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Instituto Superior de Agronomia
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Phytochemical and Genetic Diversity in *Mentha* species: Assessment, Valorization and Conservation

Diversidade Genética e Fitoquímica em Mentas: Caracterização, Valorização e Conservação

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Caneta sobre papel

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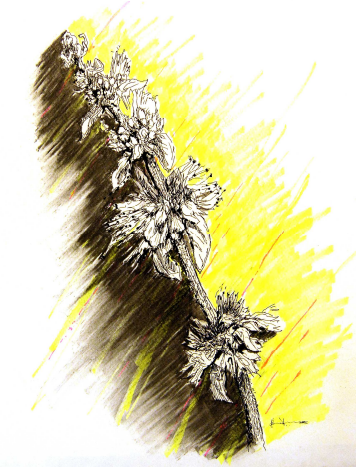

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Not everything that can be counted counts,
and not everything that counts can be counted.
Albert Einstein

À minha família
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Cada novo amigo que ganhamos no decorrer da vida
aperfeiçoa-nos e enriquece-nos, não tanto pelo que dá,
mas pelo que nos revela de nós mesmos.
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Resumo

Diversidade Genética e Fitoquímica em Mentas: Caracterização, Valorização e Conservação

Mentha cervina (L.) Opiz e *Mentha pulegium* L. são plantas aromáticas e medicinais tradicionalmente usadas em Portugal para fins aromáticos, condimentares e medicinais, nomeadamente no tratamento de problemas gástricos e respiratórios. O indumento de ambas as espécies, analisado por microscopia óptica e de varrimento, evidenciou tricomas glandulares e não glandulares, idênticos aos encontrados em Lamiaceae. A composição química dos óleos essenciais de ambas as espécies, analisada por cromatografia gasosa e cromatografia gasosa acoplada a espectrofotometria de massa, revelou uma grande uniformidade, para populações de diferentes proveniências, cultivadas ou silvestres e nos diferentes estádios de desenvolvimento, pertencendo todos ao quimiotipo pulegona. Os óleos essenciais de *M. cervina* mostraram maior actividade antibacteriana do que os componentes principais isoladamente, suportando a hipótese do efeito sinérgico entre eles. A actividade antibacteriana foi superior contra *Escherichia coli* e *Acinetobacter baumanni,* validando o seu uso tradicional. O baixo nível de diversidade genética e a grande estruturação entre as populações de *M. cervina*, revelada pelo uso de marcadores moleculares ISSRs, parece resultar da combinação da sua história evolutiva e das suas características biológicas, nomeadamente, modo reprodutivo, propagação clonal, baixa capacidade de dispersão e fragmentação do seu habitat. Os resultados indicam a necessidade de conservar o máximo número de populações e as populações para conservação *ex-situ*.

Palavras chave: *Mentha cervina*, *Mentha pulegium*, Tricomas, Óleos Essenciais, Pulegona, GC-MS, Diversidade Genética, ISSRs, Bioactividade, Conservação Genética.

Abstract

Mentha cervina (L.) Opiz and *Mentha pulegium* L. are medicinal and aromatic plants traditionally used in Portugal for aromatic and seasoning purposes and in folk medicine, for treatment of gastric and respiratory problems. Light and scanning electron microscopy of both species indumentum revealed non-glandular and glandular trichomes, corresponding to the common arrangement in Lamiaceae. Gas Chromatography and Gas Chromatography–Mass Spectrometry of both species essential oils (EOs) showed no chemical polymorphism in populations with different provenances, in cultivated or in wild growing conditions and at different developmental stages. All populations EOs belonged to the pulegone chemotype. *M. cervina* EOs antibacterial activity was higher than the main components alone, supporting the hypothesis of a synergistic effect of their different components. The antibacterial activity was more effective against *Escherichia coli* and *Acinetobacter baumanni*, validating their traditional use. The low levels of genetic diversity and the high structuring of *M. cervina* populations, assessed with Inter-simple sequence repeats markers, were assumed to result from a combination of evolutionary history and its unique biological traits, such as breeding system, clonal growth, low dispersion capacity and habitat fragmentation. The results point the necessity of conserving the maximum possible number of populations and sites for *ex situ* conservation.

Keywords: *Mentha cervina*, *Mentha pulegium*, Trichomes, Essential oils, GC-MS, Pulegone, Genetic diversity, ISSRs, Bioactivity, Genetic Conservation.

Índice

Agradecimentos ..... **i**

Resumo ..... **vii**

Abstract ..... **ix**

Chapter I – General Introduction **1**

Introduction **3**
Lamiaceae Family **4**
Mentha Genus 5
Mentha cervina (L.) Opiz 7
Mentha pulegium L. 8
Essential oils **10**
Chemical diversity of essential oils in *Mentha* 14
Essential Oil Bioactivity in *Mentha* 17
Genetic Diversity and Implications for Conservation **19**
Aims and Goals **23**
References **24**

Chapter II - Morphology of secretory structures and essential oil composition in Mentha cervina L. from Portugal **37**

Abstract **39**
Introduction **40**
Experimental **41**
Plant material 41
Morphological studies 42
Histochemical studies 42
Essential oil analysis 42
Data analysis 43
Results **44**
Morphological studies 44
Histochemical studies 47
Essential oil composition and quantification 48
Discussion **51**

References **53**

Chapter III - Trichomes micromorphology and essential oil variation at different developmental stages of cultivated and wild growing Mentha pulegium L. populations from Portugal **57**

Abstract **60**
Introduction **60**
Material and Methods **63**
Plant Material 63
Morphological Studies 63
Light Microscopy (LM) 63
Scanning Electron Microscopy (SEM) 66
Histochemical Studies 66
Essential Oil Analysis 66
Isolation Procedure 66
Gas Chromatography (GC) 66
Gas Chromatography – Mass Spectrometry (GC-MS) 67
Data Analysis 67
Results and Discussion **68**
Morphological Studies 68
Histochemical Studies 72
Essential Oil Composition 73
Conclusions **79**
References **79**

Chapter IV - Chemical composition and antibacterial activity of the essential oils from the medicinal plant Mentha cervina L. grown in Portugal **85**

Abstract **88**
Introduction **88**
Methods and materials **90**
Plant material 90
Essential oil isolation procedure 90
Gas-chromatography and Gas-Chromatography–Mass Spectrometry 91
Bacterial strains 91
Antimicrobial activity assay 91
Interpretation of the results 92
Results and discussion **93**
Essential oil chemical composition 93

Antibacterial activity of the *M. cervina* EOs and of the pure aromatic compounds... 95
Conclusion ... 98
References ... 98

Chapter V - Low genetic diversity and significant structuring in the endangered Mentha cervina populations and its implications for conservation ... 103

Abstract ... 105
Introduction ... 106
Materials and Methods ... 108
Plant Material ... 108
DNA Extraction and ISSR-PCR Amplification ... 109
Data Analysis ... 111
Results ... 112
Genetic Diversity ... 112
Genetic Differentiation ... 113
Genetic Relationships ... 116
Discussion ... 120
Genetic Diversity and Differentiation ... 120
Implications for Conservation ... 124
References ... 126

Chapter VI – Study of genetic diversity in Mentha cervina (L.) Opiz based on morphological traits, Essential oils profile and ISSRs markers ... 135

Abstract ... 137
Introduction ... 138
Materials and Methods ... 140
Plant Material ... 140
Morphological Study ... 140
Phytochemical Study ... 142
Genetic Study ... 143
Results and Discussion ... 143
Morphological study ... 143
Phytochemical Study ... 146
Molecular Study ... 151
Correlation between morphological, phytochemical and molecular traits ... 154
References ... 155

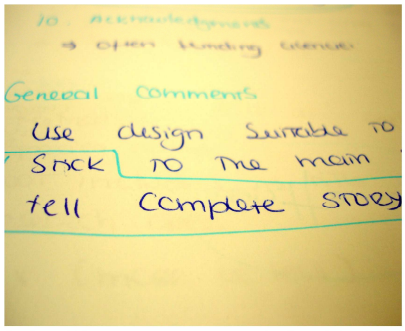
Chapter VII – General Discussion and Conclusions 161

Micromorphology of the Essential Oil Secretory Structures 163
Phytochemical Diversity of essential oils in *Mentha cervina* and *Mentha pulegium* 164
Bioactivity of *Mentha cervina* Essential Oils 165
Genetic Diversity in *Mentha cervina* and Consequences for Conservation 166
References 168

Chapter VIII – Future Perspectives 171

Population Genetic Studies in the *Mentha* Genus 173
Reproductive Mode in *Mentha cervina* 174
Taxonomy of the *Mentha* Genus 174
Use of Essential Oils in the Control of Pests and Diseases in Agriculture and Forest 175
References 177

Apendix I – Scientific Outputs 181



CHAPTER I

INTRODUCTION

Introduction

Perfumes are the feelings of flowers
Heinrich Heine

Popular knowledge of plants used by humans in therapeutic sense is based on thousands years of experience. By *trial and error*, people learned how to recognize and use plants for their consumption as well as for their health care. This popular knowledge was widespread in ancient civilizations and until the middle of the 19th century plants were the main therapeutic agents used by humans, and even today their role in medicine is still relevant. According to the World Health Organization (2008), more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs. Moreover, in many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine (WHO, 2008).
In the last decades, the consumption of herbal medicines has increased and there has been a growing interest in the investigation of natural products, in particular the essential oils extracted from plants, for the discovery of active compounds that can be applied to the food industry. The fact that they can ally their aromatizing capacity with other functional uses, such as antimicrobial, antifungal, antioxidant and insecticide properties, have contributed to this (Ormancey *et al*., 2001; Figueiredo *et al.*, 2008). As consumers are avoiding the consumption of products with synthetic additives or preservatives, the natural products constitute an alternative, mainly because they are considered safe, natural and biodegradable, with low toxicity to mammals. Adding to this, there is also a trend interest oriented to the analysis of metabolites from food (vegetables and spices) with bioactivities, recently named *nutraceuticals* and *phytochemicals*. These metabolites have a great potential in the food industry because they can combine nutritional and medicinal benefits, to produce the so-called *Functional Foods*. The antimicrobial activity in particular has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. This aspect assumes a particular relevance due to an increased resistance of some bacterial strains to the most common antibiotics and antimicrobial agents for food preservation (Sivropoulou *et al.*, 1995). Furthermore, essential oils can be used to prevent, control, or eradicate pest and disease in agriculture and forest, with the advantage of not accumulating in the

environment and having a broad range of activities, which diminishes the risk of developing resistant pathogenic strains (Figueiredo *et al.*, 2008). The search for organic compounds responsible for those activities has increased scientific studies on aromatic and medicinal plants used in different regions of the world, which is the case of plants from the Lamiaceae Martinov (= Labiatae Juss.) family, because they have a very characteristic set of chemical compounds which give distinctive odours and flavours to the foliage of many well-known species, such as mint (*Mentha*), savory (*Satureja*), thyme (*Thymus*), oregano (*Origanum*) or bergamot (*Monarda*) (Harley & Reynolds, 1992).

Lamiaceae Family

With about 240 genera and 7200 species (Harley *et al.*, 2004), the Lamiaceae family is regarded as being one of the most highly evolved plant families (at least from the viewpoint of floral structure) (Hedge, 1992). It has a cosmopolitan distribution and is often associated with the Mediterranean region. Indeed, according to Zeven & Zhukovsky (1975), this is the primary centre of diversity and probably the place of origin for the cultivation of several species and hybrids of mint. Other smaller centres in the Old World are Ethiopia, Madagascar, southern areas of Africa and India, Sri Lanka, and oceanic regions eastward. Centres of distribution in the New World, range from the mountains in central Mexico into Argentina and Chile with secondary centres radiating northward and eastward (Hedge, 1992).

The taxonomy of this family, has been controversial, and according to the most recent study (Harley *et al.*, 2004), the family is divided into seven subfamilies of which subfamily Nepetoideae has the most genera. The vast number of genera and species are placed in tribe Mentheae which itself is subdivided in three subtribes: Salviinae, Nepetinae and Menthinae. The latter includes 43 out of 66 genera in Mentheae, among them many spices and medicinal herbs, such as mints.

This family is of outstanding importance in its use in indigenous medical systems, being ranked third in ethnobotanical importance in studies of aromatic and medicinal plants used by North American Indian cultures, and probably the same picture would be found in rural and primitive societies elsewhere in the world (Heinrich, 1992). Species of this family are long know as important sources of essential oils used in food, perfume, cosmetic and pharmaceutical industries because of their culinary, fragrance and

antimicrobial properties (Lis-Balchin & Deans, 1997; Ohloff, 1994). They are used for treatment of different disorders of gastrointestinal tract, migraine, and diseases of upper respiratory tract and those related to the cardiovascular system (Bisset & Wichtl, 2001). Indeed, several of these plants are official drugs described in numerous pharmacopoeias and important raw material for pharmaceutical, cosmetic, perfume and food industry (Council of Europe, 2002).

Mentha Genus

Mentha L. is the most important genus in the Lamiaceae family because it contains a number of essential oil producing taxa that have achieved a high economic value, indeed it is the second most important essential oil producing genus, after *Citrus* L. (Mucciarelli *et al*., 2001). Most of the *Mentha* species are characterized by a great morphological variation reflected in the high number of different taxonomic rank names attributed by taxonomists during the past 200 years. In fact, the understanding of the *Mentha* genus taxonomy has been extremely challenging and its current status remains uncertain. Taxonomic difficulty may be attributed to diverse morphology, variation in base chromosome number, phenotypic plasticity, high incidence of polyploidy and frequent interspecific hybridization, both in wild populations and in cultivation (Harley & Brighton, 1977; Kokkini, 1992), stabilized by vegetative propagation, leading to the complex variation pattern characterizing most of these species. Taxonomic treatments of *Mentha* L. have recognized 13–25 species (Briquet, 1897 (17); Harley & Brighton 1977; Tucker & Naczi, 2007), with more than 3000 epithets leading to missidentification of some taxa. As an example, according to Lawrence (2007), the correct taxonomic source of cornmint oil, one of the most popular mint producing oil species, is *Mentha canadensis* L. not the synonyms *M. arvensis* L. or *M. arvensis* f. *piperascens* Malinv.ex Holmes, used by some authors.

Most of these taxa have been naturalized and have spread in temperate zones of the northern hemisphere from four domestication centres: Mediterranean, North America, China-Japan, and Europe/Siberia (Zeven & Zhukovsky, 1975). *Mentha* species are found in Europe and Asia (Briquet, 1897; Harley 1972), yet six species are found in Australia and Tasmania, *M. cunninghamii* Benth. is a New Zealand endemic, and the North American *M. canadensis* L. is the only species native to the New World (Tucker & Naczi, 2007).

The diverse morphology in this genus implies the use of several diagnostic traits to characterize each section of *Mentha*, and thus the circumscription and infrageneric classification of *Mentha* has been problematic. *Mentha* has been divided in four to six groups and some species have been in and out of this genus according to the classifications (Harley, 1972; Harley & Brighton 1977; Rösch *et al.*, 2002; Tucker & Naczi, 2007). *M. cervina* (L.) Opiz was placed in the genus *Preslia* Opiz by Briquet (1897) and Tucker & Naczi (2007) based on morphological, karyological, and chemical characters replaced it in the *Mentha* genus but, on the other hand, excluded *M. cunninghamii*. The latter study of Bunsawat *et al.* (2004), used chloroplast DNA sequences and demonstrates that all *Mentha* species sampled belong to a single well-supported clade, including *M. cunninghamii* and *M. cervina*, but also suggests that none of the previously recognized sections is monophyletic, which imply a revision of the infrageneric classification of the *Mentha* genus (Bunsawat *et al.*, 2004). This means that the phylogenetic relationships within the genus *Mentha* remain unresolved, despite intensive morphological, molecular and cytological studies.

