophilic components. The presence of these compounds was independent of the organ and developmental stage, but dependent on the trichome type (Table 2). The secretion of capitate hair cells was more hydrophilic, while secretion accumulated under the cuticle of peltate hairs displayed both a hydrophilic and a lipophilic nature, with a predominance of lipophilic content. The secreted material of both types of trichomes also stains positively to non-cellulosic polysaccharides, as shown by the ruthenium red test.

Essential Oil Composition and Quantification

In the 20 populations surveyed, at full flowering the oil yield was in the range 2.4–4.0% w/w. Twenty-nine components were identified in the *M. cervina* populations studied, with an identification range between 87% and 99% (Table 3). Both cultivated and wild-collected populations were dominated by oxygen-containing monoterpenes (78–88% and 89–91%, respectively). Pulegone was the main component of this fraction, as well as the dominant component of all oils; it was in the range 62–78% in oils isolated from cultivated plants, and 73–80% in those from the wild populations. Isomenthone was the second main component of *M. cervina* oils (range 3–18%). The monoterpene hydrocarbon limonene varied between 3% and 7% and was the third main component of the oils isolated from the 20 populations.

Only two sesquiterpenes were detected in all oils. β-Caryophyllene oxide was the most representative component of the oxygen-containing sesquiterpenes, attaining a maximum of 2%.

A third fraction of non-terpenic compounds, designated 'others' (Table 3), also attained a maximum relative amount of 2% in all the populations studied.

The cluster analysis of the percentage composition of essential oils clearly showed a major chemical homogeneity, supported by the very high correlation between all oils ($S_{corr} > 0.98$), despite the fact that some were obtained from cultivated plants and others from plants grown in the wild.

Discussion

The glandular trichomes of *M. cervina* are similar to the two main types occurring in other members of the family Lamiaceae, the peltate and capitate types.[28] For many Lamiaceae species, the head of a peltate trichome consists of two more-or-less distinct circles of cells, four in the middle, and a variable number of cells surrounding them.[29–33] In *M. cervina*, two circles of cells was the most common arrangement, although peltate trichomes with eight-celled heads could also be seen, as reported for *M. piperita*.[34]

Unlike peltate trichomes, which possess a rather uniform morphology, the capitate trichomes found differ in terms of stalk length and head shape and correspond to the capitate types I and II described by Werker *et al.*[31]

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Table 3. Composition of the essential oils, isolated by hydrodistillation, from the aerial parts of *Mentha cervina* collected in the flowering stage