Many members of this genus are cultivated for ornamental purposes, but above all for their essential oils, which are secreted by the glandular trichomes distributed across the vegetative and reproductive organs. Indeed, leaves, flowers and stems of *Mentha* species are frequently used in herbal teas or as additives in commercial spice mixtures for many foods to offer aroma and flavour (Kothari & Singh, 1995; Moreno *et al.*, 2002). In addition, *Mentha* species have been used as a folk remedy and in official medicine for treatment of nausea, bronchitis, flatulence, anorexia, ulcerative colitis, and liver complaints due to its antinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue, hepatoprotective, antiviral, anticarcinogenic and anticatharrhal activities (Naigre *et al.*, 1996; Bruneton, 1999; Flamini *et al.*, 1999; Işcan *et al.*, 2002; Moreno *et al.*, 2002, Mimica-Dukic, 2003; Mimica-Dukic & Bozin, 2008). As such, mints are valuable crops with a substantial importance in the botanical economy and to the pharmaceutical industry. In this genus, cornmint (*M. canadensis* L.), along with peppermint (*M. piperita* L.) and spearmints (*M. spicata* L. and *M. cardiaca* Baker), are widely cultivated species, because of their essential oil compositions rich in the cyclic monoterpenes menthol, menthone and carvone (Kokkini, 1991). Globally, mint species are responsible for more than 33% of the 111000 tons of essential oils produced and commercialized in 2008 (Schmidt, 2010).

Mint oils are produced in different parts of the world. For example, peppermint, Native spearmint, and Scotch spearmint oil is produced in North America, whereas almost all the cornmint oil and natural menthol are produced in China and India (Lawrence, 2007). India is the major global producer and supplier of mint oil and its derivatives, accounting for more than 75 % of total current menthol mint production in the world (Varshaney, 2000), with 0.15 million ha under cultivation and an annual production of 20,000 tons of essential oil (Chauhan *et al*., 2012). In Portugal, despite of suitable climate, mint oil is not produced commercially, but the recently renewed interest in these aromatic species has induced local farmers to cultivate them for selling either as a living plant or as dried material (aerial parts) (Politi *et al*., 2008). Besides these well known taxa, there are other mints that can be potential sources of chemical components. In Portugal, there are at least nine *Mentha* species and several hybrids, they are *M. requienii* Bentham, *M. pulegium* L., *M. cervina* (L.) Opiz, *M. arvensis* L., *M. aquatica* L., *M. piperita*, L., *M. spicata* L., *M. suaveolens* Ehrh., and *M. longifolia* (L.) Hudson (Franco, 1984). Two of them, *M. pulegium* and *M. cervina*, are of particular interest because they are aromatic plants traditionally used in Portugal to flavour food dishes and for its medicinal properties, preventing different gastric disorders and inflammations of the respiratory tract (Póvoa *et al.*, 2006; Monteiro *et al.*, 2007a,b).

Concerned with the sustainable utilization of unexploited Portuguese aromatic flora and aiming to explore new sources of aromatic plants, this PhD project intends to characterize the glandular structures, address the phytochemical diversity of the essential oils and the genetic diversity of *M. pulegium* and *M. cervina* species in Portugal. The following paragraphs of the Introduction provide an overview about these two species and the current knowledge about their phytochemical and genetic diversity. In the subsequent parts of the thesis, the results, major conclusions and future perspectives are addressed.

Mentha cervina (L.) Opiz

Mentha cervina (L.) Opiz commonly known as hart's pennyroyal (*hortelã-da-ribeira, erva-peixeira* or *poejo-fino* in Portuguese) and previously named *Preslia cervina* (L.) Fresn., is a spreading herbaceous perennial (hemicryptophyte) with slender, lance-shaped, mid-green leaves and whorls of white or lilac flowers from midsummer into autumn (Fig. 1.1). In mainland Portugal this species blooms from June to September, and completes its vegetative life cycle in 7 months (Coutinho, 1974; Rosselló-Graell, 2003).

Figure 1.1. *Mentha cervina* (L.) Opiz. A. Botanical illustration adapted from Valdés *et al*. (1987). B. Whole plant. C. Detail of the Inflorescence.

M. cervina has a western steno-Mediterranean distribution. Although it has a restricted area of occupancy (approximately 600 km^2), the extent of occurrence is large (800.000 km^2) and it is found in Portugal, Spain, France, Morocco, Algeria and is presumed extinct in Italy (Valdés *et al*., 1987; Rhazi & Grillas, 2010). *M. cervina* is a heliophilous species, sensitive to the shade and to the modification of water regimes. It occurs, mainly in river banks and other wetlands and temporarily flooded habitats. According to the Natura 2000 Network Initiative, in Portugal mainland, *M. cervina* is a bioindicator of the priority habitat NATURA 3170 (Mediterranean temporary ponds) and NATURA 3130pt5 (deep seasonal ponds) (Silva *et al*., 2009). Contrary to other mint species, this plant is not so competitive and it is currently difficult to find it growing spontaneously in the natural habitat.

Mentha pulegium L.

Mentha pulegium L., known as European, or Old World, pennyroyal (*poejo* in Portuguese), is an aromatic perennial herbaceous plant reaching up to 40 cm height

(Stengele & 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 (Tutin *et al.*, 1972; Chalchat *et al.*, 2000). Morphologically the plant is a low growing, prostrate spreading herb with leaves that range in shape from ovate to orbicular. It shows lilac-coloured flowers, from June to August in Portugal (Coutinho, 1974; Franco, 1984). Although the flowers seem to set seed well, and are self-compatible the plant tends to spread by rooting from the stems. It is cultivated in Europe and grows particularly well in fertile, moist soils in Mediterranean coastal regions (Fig. 1.2).

This species was thought of as something of a *cure-all*, it was a common feature of cottage gardens and was used as a treatment for many ailments including colds, and as a flea repellent. Indeed, the Latin name *pulegium* was given to the plant by the Romans who knew that the leaves kept the fleas away, *pulex* being the Latin for flea (Grieve, 1931).

Figure 1.2 *Mentha pulegium* L.. A. Botanical illustration adapted from Valdés *et al*. (1987). B. Whole plant. C. Detail of the Inflorescence.

In folk medicine *M. pulegium* is used as an infusion, preventing different gastric disorders and inflammations of the respiratory tract (Mkaddem *et al*., 2007; Camejo-Rodrigues *et al.*, 2003; Póvoa *et al.*, 2006). Nevertheless, there are no approved medicinal uses for

pennyroyal essential oil (Barceloux, 2008). Because of the strong aromatic, mint-like pungent smell, pennyroyal oil is a constituent of alcoholic beverages and a frequently used raw material in flavourings, confectionery and cosmetics (Barceloux, 2008; Hayes *et al.*, 2007 Monteiro *et al*., 2007a; Mkaddem *et al.,* 2007).

Essential oils

Plants are the source of an enormous structural diversity of metabolites, generally classified as primary or secondary according to their involvement in basic life functions such as cell division and growth, respiration, storage, and reproduction and their universal distribution in plants. The term *secondary metabolites* had for a long time an unprestigious meaning, since *secondary* assumes that the *primary* ones are more important. This meaning is no longer acceptable and several examples show that the opposite may also be the case (Figueiredo *et al*., 2008). In nature, many secondary metabolites play an important role in the protection of the plants, functioning as antibacterial, antiviral, antifungal, insecticidal and also against herbivores by reducing their appetite for such plants. They also may attract some insects to favour the dispersion of pollen and seeds, or repel undesirable predators and also serve as signal in plant to plant communication (Pichersky & Gershenzon, 2002; Dudareva & Pichersky, 2008; reviewed in Bakkali *et al.,* 2008 and in Maffei, 2010). They are often synthesized from primary metabolites by all plant organs, such as buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and since many of the constitutive defence compounds may be toxic at high concentrations to the plant itself, the plant must be able to generate and store such substances without poisoning itself. The obvious strategy to overcome this problem is to store this substances as inactive precursors, for instance as glycosides (Jerkovic & Mastelic, 2001), or in extracellular compartments, as cavities, canals or glandular trichomes (Bakkali *et al.,* 2008). Glandular trichomes are present in several plant families and their morphology, distribution and frequency are considered distinctive characteristics among species (Werker, 2000). According to Fahn (1979), glandular trichomes secreting essential oils are the base for the economic importance of several plant families, including the Lamiaceae. In peppermint, the site of terpene biosynthesis has been localized to the secretory cells of the glandular trichomes, mainly located on the

leaf and stem surfaces. In Lamiaceae species, although the morphology of these glandular trichomes may vary, two morphological types are frequently present, peltate glandular trichomes, with a broad head of several secretory cells, usually arranged in circles, subtended by a short stalk and a basal cell, embedded between the ordinary epidermal cells (Fig. 1.3) and capitate glandular trichomes, with a basal cell, a 1-2 stalk cells and a round to elongated secretory head cell (Fig. 1.4) (Fahn, 1979; Maffei *et al*., 2010, Werker, 2000).

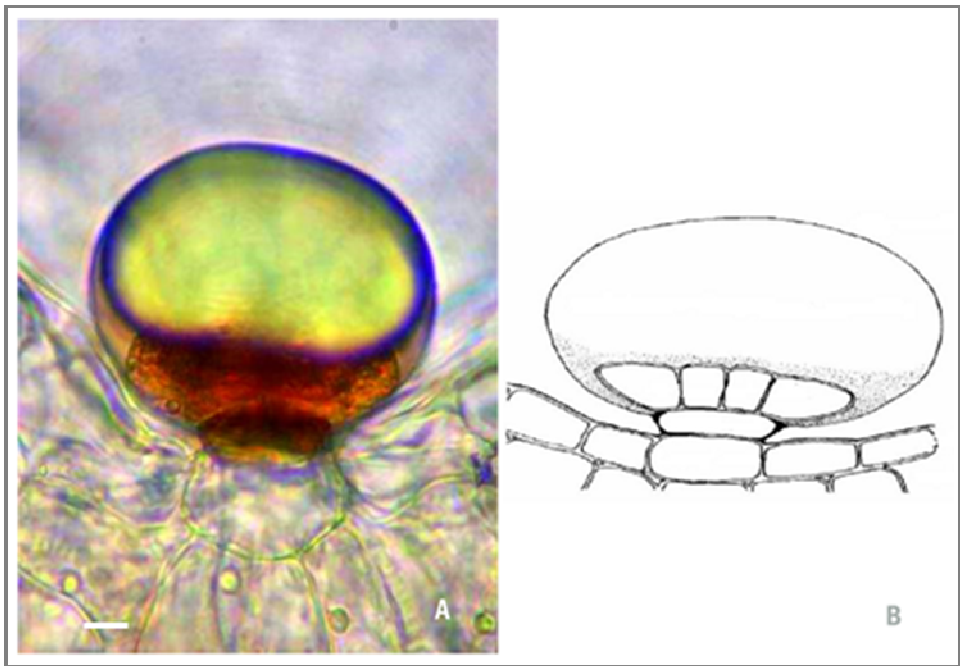


Figure 1.3. Peltate trichome consisting in a basal epidermal cell, a short stalk cell and a secretory head with several secretory cells. A: In *M. cervina* (L.) leaf. B: Illustration adapted from Turner *et al*. (1999). Scale bar =10 µm.

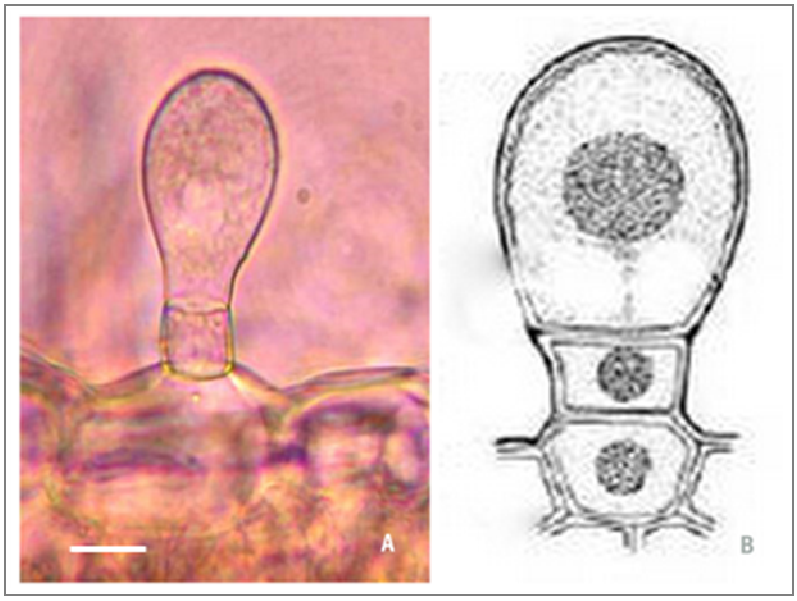


Figure 1.4. Capitate trichome consisting in a basal cell, one stalk cell and one secretory cell. A. In *M. cervina* (L.) Opiz. B. Illustration adapted from Fahn (1979). Scale bar =10 µm.

Secondary metabolites are accumulated in concentrations that differ between plants and have a distribution which is sometimes confined to a genus or species (Croteau *et al*., 2000; Bourgaud *et al*., 2001; Figueiredo *et al*., 2008). This narrower distribution of secondary compounds constitutes the basis for chemotaxonomy. Among the different type of secondary metabolites, volatiles present in a plant consist of a complex mixture of chemical compounds, each of which has certain chemical and physical properties that in combination with their different proportions represents the aroma of the aromatic plants (Figueiredo *et al*., 2008). Today more than 1700 volatile compounds have been isolated from more than 90 plant families, constituting approximately 1% of all plant secondary metabolites (Pichersky & Gershenzon, 2002).

Essential oils are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. Obtained by hydrodistillation, steam distillation or dry distillation, or by a suitable mechanical process without heating (for *Citrus* fruits), of a plant or of some parts of it (Council of Europe, 2008), meaning that they are include in the volatile fraction responsible for the *essence* of the aromatic species. Usually they are liquid at room temperature (and hence the term oil), slightly soluble in water and highly soluble in organic solvents (Bakkali *et al*., 2008; Rubiolo *et al.*, 2010). There are approximately 3000 essential oils known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Bakkali *et al*., 2008).

Analysis of an essential oil usually involves the separation, identification and quantitative determination of its components. The volatility and polarity of essential oil components make capillary gas chromatography (GC) the technique of election for their analysis, because essential oils are in general, formed by a complex mixtures of components with similar physicochemical characteristics at quite different concentrations. The identification of these components is generally carried out either by chromatographic data, measurable with a universal detector such as FID (Flame ionization detector) or TCD (thermo-conductivity detector), or by spectral data, mainly by mass spectrometry (GC–MS) or, better, by their combination, as required by the International Organization of the Flavour Industry (IOFI) (Rubiolo *et al.,* 2010). The essential oils are usually characterized by two or three major components at fairly high concentrations (20–70%) compared to other components present in trace amounts. Though the essential oils are a complex mixture of compounds, the terpenes (mainly mono-, sesqui- and di-terpenes) are the most representative molecules and are among the most valuable compounds produced by plants, side by side with alkaloids and phenolic substances (Bakkali *et al.,* 2008; Figueiredo *et al.,* 2008; Kirby & Keasling, 2009). In addition to terpenes, other classes of compounds found in essential oils include phenylpropanoids, polyacetylenes and even some fatty acids (Figueiredo *et al.,* 2007).

Terpenes form structurally and functionally different classes. They are synthesized via the two universal C_5 building blocks: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). These universal precursors can be produced by either of two routes: the mevalonate pathway or the 1-deoxy-d-xylulose-5-phosphate (DXP) pathway. These pathways are distributed throughout nature, and as a rule of thumb the mevalonate pathway is prevalent in eukaryotes and archaea, whereas the DXP pathway is widespread in eubacteria. The biosynthesis of terpenes consists of repetitive addition of IPPs, modification by terpene specific synthetases to form the terpene skeleton and finally, secondary enzymatic modification (redox reaction) of the skeleton to attribute functional properties to the different terpenes (Kirby & Keasling, 2009). The main terpenes are the monoterpenes (C_{10}) and sesquiterpenes (C_{15}), but hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}) also exist. The monoterpenes (C_{10}) are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures (Bakkali *et al.,* 2008; Figueiredo *et al.,* 2008; Kirby & Keasling, 2009). Generally, these major components determine the biological properties of the essential oils.

Chemical diversity of essential oils in *Mentha*

Plants belonging to the genus *Mentha* have evolved in nature through natural hybridization and selection, showing substantial variation in terms of their natural habitats, growth characteristics, and aromas (Tutin, 1972; Franco, 1984). In most of the wild growing mint species, and hybrids, a great phytochemical diversity is observed, with respect to their essential oil constituents (Kokkini, 1991, 1992; Mimica-Dukic & Bozin, 2008). Based on the monoterpene compound prevailing in the essential oil, which is reflected by the specific metabolic pathway (McConkey *et al.,* 2000), mints have been classified: the production of linalool and linalylacetate is typical for the linalool pathway; menthol, menthone and menthofuran are constitutes of the menthol pathway (Fig. 1.5); and carvone, dihydrocarvone and carveol, finally, characterize the carvone pathway (Schalk & Croteau, 2000; Lawrence, 2007). These pathways are as examples represented by *M. aquatica* var. *citrata*; *M. piperita*; and *M. spicata*, respectively. According to this classification *M. pulegium* and *M. cervina* belonged to the Menthol pathway (Lawrence, 2007). Although the composition of the essential oil can be regarded as characteristic of each mint and relationships among them can be deduced from the similarity of the essential oil pattern (Rösch *et al*., 2002), a taxonomic study by Šarić-Kundalić *et al*., (2009), showed that the anatomically and morphologically defined species exhibited a high level of phytochemical polymorphism which was largely inconsistent with the hierarchical classification. This means that distribution of secondary metabolites apparently has some value for taxonomy but have to be analysed carefully and critically, as any other adaptive trait (Wink, 2003). Moreover, the same taxon growing in different areas may have widely differing chemical components resulting in the existence of intraspecific chemical differences (chemotypes), which is very common in the *Mentha* genus (Kokkini, 1991). Indeed, the occurrence of chemotypes has been important in the selection of *Mentha* clones for agricultural purposes, as well as in horticulture, where different clones have been selected for their characteristic scent (lemon mint, orange mint, lavender mint) (Richardson, 1992). Several laboratories are already pursuing desirable clones with improved monoterpene combinations, increased accumulation of essential oil and with superior agronomic traits to be used in breeding programs to produce desired recombinant hybrid genotypes with both oil quality and yield (Khanuja *et al*., 2000).

Essential oil constituents and production can also be modulated substantially by physiological variations - organ type and development, pollinator activity cycle, type of

secretory structure, seasonal variation, mechanical or chemical injuries -, environmental conditions - climate, pollution, diseases and pests, edaphic factors -, geographic variation and genetic factors, resulting in highly variable commercial yields and compositions (Voirin *et al*., 1990; Masotti *et al*. 2003; Angioni *et al*. 2006; Figueiredo *et al*., 2008).

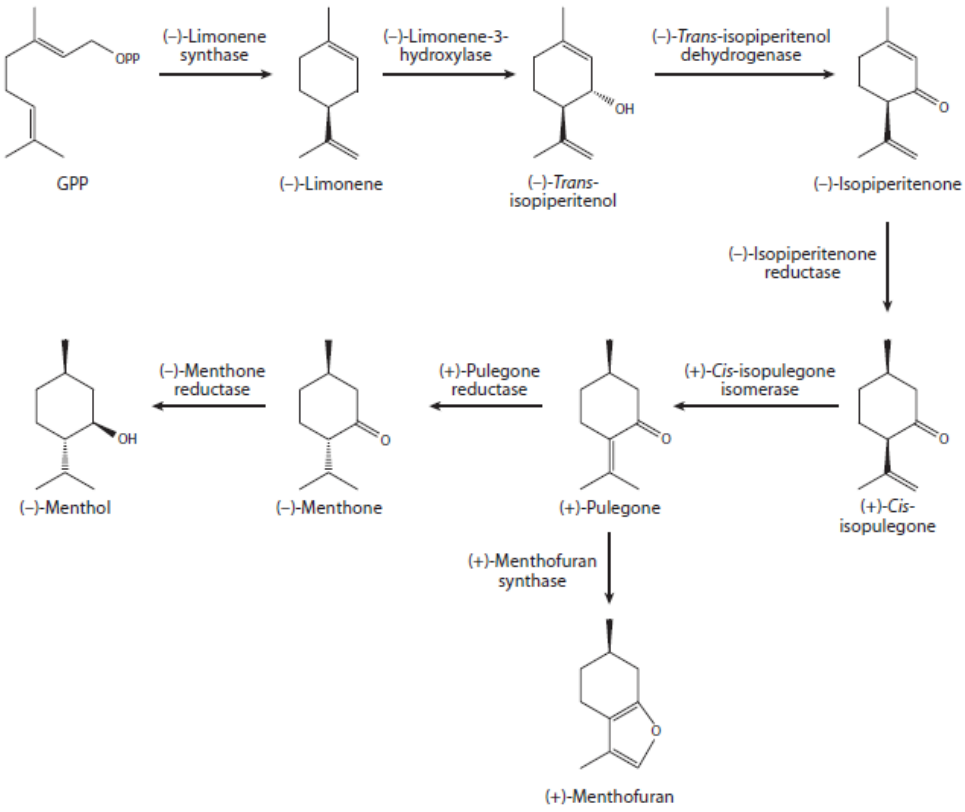


Figure 1.5. The Menthol biosynthetic pathway of peppermint (*M. piperita*), adapted from Croteau *et al.* (2005).

So, in order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season (Bakkali *et al*., 2008). Most of the studies performed so far on *M. pulegium* essential oils were carried out in different regions of the world, including Turkey (Müller-Riebau *et al*., 1997), Yugoslavia (Chalchat *et al*., 2000), Urugay (Lorenzo *et al*., 2002), Bulgaria (Stoyanova *et al*., 2005), India (Agnihotri *et al*. 2005), Egypt (El-Ghorab, 2006), Greece (Kokkini *et al*., 2004; Cook *et al*., 2007), Iran (Aghel *et al*., 2004; Mahboubi & Haghi, 2008; Hassanpouraghdam *et al*., 2011), Spain (Maroto-Diaz *et al*., 2007), Tunisia (Mkaddem *et al*., 2007), and Portugal (Monteiro *et al*., 2007b; Lopes *et al*., 2010; Teixeira *et al*., 2012),

and were focused mainly on its chemical composition.

The literature suggests that *M. pulegium* is a chemical polymorphic species, both in the qualitative and/or quantitative oil composition, with four chemotypes reported: (1) pulegone, (2) piperitenone/piperitone, (3) isomenthone/neoisomenthone types and (4) menthone with appreciable amounts of menthol and neomenthol from Northwest Iran, being pulegone the most common chemotype. Despite these reports from different regions of the world, and from three studies in cultivated populations (Monteiro *et al.*, 2007b; Lopes *et al.*, 2010; 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 is observed (Kokkini, 1992; Mimica-Dukic & Bozin, 2008).

M. cervina is a less-known species, and to date, not counting with the studies here in reported, previous studies have been mainly focused upon its morphology (Póvoa *et al.*, 2006), reproductive biology (Monteiro, 2006), antifungal activity of the essential oils (Gonçalves *et al.*, 2007) and habitat characterization (Silva *et al.*, 2009). Concerning the essential oils characterization, only two studies on chemical composition were published (Vidaurreta *et al.*, 1992; Gonçalves *et al.*, 2007). In both cases, few samples were used, (only one concerned the Portuguese flora) and pulegone was the main component. Pulegone is a toxic compound with potentially lethal hepatotoxic effects (Anderson *et al.*, 1996), it is metabolized by hepatic microsomal mono-oxygenases to a series of hepatotoxins that cause liver cancer (Nelson, 1995) and is involved in many reported cases of intoxication in humans and animals (Bakerink *et al.*, 1996). 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 peppermint or mint-flavoured beverages and 350 mg/kg in mint confectionery (EEC, 1988; European commission, 2002). Pulegone may not be added as such to foodstuffs (EEC, 1988). Conversely, pulegone is listed among the authorized synthetic flavouring substances in the USA (Siano *et al.*, 2005). Recommended limits of pulegone in cosmetic formulations are <1% (Barceloux, 2008). Because of these restrictions, 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.

Essential Oil Bioactivity in *Mentha*

It is well-known that plant-derived natural products are extensively used as biologically active compounds. Among them, essential oils were the first preservatives used by man, originally in their natural state within plant tissues and then as oils obtained by distillation. They are noteworthy for exhibiting a wide range of biological activities, such as antifungal, antimicrobial, antiviral and insecticidal activities that haveplayed a critical role in the development of our current pharmaceutical and agrochemical industries. Because of the great number of constituents, essential oils seem to have no specific cellular targets (Carson *et al*., 2002). As their mode of action affects several targets at the same time, generally, no particular resistance or adaptation to essential oils has been described (Bakkali *et al*., 2008). Indeed, essential oils or some of their constituents are effective against a large variety of organisms including virus, fungi, bacteria, mites, larvae, worms, insects and molluscs (reviewed by Bakkali *et al*., 2008). This property is of great importance in the applications of essential oils not only against certain human or animal pathogens or parasites but also for the preservation of food and food products.

The essential oils and extracts from *Mentha* species have been in use since ancient times for the treatment of many digestive tract diseases and in cuisines (Işcan et al., 2002), and are therefor potential candidates for exhibiting antimicrobial, antioxidant, radical-scavenging and cytotoxic activities (Yadegarinia *et al*., 2006; Gulluce *et al*., 2007). These activities are due to the presence of active constituents, mainly attributable to isoprenes such as monoterpenes, sesquiterpenes and related alcohols, other hydrocarbons and phenols (Griffin *et al*., 1999; Dorman & Deans, 2000).

There is some difficulty in comparing the different results obtained by research groups across the world since so many variables exist. Besides the experimental variations, the biological activity is dependent of the essential oil composition, and thussimilarly subject to variation (Oumzil *et al*., 2002), which explains the different results found concerning the biological properties of *Mentha* essential oils. However, there is some thread of similarity in the microorganisms against which the various members of the genus have been evaluated can be grouped together as important disease-causing bacteria, including *E. coli*, with strain O157:H7, *Salmonella*, and *Shigella* sp., *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and *Staphylococcus aureus*. It is significant that MRSA and VRE organisms are susceptible to mint oils since no development of resistance appears to occur (Deans, 2007).

The bioactivity of several mint essential oils have been studied, in particular the antimicrobial activity of *M. piperita* L. (peppermint) (Işcan *et al.*, 2002; Yadegarinia *et al.*, 2006), the antibacterial and antifungic activities of *M. suaveolens* (Oumzil *et al*., 2002), the antibacterial activity of *M. rotundifolia* (Derwich *et al.*, 2010) and the antimicrobial and antioxidant activities of *M. aquatica* and *M. longifolia* (Gulluce *et al*., 2007; Mimica-Dukic *et al*., 2003).

Most studies performed so far in *M. pulegium* essential oil were focused mainly on its chemical composition. Nevertheless, the antimicrobial activity was studied by Mahboubi & Haghi (2008) and the antiradical activity, the antioxidant activity and the acetylcholinesterase inhibitory capacity of essential oils was studied by Mata *et al*. (2007). More recently, Teixeira *et al.* (2012) studied the *in vitro* antioxidant and antimicrobial activities of *M. pulegium* essential oil and extracts, showing that the essential oils have strong antibacterial properties. In this way, *M. pulegium* extracts and essential oil have a huge potential as alternatives to synthetic preservatives in food industry.

In *M. cervina*, there is a lack of information concerning the chemical composition and bioactive properties of the essential oils. There is one study reporting the chemical composition and antifungal activity against *Candida*, *Aspergillus* and dermatophyte strains (Gonçalves *et al*., 2007). These authors suggest that *M. cervina* essential oils can be used as alternative antifungal agents in the treatment of dermatophytosis.

Nevertheless, further studies should evaluate the safety and toxicity of *M. pulegium* and *M. cervina* essential oils to human consumption before considering their use for food preservation or medicinal purposes.

Genetic Diversity and Implications for Conservation

Biological and bio-technological research for exploitation of aromatic compounds in pharmaceutical, agronomic, food, cosmetic and perfume industries relies on two conditions for their ultimate success: a rich source of biodiversity, and the knowledge of the natural relationships between species. Biodiversity will depend on a wise international conservation policy, while the second falls in the discipline of taxonomy.

Aromatic and medicinal plants represent, in number of species used, widespread nature of their use and their contribution to human health, perhaps one of the most significant ways in which humans directly reap the benefits provided by biodiversity (Farnsworth & Soejarto, 1991; Hamilton, 2004). Contrary to plant species used for food and agriculture, aromatic and medicinal plant species are a more diffuse category. The most important crops are cultivated and efforts exist towards the conservation of their genetic diversity (Fowler & Hodgkin, 2004), on the other hand, most of the aromatic and medicinal plants are found wild (40.5%) or naturalized (33.9%) while only 3.3% are cultivated (Aguilar-Støen & Moe, 2007). According to Vines (2004), approximately two thirds of the 50.000 different aromatic and medicinal plant species in use are collected from the wild and in Europe only 10% of medicinal species used commercially are cultivated. Currently, these plant populations face an incomparably growing pressure in the wild due to increasing commercial collection, largely unmonitored trade and habitat loss, which is causing loss of genetic diversity, local extinctions and habitat destruction (Canter *et al.*, 2005).

Globally, the populations of *M. cervina* are suffering severe and rapid declines throughout its range, and are therefore classified as Near Threatened in the IUCN Red List of Threatened Species (Rhazi & Grillas, 2010). The populations once known in Italy (Abruzzi) are presumed extinct. In France, it is known in six departments and is considered as vulnerable (one level upward of the Near Threatened, according to the IUCN nomenclature). In North Africa, it is considered rare in Morocco with only one locality known in the Rif (Tanger) and very rare in Algeria where it is found in the Haut Plateaux of the Regions of Algiers and Oran (Rhazi & Grillas, 2010). In Spain and Portugal, it is known in the western half of the Iberian Peninsula with some additional localities in the east. It is not very common and the populations are possibly declining because of the destruction of their habitat. The main threat is the destruction of the habitat

by anthropogenic activities such as changes of the hydrology (drainage or permanent flooding, agriculture and dams) and in habitat management such as overgrazing and over exploitation. The small number of populations, their reciprocal remoteness and their reduced strengths essentially expose them to risks of extinction. In Portugal the remaining populations are under imminent threat from the construction of dams and in one case, it has already disappeared due to the Alqueva hydroelectric dam construction (Póvoa *et al*., 2006). According to a field survey, in areas that had previously been reported in herbaria as species habitat, it was confirmed that the habitats have been severely deteriorated or fragmented largely due to anthropogenic activities (e.g., deforestation, over-exploitation, over-grazing). Also, with the growth of commercial demands in recent years, the excessive harvesting from the wild and the unfavourable conservation status of these habitats has shrunk the natural resource of *M. cervina* to a narrow distribution (Póvoa *et al.*, 2006).

In terms of conservation measures, this species is protected at national and regional level in France, and it is classed as Vulnerable in the Red Data Book (Olivier *et al.*, 1995). In North Africa, there are no conservation measures but, legal protection of the species, surveillance of the existing sites, search for new sites, monitoring of population size and dynamics, maintenance of a favourable hydrological regime and raising public awareness are actions that are recommended. In Spain and Portugal there are no records of conservations measures or actions for the species. Unlike *M. cervina*, *M. pulegium* has been widely recorded throughout much of Europe, where it is not threatened, but is known to be in decline in some countries, such as Great Britain where it is classified as vulnerable (Rhazi & Grillas, 2010).

The development and use of molecular markers has become an important tool for a broad range of plant population genetic, conservation genetic and evolutionary studies such as investigating patterns of gene flow, mating systems, population genetic structure, hybridization and effective population size. Several genetic approaches have been used to determine the genetic relationships and patterns of diversity. Fenwick and Ward (2001) studied RAPDs (Randomly amplified polymorphic DNA) ability to accurately identify peppermint and spearmint varieties. Despite testing hundreds of primers, they were unable to distinguish among six of the eight commercial peppermint and native spearmint types. Shasany *et al*. (2002) evaluated RAPD to assess the genetic relationship among 15 native spearmint accessions from India and concluded that the results of RAPD analysis were better for this purpose than the traditional approach of comparing plant

morphologies and chemistries. Althought, this technique benefits from simplicity and low cost because prior knowledge of a plant's genetic sequence is not required, few researchers currently use RAPD analysis because results are not as consistent as with other genetically based methods. Amplified fragment length polymorphism (AFLP) is another method used to characterize genetic relationships among plants, and has been used successfully to group different mint plants into major taxonomic clusters (Gobert *et al*., 2002). Like RAPD, AFLP requires no knowledge of genetic sequences, however, it is difficult to use and may not be sensitive enough to distinguish among closely related individuals. Among various molecular tools, genomic fingerprinting with inter-simple sequence repeats (ISSR) markers has gained increased interest. This markers use arbitrary primers that anneal to microsatellites (or SSRs -simple sequence repeats), and amplify the regions between adjacent, inversely orientated SSRs. The method reveals inversions, insertions, deletions, and mutational events, reflect in the length variation between the adjacent microsatellites (Zietkiewicz *et al*., 1994; Culley & Wolfe, 2001). Because they have higher annealing temperature and longer primer sequences, they yield greater reliability and reproducibility of bands when compared to RAPD (random amplified polymorphic DNA) primers (Culley & Wolfe, 2001). At the same time, the cost of the analyses is relatively lower than that of some other markers such as AFLPs (Fang & Roose 1997). Therefore, the use of ISSRs has been extensively used in population genetics studies with wide applications in genetic diversity studies of species of conservation concern (Esselman *et al.,* 1999; McGlaughlin *et al.,* 2002; Smith & Bateman 2002; Ge *et al.,* 2005; Xia *et al.,* 2007), including Lamiaceae species (Liu *et al.,* 2006, Mendes *et al.,* 2009), and are especially useful in cultivars identification (Charters *et al.,* 1996) and in detecting diversity in closely related, or even clonal, individuals (Zietkiewicz *et al.,* 1994; Esselman *et al.,* 1999; Chen *et al.,* 2006; Han *et al.,* 2007).