Components	RI	Cultivated																Wild		
		MC 1	MC 2	MC 3	MC 7	MC 10	MC 12	MC 13	MC 14	MC 15	MC 16	MC 17	MC 18	MC 19	MC 21	MC 22	MC 24	MC 29	MC 35	MC 38	MC 39
3-Methylcyclohexanone	914	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
α-Thujene	924	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	0.1	t	t	t
α-Pinene	930	0.8	1.3	0.8	1.0	0.6	1.0	0.7	0.8	0.9	0.8	0.7	0.7	0.8	1.1	0.8	0.9	0.9	0.9	0.9	0.6
Camphene	938	0.1	t	t	t	t	t	t	t	t	t	t	t	t	t	0.1	0.1	0.1	0.1	0.1	t
Sabinene	958	0.1	t	t	0.1	t	0.1	t	t	t	t	t	t	t	0.1	0.2	0.1	0.2	0.1	t	t
β-Pinene	963	0.8	1.1	0.7	1.0	0.6	0.9	0.7	0.7	0.8	0.7	0.7	0.6	0.7	1.0	0.7	0.7	0.7	1.0	1.0	0.6
2,5-Dimethyl-1-hexene	970	0.2	0.1	t	0.1	0.2	t	0.1	0.3	t	t	0.2	0.1	0.7	0.1	0.3	0.3	0.3	t	t	t
3-Octanol	974	1.7	0.1	0.1	1.7	0.6	2.1	2.1	1.2	1.8	1.8	1.2	1.1	0.8	1.8	0.2	1.2	0.7	0.4	t	0.2
Myrcene	975	t	t	0.1	0.2	0.4	0.2	t	0.1	0.1	t	t	0.1	t	0.3	0.4	0.2	1.0	t	t	t
p-Cymene	1003	t	t	t	t	0.1	0.1	t	t	t	t	t	t	t	t	0.2	t	t	t	0.1	t
1,8-Cineole	1005	t	t	t	t	t	t	t	t	t	t	t	t	t	t	0.1	t	t	t	t	t
Limonene	1009	4.1	6.7	5.6	5.1	5.2	4.6	3.4	5.2	5.2	4.1	5.9	5.0	5.7	6.2	7.4	6.6	6.7	5.0	4.5	5.2
cis-β-Ocimene	1017	t	t	0.2	t	0.1	t	t	t	t	t	t	t	t	0.2	0.4	t	t	t	t	t
trans-β-Ocimene	1027	0.3	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	0.1	t	t	0.1
γ-Terpinene	1035	0.1	t	t	t	t	t	t	t	t	t	t	t	t	0.1	t	0.1	0.1	t	t	t
n-Octanol	1045	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
cis-Linalol oxide	1045	t	0.1	t	t	t	t	t	t	t	t	t	t	t	t	t	0.1	t	0.1	t	t
trans-Limonene oxide	1112	t	t	t	0.3	0.2	0.3	t	t	t	0.2	0.2	0.2	0.3	0.1	0.1	0.2	0.2	t	t	t
Menthone	1120	1.4	1.3	1.6	1.1	3.2	1.2	1.5	1.7	0.8	1.4	1.7	1.8	1.5	1.1	1.2	1.7	1.7	2.2	t	0.2
Isomenthone	1126	6.3	6.1	5.4	3.1	15.0	5.3	8.3	7.3	4.3	7.8	8.5	5.7	5.0	3.2	4.0	8.9	9.1	6.1	18.2	10.3
Isopulegone	1134	1.6	2.0	2.1	1.4	1.4	1.5	1.5	1.4	1.5	1.4	1.5	1.5	1.5	1.5	1.4	1.4	1.5	t	t	0.6
Verbenone	1164	t	t	t	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.3	0.2	0.2	0.2	0.1	0.2	0.2	t	t	t
Myrtenol	1168	t	t	t	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.3	0.2	0.2	0.2	0.1	0.2	0.2	t	t	t
Pulegone	1210	75.4	76.4	77.9	76.5	61.5	73.4	71.8	71.9	72.9	69.9	65.4	71.1	70.3	74.1	71.8	67.8	67.0	80.1	72.5	77.1
Piperitone	1211	t	t	t	0.1	0.4	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	t	t	t
Carvotanacetone	1222	1.0	0.8	0.7	0.7	1.1	0.7	0.7	0.3	0.4	0.7	0.3	0.4	0.4	0.6	0.3	0.3	0.3	t	0.1	0.6
Piperitenone	1289	t	t	t	0.7	t	t	0.8	t	t	0.7	t	t	t	t	t	0.1	t	t	0.2	t
β-Caryophyllene oxide	1561	0.7	1.1	0.5	0.5	0.5	0.5	0.7	0.6	0.7	0.7	0.4	0.4	0.4	0.5	0.6	1.2	0.8	0.5	1.2	2.3
Humulene epoxide	1579	t	t	t	t	t	t	t	t	t	t	t	t	t	t	0.1	t	t	t	t	t
% Identification		94.6	97.1	95.7	93.8	91.3	92.2	92.6	92.1	89.9	90.5	87.4	89.2	88.6	92.5	90.6	92.4	92.0	96.5	98.8	97.8
Grouped components																					
Monoterpene hydrocarbons		6.3	9.1	7.4	7.4	7.0	6.9	4.8	6.8	7.0	5.6	7.3	6.4	7.2	9.0	10.2	8.7	9.9	7.1	6.6	6.5
Oxygen-containing monoterpenes		85.7	86.7	87.7	84.1	83.0	82.7	84.9	83.2	80.4	82.4	78.3	81.2	79.5	81.1	79.2	81.0	80.3	88.5	91.0	88.8
Oxygen-containing sesquiterpenes		0.7	1.1	0.5	0.5	0.5	0.5	0.7	0.6	0.7	0.7	0.4	0.4	0.4	0.5	0.7	1.2	0.8	0.5	1.2	2.3
Others*		1.9	0.2	0.1	1.8	0.8	2.1	2.2	1.5	1.8	1.8	1.4	1.2	1.5	1.9	0.5	1.5	1.0	0.4	t	0.2
Oil yield (w/w)		3.36	3.95	3.51	2.81	2.70	2.99	3.04	3.33	2.82	3.50	3.37	3.62	3.52	2.87	3.16	3.36	3.51	2.53	2.45	2.85