Analysis of single sequence repeats (SSRs), also called microsatellite DNA markers, is another approach to distinguish different mint plants. Microsatellites are very reliable and easy to use, but their application requires genetic sequencing information that can be difficult and expensive to obtain. Liu and Blouin (in preparation) have developed 22 SSR markers capable of differentiating among several species and varieties in the genus *Mentha*. However, additional SSR markers are needed to distinguish among other closely related mint plants (Morris, 2007).

The assessment of patterns of genetic variation in plant populations has made critical

contributions to many studies in evolutionary biology, conservation genetics, plant breeding and ecological genetics. The value in such assessments not only relates to the quantification and distribution of genetic variation in populations but also to the investigation of the processes that influence those patterns, in plants in particular because they have a huge diversity in breeding systems and contrasting life-histories. Although this knowledge is important to the formulation of effective conservation strategies for threatened species (Holsinger & Gottlieb, 1991; Escudero *et al.,* 2003; Shah *et al*., 2008), no study has targeted the genetic diversity and structure of *M. cervina* populations in Portugal.

Nevertheless, the search for natural products must not make immoderate use of the local flora. Moreover, the use of natural products also implies investing in quality, agricultural innovation and a combined effort of agriculture, industry and science for the control of diseases and pests, in the selection of the best cultivation sites, improved plant varieties, determination of the right time for collection and sustainable use of biodiversity.

Aims and Goals

The aim of this PhD project was to develop an integrative study to CHARACTERIZE the aromatic species, *M. cervina* and *M. pulegium*, in several aspects related with their essential oils (secretory structures and chemical composition and evolution) and genetic biodiversity to understand the processes driving population dynamics when subjected to pressure, in order to develop strategies for their CONSERVATION. It was also a goal to promote the VALORISATION of these species by evaluating the yield, quality and bioactivity of the essential oil against different microorganisms, such as human and food-borne pathogens.

The specific objectives of this work were:

1) Characterization of the morphology and distribution of glandular trichomes, the structures responsible for producing the essential oils, in *M. cervina* (Chapter I) and *M. pulegium* (Chapter II).

2) Quantification and chemical characterization of the essential oils, with identification of chemotypes and desirable traits, in *M. cervina* (Chapter I) and *M. pulegium* (Chapter II) populations.

3) Determination of the influence of growing conditions and developmental stages in the yield and composition of the essential oils in *M. pulegium* (Chapter III) and *M. cervina* (Chapter VI).

4) Evaluation of the antibacterial activity of *M. cervina* essential oils and corresponding pure main components, in order to validate the plant traditional uses in Portuguese folk medicine (Chapter IV).

5) Assessment of the genetic diversity and structuring of *M. cervina* populations and individuals in order to provide guidelines for the conservation and sustainable use of this threatened aromatic and medicinal species (Chapter V and VI).

These approaches bring together the genetic and the essential oil diversity, geographic distribution, ecological features and agronomical traits that will allow better conservation and management strategies of natural biodiversity and also contribute for economical production of mint related commodities with medicinal and industrial importance.

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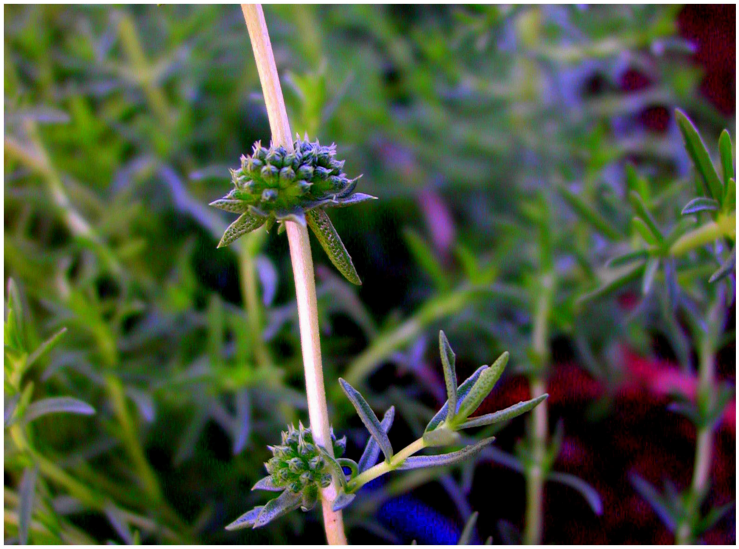
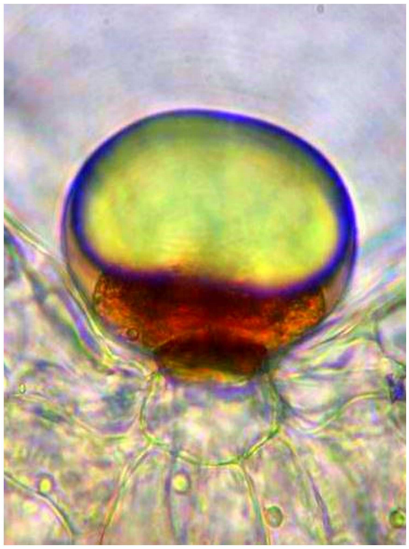

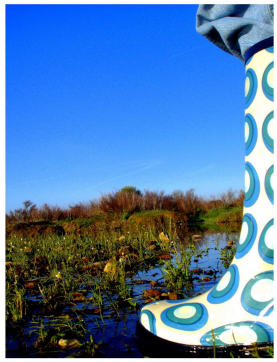


CHAPTER II

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

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Morphology of secretory structures and essential oil composition in Mentha cervina L. from 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 twenty 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, 2 populations were surveyed at different stages of their lifecycle. 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 twenty populations evaluated.

Key Words: *Mentha cervina*, Lamiaceae, essential oils, GC, GC-MS, histochemistry, trichomes, pulegone.

Introduction

Mints (*Mentha* spp.) 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 know 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 of 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 aromatherapy our 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 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 3 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).

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

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 (Tokyo, Japan) microscope 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 3 populations: MC10, MC21 and MC29 (with more contrasting agronomic behaviour). The histochemical tests included: (1) Sudan III for total lipids[22]; (2) Nile Blue for neutral and acid lipids[23]; (3) 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 one hour in a Clevenger-type according to the European Pharmacopoeia[25]. The oils were kept at a low temperature until further analysis.

GC and GC-MS Analysis. GC analysis were performed using a Perkin Elmer 8700 gas chromatograph (Perkin Elmer, Shelton, Connecticut, 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 (30m x 0.25mm i.d., film thickness 0.25µm; J & W Scientific Inc., Agilent Technologies, Santa Clara, California, USA); and a DB-17HT fused-silica column (30m x 0.25mm i.d., film thickness 0.15µm; 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 10min; injector and detector temperatures were 280°C and 290°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30cm.s^{-1}. Samples were injected using the split sampling technique, ratio 1:50, with a volume of injection of 0.1µl 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. The GC-MS unit consisted of a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, Connecticut, USA), equipped with DB-1 fused-silica column (30m x 0.25mm i.d., film thickness 0.25µm; J & W Scientific, Inc., Agilent Technologies, Santa Clara, California, USA), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer, Shelton, Connecticut, 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 30cm s^{-1}; split ratio, 1:40; ionization energy, 70eV; ionization current, 60µA; scan range, 40-300u; scan time, 1s. 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-synthesised components and commercial available standards.

Data analysis

Quantitative results of the morphological characters did not distinguish between populations growing either in the same or different locations. Therefore results are the mean value of the data.

The percentage composition of the essential oil samples was 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, Setauket, New York)[26]. Correlation coefficient was selected as a measure of similarity among the twenty populations, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition. The degree of correlation was evaluated in: very high if correlation ranged between 0.9 and 1; high, between 0.7 and 0.89; moderate, between 0.4 and 0.69; low, between 0.2 and 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 1-2 layers of palisade parenchyma cells and 4-5 layers of spongy parenchyma cells. Midribs showed a vascular bundle surrounded by 3-6 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 lifecycle.

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: **i)** unicellular, with a warty surface, a swollen basal epidermal cell and acute apices (Fig. 1-A, E), which is seen on stems and sepals and on both leaf surfaces, but is more abundant on the adaxial surface; **ii)** small multicellular, 2 to 4 cells, uniseriate, warty surface, with a swollen basal cell and acute apices (Fig. 1-B), sparse on adaxial leaf surface but common on sepals inner and outer faces; **iii)** large multicellular, long up to 8 cells, 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 (Fig. 1-C).

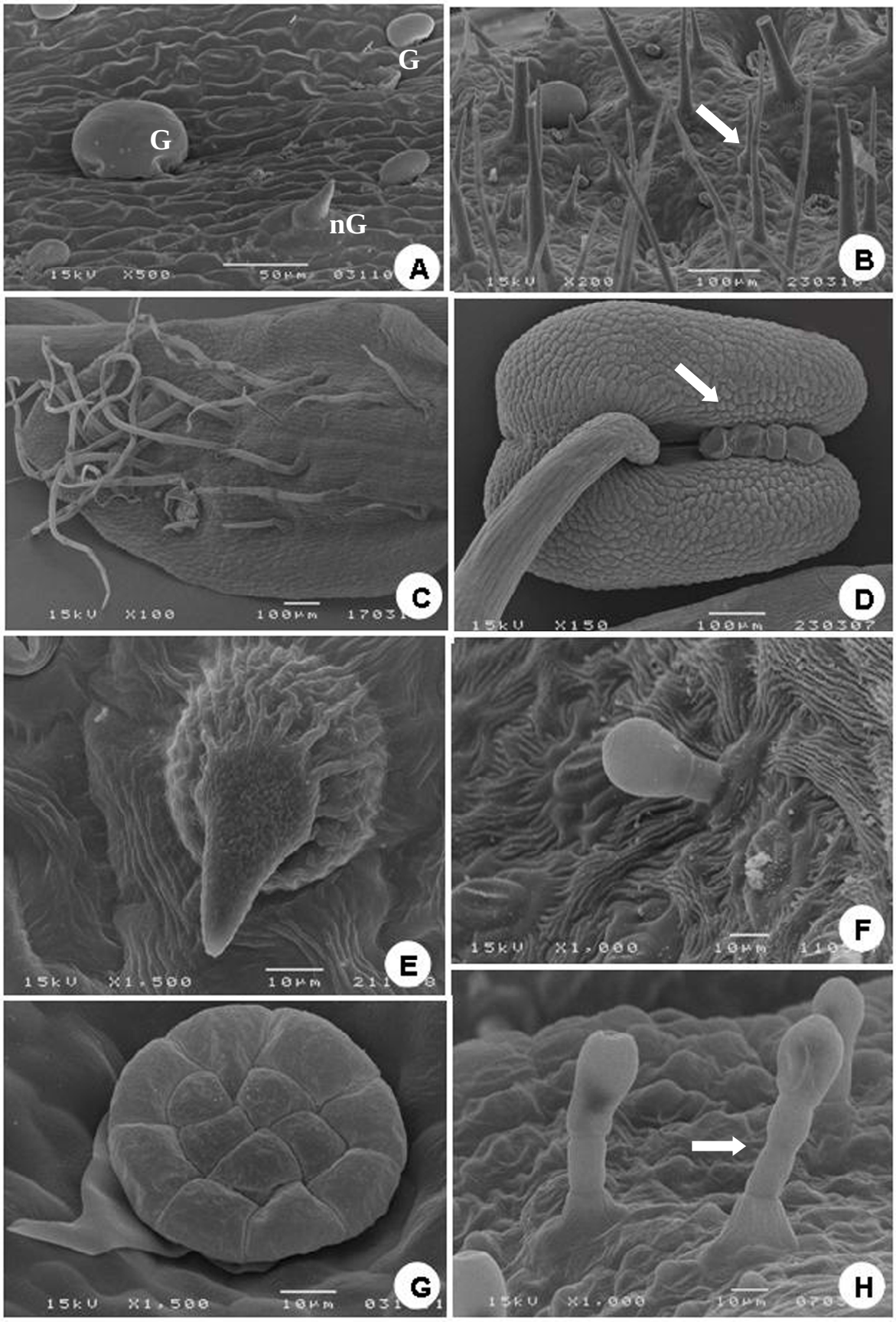
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Figure 1 A-H. SEM micrographs showing distribution and types of trichomes of *Mentha cervina*. A. Abaxial leaf surface with glandular (G) and non-glandular trichomes (nG). B. Adaxial leaf surface exhibiting small multicellular non-glandular trichomes, with 2 to 4 cells, with a regular distribution. C. Petal outer face showing large multicellular non-glandular trichomes, up to 8 cells long, leaned toward 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 trichomes with thirteen secretory cells, arranged in two circles. H. Capitate type II glandular trichome with a lower conical stalk cell, exhibiting 1 to 2 elongated neck cells.

The glandular trichomes are of two different types: peltate and capitate. Peltate are 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 (between 8 and 12 in the leaves and up to 20 in the petals) arranged in one or two circles (Fig. 1-G). 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 (±6 μm) on the corolla, compared to 89 μm (±8 μm) on the adaxial leaf surface and 98 μm (±8 μm) on the abaxial leaf surface.

The two capitate trichomes found differ from each other in the shape of the head and the length of the stalk head: **i)** capitate type I, with one stalk cell 10 μm (±0.1 μm) in length, and a round/oval secretory head cell, with a smooth surface (Fig. 1-F), 32 μm (±4 μm) in length and 22 μm (±2 μm) in diameter at the head, uniformly distributed on both leaf surfaces, calyx and stems; **ii)** capitate type II, with a lower conical stalk cell, 26 μm (±6 μm) in length and 1 to 2 elongated neck cells, 13 μm (±0.7 μm) and a round secretory head cell, with a smooth surface (Fig. 1-H), 33 μm (±2 μm) in length and 23 μm (±0.1 μm) in diameter at the head, only on the adaxial petal surface

The only trichomes on the stamens or carpel were peltate trichomes, which occurred along the lower side of the connective tissue between the two anther lobes (Fig. 1-D), 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 2 peltate glands mm^{-2} on the adaxial leaf surface and 5.25 glands mm^{-2} on the abaxial 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

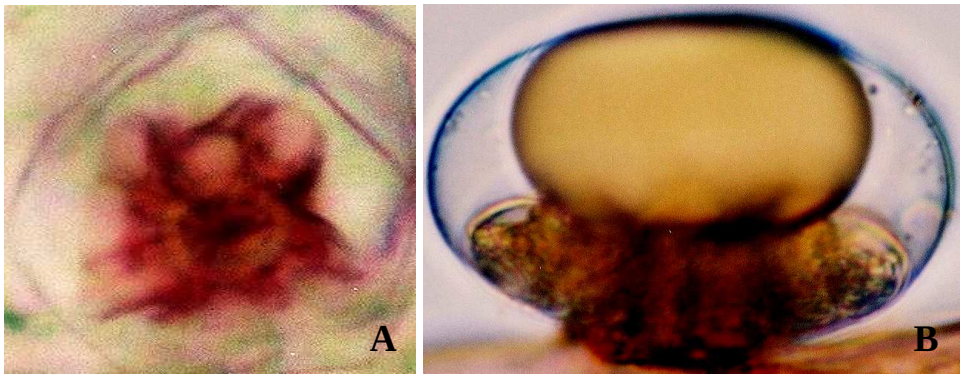
Data from histochemical tests revealed that the secreted material composition was similar in both leaves and flowers and had a complex nature, containing hydrophilic and lipophilic components. The presence of these compounds was independent of the organ and of the developmental stage, but dependent on the trichome type (Table 2).

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.

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 (Fig. 2A-B), 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.



Figs. A-B. Bright-field micrographs of *Mentha cervina* leaf trichomes. A. Peltate trichome stained with Ruthenium Red. B. Peltate hair stained with Sudan III.

Essential oil composition and quantification

In the 20 populations surveyed, at full flowering the oil yield ranged from 2.4% to 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 ranged from 62 to 78% in the oils that were isolated from the cultivated plants, and from 73 to 80% in those from the wild populations. Isomenthone was the second main component of *M. cervina* oils (ranging from 3% to 18%). The monoterpene hydrocarbon limonene varied between 3% and 7% and was the third main component of the oils isolated from the twenty 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.

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

		Cultivated																Wild		
Components	**RI**	**Mc1**	**Mc2**	**Mc3**	**Mc7**	**Mc10**	**Mc12**	**Mc13**	**Mc14**	**Mc15**	**Mc16**	**Mc17**	**Mc18**	**Mc19**	**Mc21**	**Mc22**	**Mc24**	**Mc29**	**Mc35**	**Mc38**	**Mc39**
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

		Cultivated																Wild		
Components	**RI**	**Mc1**	**Mc2**	**Mc3**	**Mc7**	**Mc10**	**Mc12**	**Mc13**	**Mc14**	**Mc15**	**Mc16**	**Mc17**	**Mc18**	**Mc19**	**Mc21**	**Mc22**	**Mc24**	**Mc29**	**Mc35**	**Mc38**	**Mc39**
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 I. 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.

Discussion

The glandular trichomes of *M. cervina* are similar to the two main types occurring in other *Lamiaceae*, the peltate and capitate types[28]. For many *Lamiaceae* species, the head of the peltate trichomes 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].

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 abaxial, of about twice the number of glands of the adaxial 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 ranged from 2.4% to 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 edaphologic conditions. The same pattern of chemical composition was obtained for the populations that grew in wild conditions, witch 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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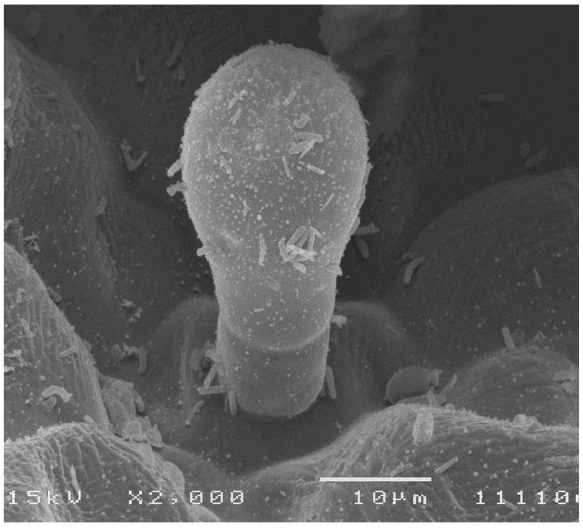

CHAPTER III

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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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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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.

Key words: *Mentha pulegium* L., Lamiaceae, Trichomes, Essential oil, Histochemistry, GC, GC-MS

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 de 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 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.

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**
Bulgaria	Aerial parts	Water and steam distillation	Pulegone 43-45	GC. GC/MS	Stoyanota 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	GC. GC/MS	Mahboubi and Haghi. 2008
Iran	Aerial parts	Hydrodistillation	Menthone 39	GC. GC/MS	Hassanpouraghdam et al.. 2011
Portugal	Aerial parts	Hydrodistillation	Pulegone 60	GC, GC/MS	Monteiro et al., 2007
Portugal	Aerial parts	Hydrodistillation	Pulegone 65-87	GC, GC/MS	Lopes et al., 2010
Portugal	Aerial parts	Hydrodistillation	Menthone 36 Pulegone 23	GC, GC/MS	Teixeira et al., 2012
Spain	Aerial parts	Hydrodistillation	Pulegone	GC/MS. GC/Olfactometry	Maroto-Diaz et al.. 2007
Tunisia	Aerial parts	Hexane extract	Pulegone 42	GC. GC/MS	Mkaddem et al.. 2007
Uruguay	Leaves	Hydrodistillation	Pulegone 73	GC-FID. GC/MS	Lorenzo et al.. 2002
Yugoslavia	Aerial parts	Steam Distillation	Menthone 31	GC. GC/MS	Chalchat. 2000

Material and Methods

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, Figure 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) where 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 m^2 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.

Morphological Studies

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

population.

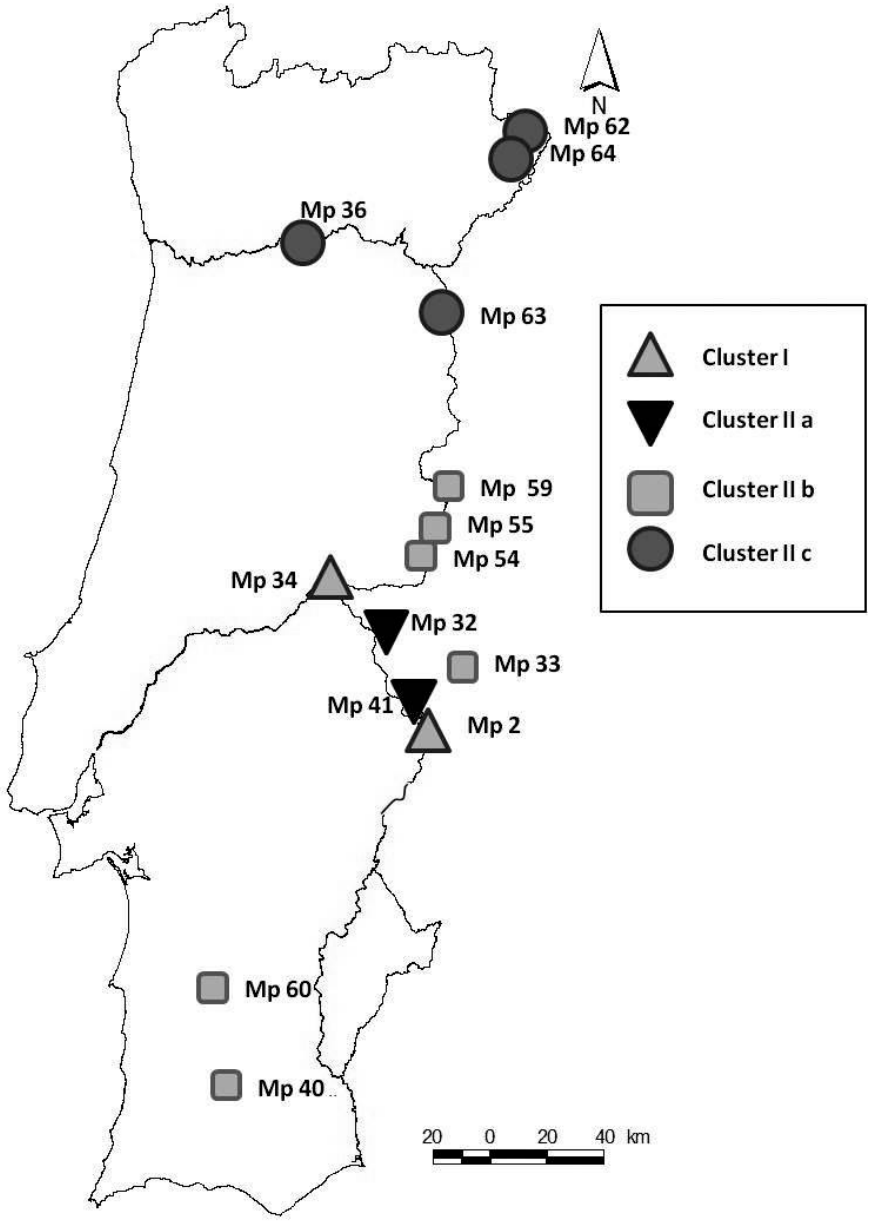


Fig. 1. Map of Portugal with collection sites of *Mentha pulegium* populations analyzed. Symbols, according to the essential oil cluster analysis (Fig. 3). For abbreviations, see Table 2.

Table 2

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

Populations	**Sample type**	**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	Bagaúste, 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

*Sample type: W - wild, C - cultivated, DS - Developmental stage.

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.

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 & Carde, 1964); and (4) Ruthenium Red for pectins (Johansen, 1940). Standard control procedures were carried out simultaneously.

Essential Oil Analysis

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

Gas Chromatography (GC)

GC analysis were performed using a Perkin Elmer 8700 gas chromatograph (Perkin Elmer, Shelton, Connecticut, 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 x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Agilent Technologies, Santa Clara, California, USA); and a DB-17HT fused-silica column (30 m x 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.

Gas Chromatography – Mass Spectrometry (GC-MS)

The GC-MS unit consisted of on Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, Connecticut, USA), equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc., Agilent Technologies, Santa Clara, California, USA), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer, Shelton, Connecticut, 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 cms^{-1}; 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_9–C_{17} 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.

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 Arithmetical 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).

Results and Discussion

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. 2-A), which is seen on stems and sepals and on both leaf surfaces, but more abundant on the adaxial surface; ii) short multicellular, 2 to 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. 2-A); 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.2-B). 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*

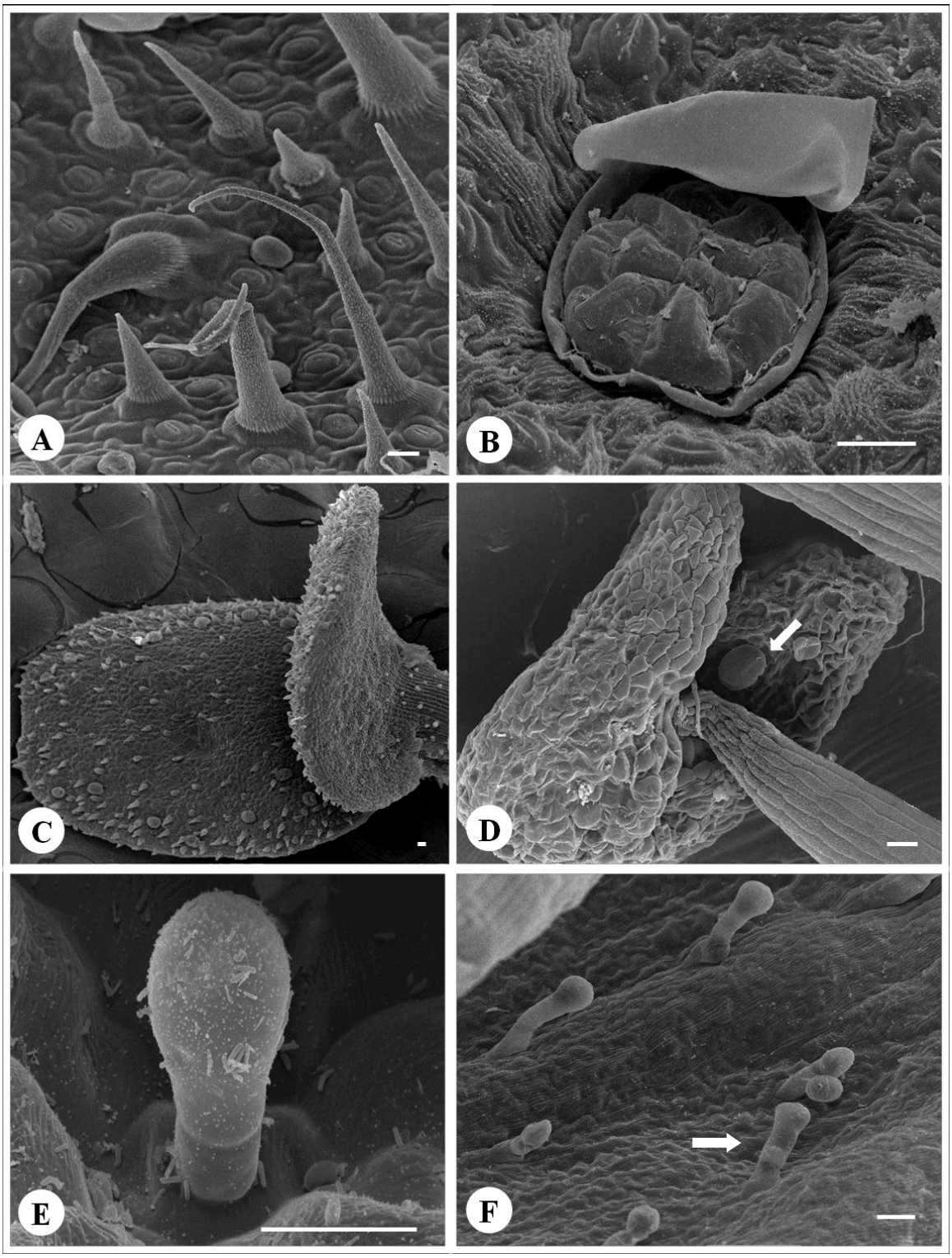
12 cells are probably the most common number in a peltate trichome of mature leaves, usually 4 in the inner circle and 4-8 in the outer (Fig.2-G). That number can reach up to 16 cells in the petals peltate trichomes, usually 4 in the inner circle and 8-12 in the outer. According to Turner et al. (2000) 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* (Gerbach, 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 (±9 μm) on the corolla, compared to 88 μm (±10 μm) on the adaxial leaf surface and 92 μm (±9 μm) on the abaxial leaf surface. Morphologically well developed peltate glandular trichomes, were also observed on cotyledons (Fig. 2-C), as in other Lamiaceae, such as *Salvia officinalis* and *Ocimum basilicum* (Croteauet 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. 2-D). 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 (±0.1 μm) in length, and a round/oval secretory head cell, with a smooth surface (Fig. 2-E), 27 μm (±2.2 μm) in length and 21 μm (±1.7 μm) 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 (±6 μm) in length and 1 to 2 elongated neck cells, 12 μm (±0.7 μm) and a round secretory head cell, with a smooth surface (Fig. 2-F), and 13 μm (±0.1 μm) 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.

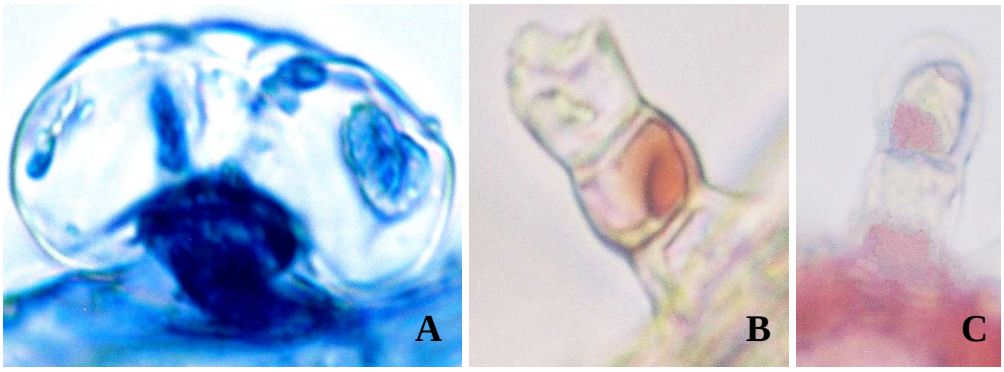
On fully expanded mature leaves of *M. pulegium*, the capitate glandular trichomes were densely distributed while the peltate were scattered among them. Similar results were found also in *M. pulegium* by Karray-Bouraoui et al. (2009) and in both *M. spicata* and *M. spicata* × *suaveolens* by Martins (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).



Figs. 2A-F. 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 to 2 elongated neck cells. G. Peltate glandular trichomes with twelve secretory cells, arranged in two circles. (bar = 20 μm).

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 trichomes stained positive with Sudan III (total lipids), Nile Blue (acidic lipids) and Nadi reagent (essential oils) (Fig.3A), while secretions from capitate trichomes only stained positive with Sudan III (Fig.3B) and Nadi reagent. Pectins were also found in the cell walls and contents of both peltate and capitate hairs (Fig.3C).



Figs. 3A-C. Bright-field micrographs of the histochemical characterization of the secretions of *Mentha pulegium* leaf trichomes. A. Peltate trichome stained with the Nadi reagent. B. Capitate type II trichome stained with Sudan III. C. Capitate type II trichome stained with ruthenium red.

The presence of total lipids in secretions of the two types of trichomes and acidic lipids only on peltate trichomes had also been 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*. The presence of pectins in both the cell walls and contents of the trichome head cells by Ruthenium Red is referenced in *Plectranthus ornatus (*Ascensão et al., 1999) and in *Satureja subspicata* (Marin et al., 2010).