For abbreviations, see Table 1. RI, retention index relative to C_9–C_{16} *n*-alkanes on the DB-1 column; t, trace (<0.05%). *Components that do not fit on the classification of terpenes or phenylpropanoids and which are mainly non-aromatic alcohols, ketones and alkenes.

The presence of peltate trichomes in the petals and on the stamens, between the two anther lobes, is a noteworthy finding, although the presence in reproductive organs was already reported for other species of Lamiaceae.[35]

As in other Lamiaceae, such as *M. piperita*, *Salvia officinalis* L. and *Ocimum basilicum* L.,[36–38] well-developed glandular trichomes could be observed on cotyledons. In *M. cervina* the measurements of the glandular secretory cells and fillings show that the maximum diameter of the secretory cells is achieved during an earlier stage of development, and that the increase in total diameter of the glandular head is due to further secretion during leaf growth. The presence of trichomes was interpreted as a functional chemical defence against predators, as well as a reward for pollinators.[28,39]

Estimates of overall peltate gland densities show a distribution, with the greatest abundance on the adaxial, of about twice the number of glands of the abaxial leaf surface, pattern reported for several Lamiaceae species.[35,40–45] Nevertheless, the densities were the lowest compared to other results in mints,[45–47] even though this is a very strong aromatic species. This may be explained by the rather unusual yield and pulegone richness of the essential oil.

In spite of the low specificity of the histochemical tests, they contribute to a better understanding of the ecological significance of glandular trichomes and are widely used to locate metabolites in glandular trichomes of other Lamiaceae.[31,35,38,39,48] Most of the essential oil is believed to be synthesized within the peltate trichomes.[49] The material secreted by the glandular cells passes through the apical walls and accumulates within a large space formed by the detachment of the cuticle of the cells of the secretory head, lifting and forming a subcuticular space to enclose secretions. The secretory product remains in this space, lending a spherical shape to each mature peltate trichome. The rupture of the cuticle occurs horizontally when the subcuticular space is filled—a process known as decapping, which leads to the collapse of the peltate trichomes.[50] In *M. cervina* the secreted product has a lipophilic nature, as shown by positive reactions to Sudan III, Nile blue and Nadi reagents, with positive staining for total and acidic lipids and essential oils. In capitate glandular trichomes much less oil is accumulated in the cell lumen and no rupture of the cuticle was observed. The lipophilic nature of the secretion is less perceptible, and only stains slightly positive to total lipids and essential oils.

At the flowering stage the essential oil yield was in the range 2.4–4.0% w/w—almost twice the yield reported in an earlier study.[16] Compared to other results in mints[7,8] and other Lamiaceae,[10] this seems to be a rather high yield. Of note, populations under cultivation showed an oil yield, in general, higher than the wild ones. The oils studied were characterized by very high pulegone content, indicating that they belong to the same, unique chemotype that has been reported to date.[16,17] Our results showed no chemical polymorphism in the essential oils obtained from populations with different provenances, collected at the same developmental stage and grown in the same ecological and edaphological conditions. The same pattern of chemical composition was obtained for populations that grew in wild conditions, which suggests that there is also not much variation in populations from different ecological conditions. The uniformity found in the essential oil contents is in contradiction to almost all the studies involving mints, since the existence of different chemotypes is a common feature in most *Mentha* species and hybrids.[19] The low chemical variation suggests a lack of variability that may be explained by the reproduction process, since this species is generally propagated vegetatively. Further studies to assess genetic diversity should be undertaken to clarify the reasons for this uniformity, in a species of a rather polymorphic genus, and also to develop strategies for biodiversity conservation.

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