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	+	+	+

- negative; + positive

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 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 92-99% 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.

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					
				a		b		c	
		Min	Max	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
p-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
trans-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
cis-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

Components	RI	Cluster I		Cluster II					
				a		b		c	
		Min	Max	Min	Max	Min	Max	Min	Max
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 relatite to C_9-C_{17} *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.

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 ($S_{corr} \geq 0.9\%$) 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 (≤ 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 60-90%. 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).

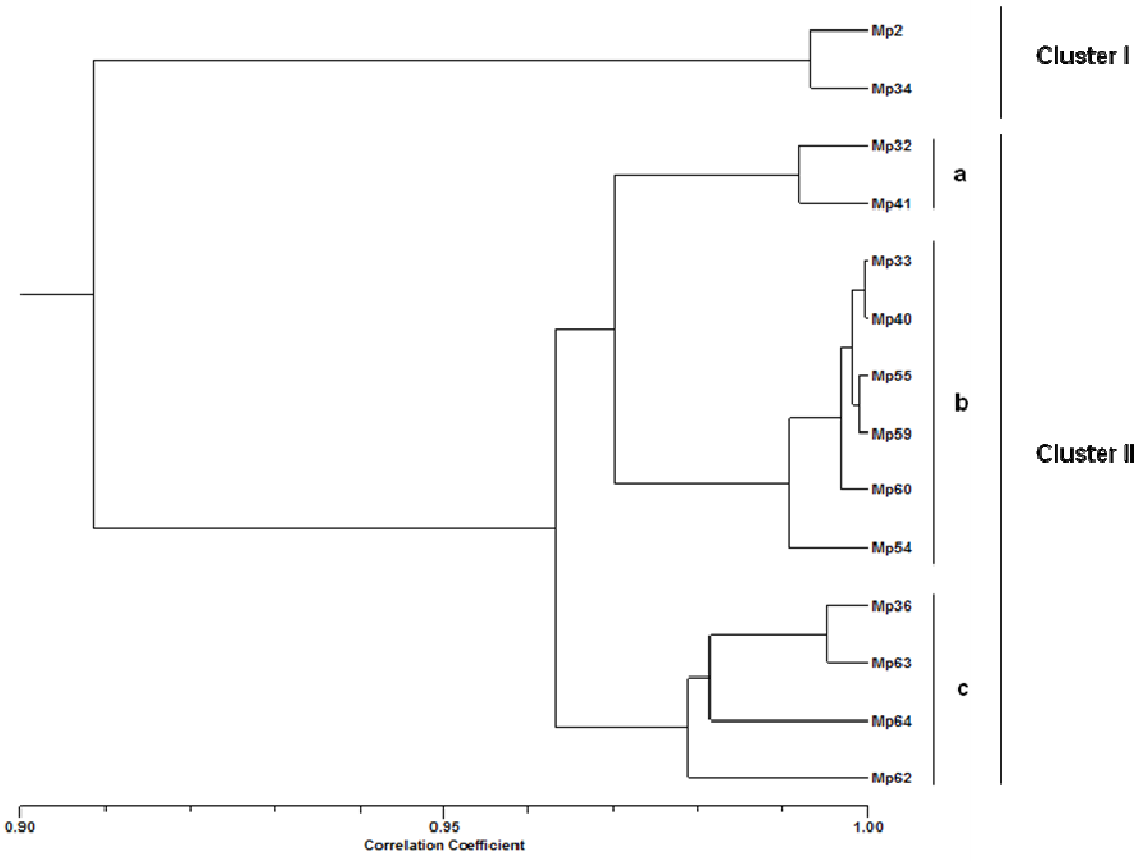


Fig. 3. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the *Mentha pulegium* samples examined, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA). For abbreviations, see Table 2.

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).

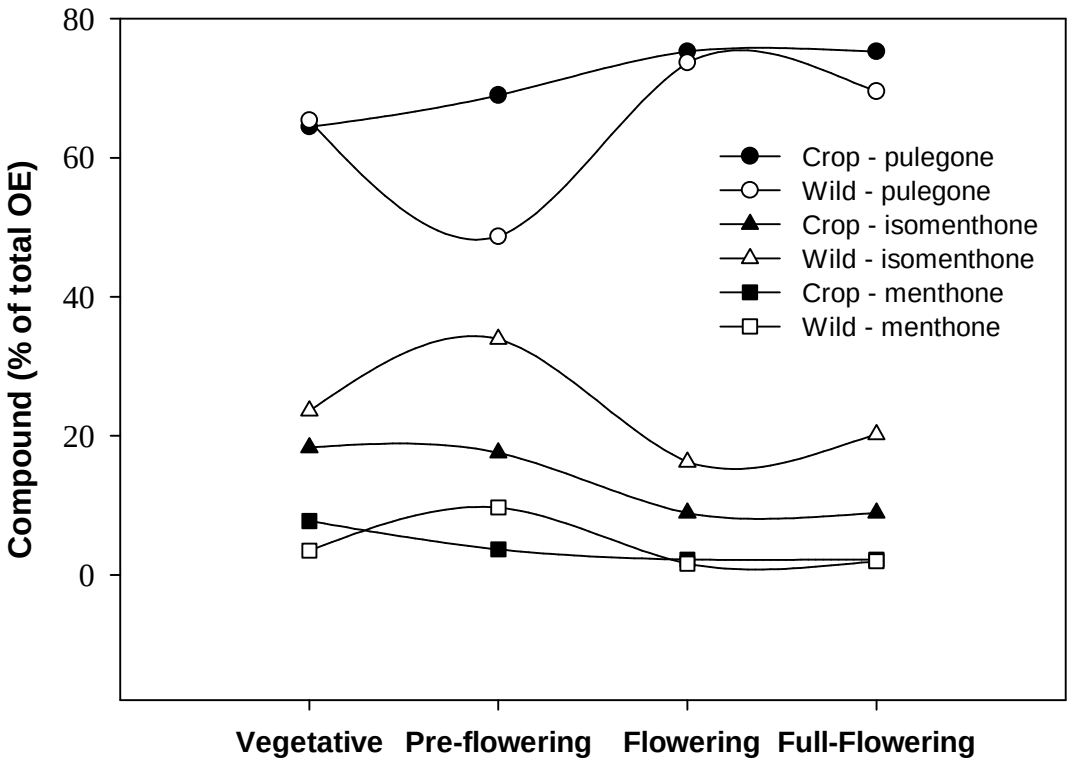


Fig. 4. Time-course study of the main components of the *Mentha pulegium* essential oils isolated in wild (open symbols) and cultivated (closed symbols) growing conditions at different developmental stages. Given the extended period of flowering, two sampling points, one month apart, were considered 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.

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 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 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 trough the life cycle may reflect some degree of substrate specificity or other environmental and / or physiological condition.

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.

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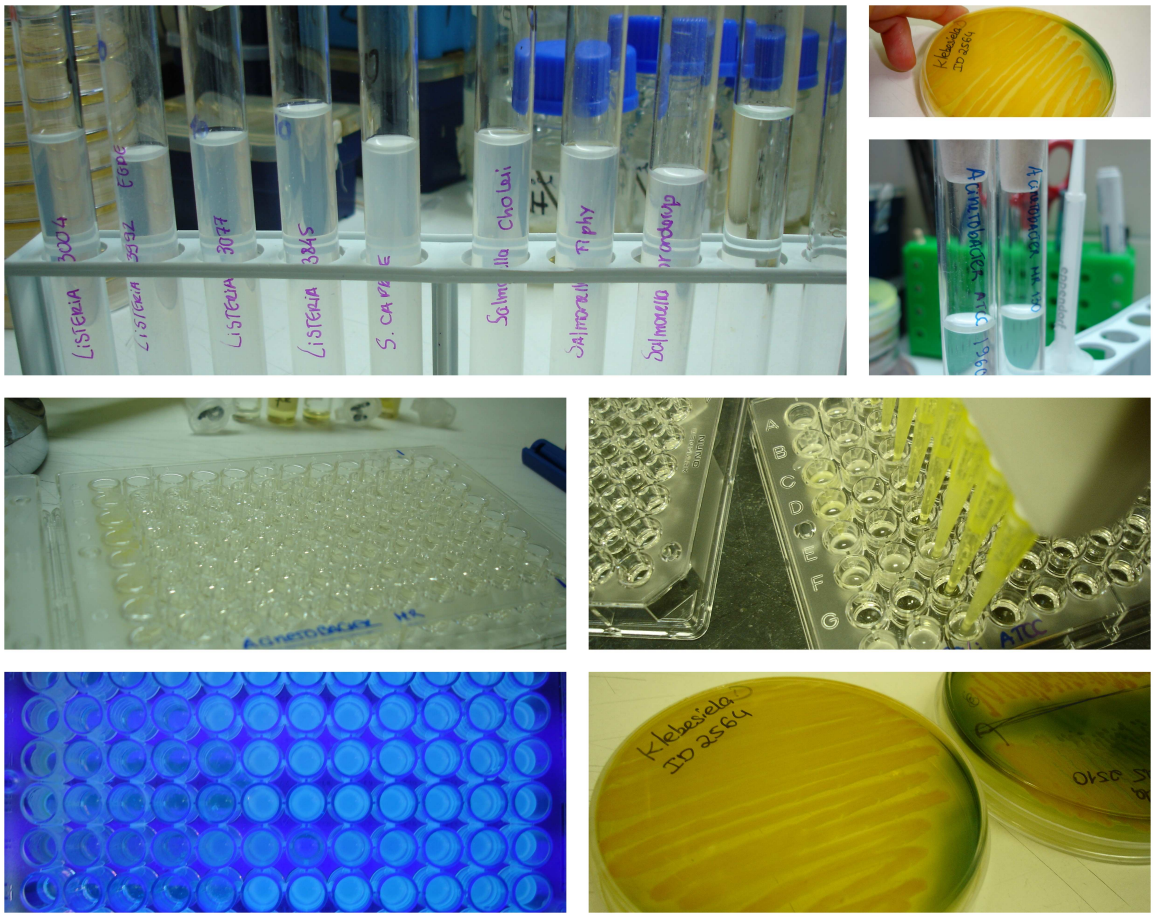

CHAPTER IV

Chemical composition and antibacterial activity of the essential oils from the medicinal plant *Mentha cervina* L. grown in Portugal

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Chemical composition and antibacterial activity of the essential oils from the medicinal plant *Mentha cervina* L. grown in Portugal

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Abstract

Mentha cervina is a medicinal plant traditionally used in Portugal in folk medicine, in different gastric disorders and inflammations of the respiratory tract. In order to validate those traditional uses, *M. cervina* essential oils (EOs) were characterized by GC and GC-MS and their antimicrobial activity was tested against 23 bacterial strains (including multiresistant strains). The EOs were dominated by the monoterpenes pulegone (52–75%), isomenthone (8–24%), limonene (4-6%) and menthone (1-2%). The antibacterial activity of these EOs was compared to that of the main components standards. The most effective antibacterial activity was expressed by the EOs against the Gram-negative bacteria, *Escherichia coli* and *Acinetobacter baumanni*, with MIC values of 1 mg/mL. The EOs complex mixtures were more active than the individual aromatic components supporting the hypothesis that the EOs antibacterial activity is a function of the synergistic effect of their different aromatic components. These results show the potential role of *M. cervina* EOs as antibacterial agents and validate the traditional use of this plant.

Keywords Lamiaceae, GC, GC-MS, essential oils, monoterpenes, antimicrobial activity, MIC.

Introduction

Essential oils (EOs) and their components are gaining increasing interest in the food, cosmetic and pharmaceutical industries, because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey *et al*., 2001). In this view, there is an ongoing effort to screen plants medicinally used in different regions of the world, which is the case of plants from the Lamiaceae family that are long know as important sources of EOs bearing plants used in food, perfume, cosmetic and pharmaceutical industries because of their culinary, fragrance and antimicrobial properties (Lis-Balchin and Deans, 1997; Ohloff, 1994;). The EOs from *Mentha* species have been in use since ancient times for the

treatment of many digestive tract diseases and in culinary (Iscan *et al*., 2002), and they are known to have antimicrobial properties (Flamini *et al.*, 1999; Naigre *et al.*, 1996). As such, mints are valuable crops with a substantial importance in the botanical economy and to the pharmaceutical industry. Concerning the antimicrobial properties of mint EOs, several species of *Mentha* have been studied, in particular *Mentha piperita* L. (peppermint) (Iscan *et al.*, 2002; Yadegarinia *et al.*, 2006), *M. suaveolens* Ehrh. (Oumzil *et al*., 2002), *M. rotundifolia* (L.) Hudson (Derwich *et al.*, 2010), *M. pulegium* L. (Mahboubi and Haghi, 2008 and citations there in), *M. aquatica* L. and *M. longifolia* (L.) Hudson (Gulluce *et al*., 2007; Mimica-Dukic *et al*., 2003). These studies yielded results that are difficult to compare, mainly due to the great variation found in the chemical composition of mint EOs and to a lesser extent to differences in the experimental techniques applied. Different *Mentha* species show differences in their pattern of oil composition, which are the result of their specific metabolic pathways (McConkey *et al*., 2000). Also, the same taxon growing in different areas may have widely differing chemical components resulting in the existence of intraspecific chemical differences (chemotypes), which is very common in the *Mentha* genus (Kokkini, 1991). Biological activity, which is dependent on the chemical composition, is similarly subject to variation, explaining the conflicting results concerning their biological properties (Oumzil *et al*., 2002).

Mentha cervina L., commonly known as hart's pennyroyal, is an aromatic plant traditionally used in Portugal to flavor recipes and in folk medicine, where it is used as an infusion, preventing different gastric disorders and inflammations of the respiratory tract (Monteiro *et al*., 2007; Póvoa *et al*, 2006; Rodrigues *et al.*, 2008). This plant is native of the Iberian Peninsula and North Africa, and in Portugal it can be found in streams, bogs and humid places, that are representative of the priority habitat Natura 3170 "temporary Mediterranean ponds" (Silva *et al*., 2009). The unfavorable conservation status of this habitat, the excessive harvesting for consumption and overgrazing are leading to the disappearance of this species from natural settings (Póvoa *et al.*, 2006).

In a previous study, the *M. cervina* EOs extracted from cultivated populations, were characterized as belonging to the same chemotype – the pulegone chemotype (Rodrigues *et al*., 2008). Considering the bioactivity of *M. cervina* EOs, there is only one study reporting the antifungal activity against *Candida*, *Aspergillus* and dermatophyte strains (Gonçalves *et al*., 2007). These authors suggest that *M. cervina*

EOs can be used as alternative antifungal agents in the treatment of dermatophytosis. Nevertheless, studies on the antibacterial activities of these EOs are missing.

Given the lack of knowledge on the antibacterial activity of the EOs from *M. cervina* grown in Portugal, the antibacterial capacity of three *M. cervina* EOs was tested against 23 bacterial strains, some of them responsible for digestive and respiratory human diseases and including multiresistant strains. The antibacterial activity of standards from the three main oxygen-containing monoterpenes from the EOs was evaluated. To our knowledge, this is the first report on the antibacterial activity of *M. cervina* EOs from Portuguese populations.

Methods and materials

Plant material

This study was based on three populations of *M. cervina* collected from natural habitats and kept under culture in the essay field of the Instituto Superior de Agronomia (Lisbon). Voucher specimens from the 3 populations have been deposited in the LISI herbarium under the voucher numbers 532/2005 (MC1), 523/2005 (MC2) and 520/2005 (MC3).

Essential oil isolation procedure

For each EO sample, 20 g of full flowering aerial parts were subjected to hydro distillation for 1 h in a Clevenger-type apparatus according to the European Pharmacopoeia method (Council of Europe, 2007). The EOs were stored at -20ºC in the dark until analysis.

Gas-chromatography (GC) and Gas-Chromatography–Mass Spectrometry (GC–MS)

GC and GC-MS analysis were performed according to Rodrigues *et al*. (2008). The identity of the components was assigned by comparison of their retention indices, relative to a C_9–C_{16} 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.

Bacterial strains

Twenty three bacterial strains were tested most of them pathogenic for humans and showed multiresistance to antibiotics. The Gram-positive strains are: *Staphylococus aureus* (ATCC and MRSA - Meticillin Resistant *Staphylococcus aureus*), *S. caprae*, *Enterococus faecalis (*VRE - Vancomycin Resistant *Enterococcus*), *E. faecium, E. hirae* and four *Listeria monocytogenes* strains. The Gram-negative strains are: *Escherichia coli* (ATCC and β-lactamase CTX-M-15 producers), *Salmonella* Braenderup; *S.* Typhimurium; *S.* Choleraesuis, *Klebsiella pneumoniae* (CIP and Extended Spectrum β-lactamases-ESBL producer), *Acinetobacter baumannii* (ATCC and multiresistant European clone II strain and the metallo-β-lactamase IMP-5 producer), *Pseudomonas aeruginosa* (ATCC and multiresistant strain by efflux pump). The microorganisms were derived from reference cultures (ATCC and CIP) and stock cultures from CBLBFF (Colecção de Bactérias do Laboratório de Bacteriologia da Faculdade de Farmácia) andCBISA (Colecção de Bactérias do Instituto Superior de Agronomia).

Antimicrobial activity assay

The bacterial strains were challenged with the three different *M. cervina* EOs and also with pure standards of the three main components of these EOs, menthone, isomenthone and pulegone (Fluka) in order to evaluate their antimicrobial activities. Since a preliminary control test with the solvent DMSO, in a range of 125-250 mg/mL yielded

no effect on microbial growth, the EOs and the standards were solubilized in this solvent (ratio of 1:1) and then diluted in culture media for use. The minimum inhibitory concentration (MIC) values were determined by the microdilution broth method, as reported in NCCLS (2006). Microdilution broth test was performed in Mueller-Hinton broth medium, in 96-well micro plates, as follows: 100 µl of Mueller-Hinton broth was added into each well of the micro plate and 100 µl of each EO or pure standards diluted in DMSO (1:1) were respectively added to the first row of the micro plate and then serially twofold diluted in a final volume of 100 µL, with concentrations ranging from 250 to 0.25 mg/mL. The wells were then inoculated with 10 µL of each bacterial suspension, adjusted to 0.5 McFarland (about 10^7-10^8 CFU/mL). The last row containing the bacterium in Mueller-Hinton broth without the test sample was used as a control for strain viability. Ampicillin and Riphampicin were used as a reference compound for antibacterial activities of Gram-negative and Gram-positive bacteria, respectively. The microplates were covered and incubated for 24 h at 37°C. Each experiment was performed in triplicate. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the product inhibiting the growth of the microorganisms.

Interpretation of the results

The interpretation of microbial growth was based on the following criterion: the result was considered positive, thus presenting microbial growth, when at least two of three replicates presented visible growth. When visible growth was detected only in one or in none of the three replicates, the result was considered negative, thus indicating absence of microbial growth.

Results and discussion

Essential oil chemical composition

Thirty-three components were identified in the *M. cervina* evaluated EOs, covering 88-97% of the total (Table 1). Oxygen-containing monoterpenes constituted the main fraction in all EOs (80-88%), pulegone (52-75%), isomenthone (8-24%), limonene (4-6%) and menthone (1-2%) being the main components (Table 1). These results are in accordance with previous studies, which have shown high chemical correlation for Portuguese *M. cervina* EOs (Gonçalves *et al.*, 2007; Rodrigues *et al*., 2008), although some variability was shown in the EOs isolated from this species grown in other countries (Lawrence, 2007). Despite the chemical homogeneity between the evaluated oils, three different profiles were considered, a pulegone-rich with the lowest level of isomenthone (MC1 in Table 1) and two pulegone-rich with similar high levels of isomenthone (MC2 and MC3 in Table 1) but that showed slight differences in the relative amount of the minor EOs components.

Table 1. Composition of the essential oils isolated by hydrodistillation from the flowering aerial parts of three *Mentha cervina* cultivated populations.

Components	**RI**[a]	***Mentha cervina* populations**		
		MC1	**MC2**	**MC3**
3-Methyl cyclohexanone	914	t	t	t
α-Thujene	924	t	t	t
α-Pinene	930	0.5	0.6	0.1
Camphene	938	t	t	0.1
Sabinene	958	0.3	0.1	t
1-Octen-3-ol	961	t	t	t
β-Pinene	963	0.2	0.8	0.2
2.5-Dimethyl-1-hexene	970	t	t	t
3-Octanol	974	1.0	0.6	2.4
β-Myrcene	975	0.6	0.4	t
p-Cymene	1003	t	0.1	t
1.8-Cineole	1005	0.3	0.3	0.1
Limonene	1009	6.1	5.6	4.0
cis-β-Ocimene	1017	0.1	t	t
trans-β-Ocimene	1027	t	t	0.1
γ-Terpinene	1035	t	t	t
n-Octanol	1045	t	t	0.1
cis-Linalool oxide	1045	0.1	t	t
trans-Limonene oxide	1112	0.1	t	0.1
Menthone	1120	0.9	2.2	1.8
Isomenthone	1126	8.3	21.2	24.4
Menthofuran	1134	t	t	t
cis-Isopulegone	1134	1.0	0.9	1.2
Terpinen- 4-ol	1148	t	t	t
Verbenone	1164	t	t	t
Myrtenol	1168	t	t	t
Pulegone	1210	74.9	62.7	52.2
Piperitone	1211	t	0.2	t
Carvotanacetone	1222	t	t	0.6
Piperitenone	1289	1.5	0.6	t
β-Caryophyllene	1414	0.3	0.2	t
β-Caryophyllene oxide	1561	t	0.8	1.0
Humulene epoxide	1580	0.1	t	t
% Identification		96.3	97.3	88.4
Grouped components				
Monoterpene hydrocarbons		7.8	7.6	4.5
Oxygen-containing monoterpenes		87.1	88.1	80.4
Sesquiterpene hydrocarbons		0.3	0.2	t
Oxygen-containing sesquiterpenes		0.1	0.8	1.0
Others		1.0	0.6	2.5

[a]Retention Index relative to C_9-C_{16} *n*-alkanes on the DB-1 column; t - trace (<0.05).

Antibacterial activity of the *M. cervina* EOs and of the pure aromatic compounds

The antibacterial capacity of the three different EOs profiles was compared among each other and to standards from the EOs oxygen-containing monoterpene main components, pulegone, isomenthone and menthone. The results showed that the antibacterial activity of the *M. cervina* EOs and the standard compounds is dependent of the type of microorganisms and in different degrees on the EO profile and the pure compound used (Table 2).

In general, Gram-negative bacteria were more sensitive than Gram-positive bacteria (Table 2). Gram-negative bacteria showed MIC values of 1 mg/mL, using the complex mixture of EOs. The OEs activity against Gram-positive bacteria was less noteworthy with the lowest MIC values equal or more than 7.8 mg/mL, with exception for *S. aureus* ATCC 6533 that was more susceptible (2 mg/mL with MC3). With the pure compounds, we had the same behaviour, the Gram-positive showed MIC values equal or more than 62.5 mg/mL (also with exception for *S. aureus*), and in the Gram-negative bacteria we could find MIC values of 2 mg/mL.

Although it has been established that Gram-positive bacteria are much more sensitive to drug action than Gram-negative bacteria (Cos *et al*., 2006), because of their less complex membrane structure (Cosentino *et al*., 1999; Karaman *et al*., 2003; Sahin *et al*., 2002), the results presented in this study with *M. cervina* EOs are not in accordance with this. The same results were obtained in other studies using *M. pulegium* and *M. longifolia* EOs (Gulluce *et al*., 2007; Hajlaoui *et al*., 2009; Hafedh *et al*., 2010; Mahboubi *et al*., 2008). The results obtained in our study are promising because the *M. cervina* EOs could be important in future formulations for treatment of multiresistant Gram-negative pathogens, including *Acinetobacter* spp., *Pseudomonas aeruginosa* and, because of their production of extended-spectrum β-lactamase, Enterobacteriaceae, responsible for serious infections in community and hospital patients (Slama, 2008).

Table 2. Minimum inhibitory concentration (MIC, mg/mL) of *Mentha cervina* essential oils, pure standard compounds and DMSO, against different bacterial strains.

Bacteria strains	MIC (mg/mL)							MIC (µg/mL)
	Essential oils			Pure compounds and Solvent				
	MC1	MC2	MC3	Pulegone	Isomenthone	Menthone	DMSO	Antibiotics[1]
GRAM -								
Pseudomonas aeruginosa								
ATCC 10554	125	62.5	15.6	125	125	125	>250	<0.25
MR (ID 1833)	125	31.3	31.3	125	125	125	>250	>250
Escherichia coli								
ATCC 11105	2.0	1.0	1.0	3.9	15.6	31.3	125	<0.25
CTX (ID2511)	7.8	1.0	2.0	3.9	62.5	62.5	125	62.5
Acinetobacter baumannii								
ATCC 19606	3.9	2.0	3.9	2.0	31.3	62.5	125	<0.25
MR (ID130)	2.0	1.0	1.0	2.0	15.6	15.6	125	15.6
IMP5 (ID65)	1.0	1.0	2.0	2.0	15.6	15.6	125	<0.25
Klebsiella pneumoniae								
KPC (ID2564)	62.5	3.9	7.8	62.5	62.5	62.5	125	-
CTX-M-15 (ID2510)	31.3	7.8	15.6	62.5	125	125	125	-
TEM-10 (ID683)	62.5	15.6	15.6	31.3	62.5	125	125	-
***Salmonella* Thyphimurium**								
CBISA 3969	62.5	31.3	15.6	62.5	7.8	31.3	125	<0.25
***Salmonella* Braenderup**								
CBISA 3991	31.3	31.3	7.8	31.3	62.5	62.5	125	<0.25
GRAM +								
Staphylococcus aureus								
ATCC 6533	31.3	15.6	2.0	62.5	15.6	15.6	>250	<0.25
MRSA CIP 106760	62.5	15.6	7.8	62.5	125	125	>250	-
Staphylococcus caprae								
CBISA 3572	62.5	-	7.8	125	125	125	>250	<0.25
Enterococcus faecalis								
CIP 104476	62.5	31.3	15.6	125	125	125	>250	125
Enterococcus faecium								
ID 435628	62.5	62.5	15.6	125	125	125	>250	2.0
Enterococcus hirae								
CIP 5855	62.5	62.5	15.6	125	125	125	>250	2.0
Listeria monocytogenes								
EGDe (CBISA 3992)	62.5	-	7.8	125	125	125	125	<0.25
CECT (CBISA 3004)	62.5	-	7.8	125	125	125	125	<0.25
CBISA 3845	62.5	-	7.8	125	125	125	125	<0.25
CBISA 3077	125.0	-	15.6	125	125	125	125	<0.25

[1] Ampicillin and riphampicin were used as reference compounds for antibacterial activities of Gram-negative and Gram-positive bacteria, respectively

The most considerable antibacterial activity was obtained against *E. coli* and *A. baumannii*, using the EOs complex mixtures. The EO MC2 showed the lowest MIC value of 1 mg/mL for both *E. coli* strains (ATCC and the multiresistant) and the *A. baumannii*. Although we can find in the literature studies using mint EOs, the results are

difficult to compare because the methodologies and the bacterial strains used are different among studies. Moreover, the same species may also present different chemotypes. For *E. coli* strains, we could find MIC values of 0.78 and 2.25 mg/mL using *M. longifolia* EO (Hafedh *et al*., 2010 and Hajlaoui *et al.*, 2009, respectively), 2.25 and 4 mg/mL with *M. pulegium* EO (Hajlaoui *et al.*, 2009 and Mahboubi *et al*., 2008, respectively), and 250 mg/mL with *M. rotundifolia* EOs (Derwich e*t al*., 2010). Interestingly the plant species with more pronounced antibacterial activity were the ones presenting EOs with high content in the monoterpenes pulegone, menthone and isomenthone (*M. pulegium*, *M. longifolia* and *M. cervina* in this study).

The antibacterial activity of the EOs was higher (Table 2) when compared with the pure standard compounds. Considering the bioactivity of the pure standards alone, in general, isomenthone and menthone, were less active than pulegone (its precursor), with the exceptions for *S.* Thyphimurium and *S. aureus* ATCC (Table 2). Similar results were obtained by other authors that reported pulegone as showing a more potent bioactivity (Flamini *et al.*, 1999; Gulluce *et al*., 2007; Hajlaoui *et al.*, 2008; Naigre *et al.*, 1996; Oumzil *et al*., 2002; Mimica-Dukic *et al*., 2003; Oyedeji and Afolayan, 2005). Considering the results with the three EOs profiles, MC3 (the EO with less content in pulegone), was the one who exhibited higher antibacterial activity against all Gram-positive and Gram-negative bacteria (Table 2). In general, the order of efficacy was MC1 < MC2 < MC3, which appears to be related to the decrease in pulegone content. So, although pulegone showed the higher antimicrobial activity (considering the pure standard compounds alone), these results do not agree with the data obtained with the EOs complex mixtures, where the EO with less content in pulegone exhibited the best results. Using *M. cervina* EOs for antifungal activities against *Candida, Aspergillus* and dermatophyte strains, Gonçalves *et al.* (2007) also obtained the highest activity with the sample containing lower amounts of pulegone. The same type of response of EO complex mixtures and individual components was reported in other species, using *M. piperita* EO against *E. coli*, *S. aureus* and *Candida albicans* (Yadegarinia *et al.*, 2006) and with *M. spicata* EO against *L. monocytogenes* (Leonard *et al*., 2010). Given the heterogeneous composition of EOs and the different antimicrobial activities of its components, it seems that different components may have different modes of action and that the activity could be attributed to the presence of minor components or at least to a synergistic effect between components.

Conclusion

This study demonstrated the potential use of *M. cervina* EOs as well as their components as antibacterial agents, in particular against Gram-negative bacteria, such as *E. coli* and *A. baumannii*, providing an explanation for the reported traditional use of this plant. These results also support the hypothesis that the antibacterial activity of the *M. cervina* EOs is a function of the synergistic effect of their different aromatic monoterpene constituents. The extraction of active compounds in single or combined forms, from this plant, may lead to their use as food preservatives as well as in pharmaceutical and natural therapies for the treatment of infectious diseases. Nevertheless, further research is required to evaluate the practical value of *M. cervina* EOs applications.

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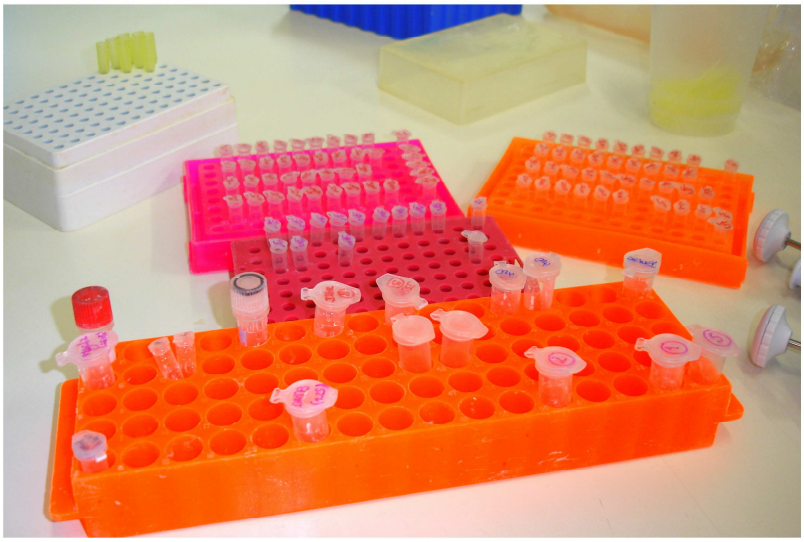

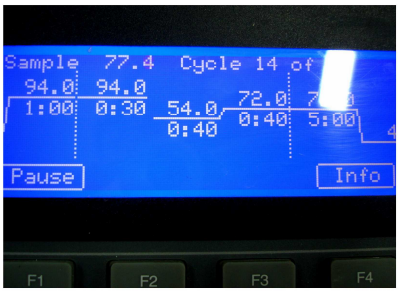

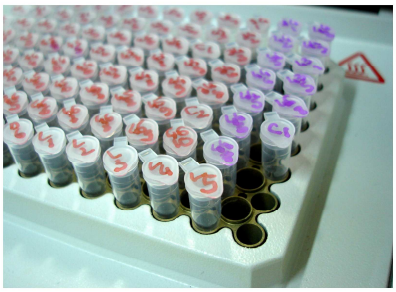

CHAPTER V

Low genetic diversity and significant structuring in the endangered *Mentha cervina* populations and its implications for conservation

Submited *(*2012a)

Low genetic diversity and significant structuring in the endangered *Mentha cervina* populations and its implications for conservation

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Abstract

Eighteen populations of the endangered aromatic and medicinal plant *Mentha cervina* (Lamiaceae) were sampled across its natural range, in the western half of the Iberian Peninsula, and inter-simple sequence repeats (ISSRs) markers were used to assess genetic diversity and population structure. *M. cervina* populations exhibited a relatively low genetic diversity (percentage of polymorphic loci *PPB* = 14.2-58.3%, Nei's genetic diversity H_e = 0.135-0.205, Shannon's information index *I* = 0.08-0.33). However, the genetic diversity at species level was relatively high (*PPB* = 98.3%; H_e= 0.325; *I* = 0.23). The results of the analysis of molecular variance indicated very structured populations, with 50% of the variance within populations, 44% among populations and 6% between regions defined by hydrographic basins, in line with the gene differentiation coefficient (G_{ST} = 0.532). A Mantel test did not find significant correlation between genetic and geographic distance matrices (r = 0.064), indicating that isolation by distance is not shaping the present genetic structure. The levels and patterns of genetic diversity in *M. cervina* populations were assumed to result largely from a combination of evolutionary history and its unique biological traits, such as breeding system, low capacity of dispersion, small effective size and habitat fragmentation. The high genetic differentiation among populations indicates the necessity of conserving the

maximum possible number of populations. The results also provide information to select sites for *ex situ* conservation. Optimal harvesting strategies, cultivation and tissue culture should also be developed as soon as possible to guarantee sustainable use of the species under study.

Keywords: *Mentha cervina*, Genetic diversity, Population structure, Conservation genetics, Endemic species, ISSR

Introduction

Long-term survival and evolution of species depend on the maintenance of sufficient genetic variation within and among populations to accommodate new selection pressures resulting from environmental changes (Barrett and Kohn, 1991). Genetic diversity maintained in a plant species can be influenced by many processes, such as the long-term evolutionary history and the characteristics of the species, such as reproductive mode and mating system (Hamrick and Godt, 1989). An accurate estimate of genetic diversity of a species can provide insights into the evolutionary processes as well as useful information for developing conservation plans.

Plants belonging to the genus *Mentha* (Lamiaceae) have evolved in nature through natural hybridization and selection, showing substantial variation in terms of their natural habitats, growth characteristics, and aromas (Tutin, 1972; Franco, 1984). In this genus, which includes many herbs that are used for medicines, cosmetics, and spices, particular attention has been given to some cultivated species, namely peppermint (*M. piperita* L.) and spearmints (*M. spicata* L. and *M. cardiaca* Baker) because of their essential oil (EO) compositions (Kokkini, 1991). The EOs from *Mentha* species have been used since ancient times for the treatment of many digestive tract diseases and in culinary (İşcan et al., 2002), and they are known to have antimicrobial properties (Flamini et al., 1999; Naigre et al., 1996). As such, mints are valuable crops with a substantial importance in the botanical economy and to the pharmaceutical industry.

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, preventing different gastric disorders and inflammations of the respiratory tract

(Monteiro et al., 2007; Póvoa et al., 2006; Rodrigues et al., 2008). It is a spreading herbaceous perennial with slender, lance-shaped, mid-green leaves and whorls of white or lilac flowers from midsummer into autumn. Although there are no reports about the breeding system of *M. cervina*, we infer that it is likely outcrossing based on the breeding features of its closely related species (Judd et al., 1999). *Mentha cervina* has a western steno-Mediterranean distribution. It is found in France, Portugal, Spain, Morocco, Algeria and is presumably extinct in Italy (Rhazi & Grillas, 2010). It occurs mainly in river banks and other damp and wet places, which require a longer flooded period that is characteristic of the priority habitat Mediterranean temporary ponds (3170) (Silva et al., 2009). According to our field survey, areas that had previously been reported in herbaria as species habitats have now been severely deteriorated or fragmented largely due to anthropogenic activities (e.g., deforestation, over-exploitation) and in one case the population had completely disappeared due to a hydroelectric dam construction (Póvoa et al., 2006). With the growth of commercial demands in recent years, the excessive harvesting from the wild (main source of plant material), overgrazing and the unfavorable conservation status of these habitats have shrunk the natural resources of *M. cervina* to a narrow distribution (Póvoa et al., 2006). Nowadays, it is considered to be decreasing in number and classified as Near Threatened in the IUCN Red List of Threatened Species (Rhazi and Grillas, 2010). A previous study (Rodrigues et al., 2008) found no chemical polymorphism in the EOs obtained in populations from different provenances, which disagrees with almost all the studies involving mints, in which the existence of different chemotypes is a common feature in most *Mentha* species and hybrids (Kokkini and Vokou, 1989). This surprising uniformity, in a species of a rather polymorphic genus, suggested a lack of variation and a need to assess genetic diversity. Up to date, previous studies of *M. cervina* have been mainly focused upon its morphology (Póvoa et al., 2006), reproductive biology (Monteiro, 2006), essential oils composition (Rodrigues et al., 2008) and their bioactivity (Gonçalves et al., 2007; Rodrigues et al., 2012), and habitat characterization (Silva et al. 2009). No study has targeted the genetic diversity and structure, although this information is essential for the formulation of effective conservation strategies in threatened species (Holsinger and Gottlieb, 1991; Escudero et al., 2003; Shah et al., 2008).

The use of molecular markers to evaluate neutral genetic variation has become an important tool to study population genetics. Inter-simple sequence repeats (ISSR) use

repeat-anchored primers to amplify DNA sequences between two inverted simple sequence repeats (SSRs or microsatellites), revealing data that reflect the length variation between the adjacent microsatellites (Culley and Wolfe, 2001; Zietkiewicz et al., 1994). Among various molecular tools, ISSRs have gained increasing interest because they have higher annealing temperature and longer primer sequences, and hence yield greater reliability and reproducibility of banding patterns when compared to RAPD (random amplified polymorphic DNA) primers (Culley and Wolfe, 2001). At the same time, the cost of the analyses is relatively lower than that of some other markers such as AFLPs, because of the much simpler protocols (Fang and Roose, 1997). Therefore, the recent use of ISSRs has been extensive in population genetics studies with wide applications in genetic diversity studies of species with conservation concerns (Esselman et al., 1999; Ge et al., 2005; McGlaughlin et al., 2002; Smith and Bateman, 2002; Xia et al., 2007), including Lamiaceae species (Liu et al., 2006, Mendes et al., 2009). ISSRs are especially useful in detecting diversity in closely related, or even clonal, individuals (Chen et al., 2006; Esselman et al., 1999; Han et al., 2007; Zietkiewicz et al., 1994).

Because of the medicinal and aromatic potential of this species and current threats for its conservation, we in the present study use ISSRs to assess levels of variation, identify the degree of genetic differentiation among populations and provide guidelines for the conservation and sustainable use of this species in the range sampled.

Materials and Methods

Plant Material

In 2009 and 2010, several field trips were conducted across the geographic range of *M. cervina*. A total of 192 individuals, which correspond to 18 populations with different geographic origins were included in the analysis (Table 1). It is important to mention that despite our efforts to collect samples from a higher number of populations, no more than 18 were found. Geographic distances between populations vary from 10 (between Mc37 and Mc38) to 487 km (between Mc33 and Mc46). Each population was evaluated by analyzing 5-16 individuals sampled randomly throughout the entire range of each

location. Due to the limited availability of individuals, sample sizes of some populations in this study were relatively small. From each sampled individual, fresh leaves were collected and dried in silica gel for subsequent DNA extraction. Vouchers for each population were deposited in the LISI Herbarium (Table 1).

DNA Extraction and ISSR-PCR Amplification

Total DNA was extracted from silica gel-dried leaves following the protocol of the plant mini kit (Quiagen), using 100-200 mg of leaf material grounded to fine powder in liquid nitrogen. The quantity of DNA extracted was evaluated by electrophoresis in 0.8% agarose gels in TBE buffer (50 mM Trisma, 50 mM boric acid, 2.5 mM EDTA, pH 8.3), and each DNA sample was diluted to 10 ng/µl for PCR amplification.

All the tested primers were synthesized by Stab Vida (Lisbon, Portugal). Twenty primers were initially screened, and 10 of them, which yielded bright and discernible bands, were used for the analysis of all 192 samples.

PCR reactions were standardized and run on a I-cycler, Bio-Rad thermalcycler. For every 20 µL reaction, 10 ng of DNA, 1× reaction buffer, 0.5 µM of primer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 2% of DMSO and 0.5 units Taq DNA polymerase were included. The amplification conditions were performed with the following program: initial denaturation at 94ºC for 4 min, followed by 40 cycles of 30s at 94ºC to denature, 45s at 48ºC to anneal the primers and 2 min at 72ºC to extend the primers. The last cycle was followed by 45s at 94ºc, 45s at 44ºC and a final extension at 72ºC for 5 min. A negative control with no DNA added was included in each PCR reaction. The amplified products were separated by electrophoresis in horizontal 1.5% agarose gels in 1× TBE buffer, at 100 volts constant for 2h. The gels were stained with ethidium bromide (0.5 µg/ml), and visualized in ultraviolet light by using GEL DOC 2000 (Bio-Rad Gel Documentation System). The size of the amplified products was determined by comparison with 100 bp ladder. To verify the repeatability of the results, each PCR amplification and gel running was repeated twice, and only the amplified ISSR fragments present in both runs where considered.

Table 1. Location of *Mentha cervina* populations and number of individuals sampled in the present study.

Populations	**sample sizes**	**Specific sample collection sites**				
		localization	Altitude (m)	Latitude	Longitude	Hidrographic basin
Mc10	9	Ouguela, Campo Maior	207	39° 4'54.96"N	7° 0'4.33"W	Guadiana
Mc29	9	Ponte da Ajuda, Elvas	166	38°46'32.02"N	7°10'29.61"W	Guadiana
Mc32	12	Vilar seco, Miranda do Douro	725	41°31'25.48"N	6°24'5.56"W	Douro
Mc33	13	Póvoa, Miranda do Douro	750	41°34'22.71"N	6°19'17.53"W	Douro
Mc34	5	Bagaúste, Peso da Régua	50	41° 9'0.41"N	7°45'2.24"W	Douro
Mc35	14	Escarigo, Figueira de Castelo Rodrigo	560	40°50'34.73"N	6°49'33.62"W	Douro
Mc36	9	Segura, Idanha-a-Nova	235	39°49'11.06"N	6°58'52.99"W	Tejo
Mc37	16	Salvaterra do Extremo, Idanha-a-Nova	253	39°53'37.50"N	6°54'18.38"W	Tejo
Mc38	12	Monfortinho, Idanha-a-Nova	255	39°59'9.96"N	6°52'50.23"W	Tejo
Mc39	9	Oledo, Idanha-a-Nova	335	39°58'10.64"N	7°18'27.85"W	Tejo
Mc40	9	Montalvão, Nisa	116	39°39'50.86"N	7°32'19.27"W	Tejo
Mc41	9	Valência de Alcântara	313	39°28'1.17"N	7°12'24.16"W	Tejo
Mc42	8	Torrão, Alcácer do Sal	50	38°17'0.32"N	8°13'57.81"W	Sado
Mc43	15	Entradas, Castro Verde	154	37°44'36.51"N	7°58'44.60"W	Guadiana
Mc44	9	La Codosera	298	39°16'48.08"N	6°52'20.89"W	Guadiana
Mc45	9	Alburquerque	234	39°11'0.69"N	7° 1'59.03"W	Guadiana
Mc46	14	Gomes Aires, Almodôvar	200	37°30'58.11"N	8°11'5.17"W	Guadiana
Mc47	11	Castro Marim	50	37°11'21.63"N	7°27'27.81"W	Guadiana

Data Analysis

Since ISSR markers are dominantly inherited, each band was assumed to represent the phenotype at a single diallelic locus (Williams et al. 1990). Consistently reproducible amplified ISSR fragments, between 300 and 1800 bp, were scored as present (1) our absent (0), each of which was treated as an independent character regardless of its intensity. Fragments of the same molecular weight were considered as the same locus.

The binary matrix (1/0), constructed with GELCOMPARII (Applied Maths), was used in parameter estimation with multiple algorithms and methodologies.

POPGENE 1.32 (Yeh et al., 1997) was used to calculate various genetic diversity parameters: percentage of polymorphic bands (PPB), expected heterozygosity (H_e), genetic diversity among populations (D_{ST}), Nei's (1973) coefficient of gene differentiation (G_{ST}) and Shannon's index of diversity (I).

Genetic diversity was also estimated as heterozygosity using the Bayesian approach of Holsinger et al. (2002), with the analysis program HICKORY version 1.1 (Holsinger and Lewis, 2003). Several runs were carried out with default sampling parameters (burn-in = 5,000, sample = 25,000 and thin 5) to ensure consistency of results and the full model was selected.

Grouping of the individuals using the Principal Coordinates Analysis (PCoA) was done with GENALEX 6 program (Peakall and Smouse 2006). The additional measurement of partitioning genetic variation was obtained with the hierarchical analysis of molecular variance (AMOVA) analysis, using GENALEX 6 program, with 9999 permutations.

To illustrate the relationship among populations, the UPGMA (Unweighted Pair Group Method with Arithmetic mean) dendrograms were generated using the software package AFLPSURV (Vekemans et al., 2002) and PHYLIP (Felsenstein, 1989) based on Nei's genetic distance. This method was implemented with bootstrapping (1000 replicates), to assess the statistical support of each branch, and then majority-rule consensus trees were generated for each type of distance, using the modules NJ and CONSENSE in the PHYLIP package. The trees were viewed and drawn using TREEVIEW program (Page, 1996).

To further understand the relationships among populations, a bayesian analysis with the software STRUCTURE (Pritchard et al., 2000) was used to reveal the number of genetic pools, assign individuals to populations and identify migrants and admixed individuals.

Several runs were carried out with default sampling parameters (burn-in = 50.000, number of MCMC runs after burn-in=500.000, using the admixture model and allele frequencies correlated) and K calculated by Evanno et al. (2005).

Geographical distances were calculated by Google Earth program. To test the correlation between Nei's genetic distance (*D*) between populations and geographic distances (in km) among populations, a Mantel (Mantel 1967) test was performed using GENALEX 6. Clonality was also tested using the multilocus genotypes analysis of the GENALEX 6 program.

Results

Genetic Diversity

Among the 20 ISSR primers tested for their ability to detect polymorphic bands (putative loci) in a subset of *M. cervina* samples, 10 primers generated interpretable polymorphic amplifications. In these 10 selected primers, the number of bands per primer (loci) varied from 14 (ISSR3) to 21 (ISSR1) with an average of 17.5 bands per primer (Table 2).

Genetic diversity estimates from ISSR are summarized in Table 3. ISSR amplification of the 192 individuals, gave a total of 175 bands that could be scored, corresponding to an average of 79.9 fragments per individual. Of these bands, 172 were polymorphic. All the primers produced polymorphic bands when all 18 populations were considered. Private bands, absent in all populations except one, were not observed. The proportion of polymorphic bands at the population level varied from 14.2% (Mc34) to 64.6% (Mc43), with a mean of 44.4%. This figure was 98.3% at the species level. Nei's gene diversity (H_e) and Shannon's information index (I) showed a similar trend to PPB. As indicated by these three indices, the least genetically diverse populations are Mc29 (H_e=0.0802, I=0.117, PPB=20.6%) and Mc34 (H_e=0.0512, I=0.076, PPB=14.2). The most diverse populations are Mc33 (H_e=0.222, I=0.332, PPB=64%) and Mc43 (H_e=0.219, I=0.328, PPB=64.6) (Table 3).

Table 2. Primers used in ISSR analysis of *Mentha cervina* and number of reproducible bands.

Primer code	Sequence[a]	Reproducible bands
ISSR1	(CA)8RG	21
ISSR3	(GA)8YT	14
ISSR4	(GA)8YC	16
ISSR5	(GA)8YG	18
ISSR6	(AG)8YT	15
ISSR7	(AG)8YC	18
ISSR8	(AC)8YA	16
ISSR10	(GT)8YC	19
ISSR15	(GACAC)3	20
ISSR898	(CA)6-RY	18
	Mean	17,5

[a]Y = C or T; R = A or G

Genetic Differentiation

According to Nei's analysis of gene diversity, the percentages of genetic variation among *M. cervina* populations were 53.2% (G_{st}) and 46% (Theta-B value, an estimate of G_{st} obtained by HICHORY analysis) which indicated elevated interpopulation genetic differentiation. The AMOVA that considered only one hierarchical level (Table 4) showed that most of the variation was found within populations (51%), the 49% among populations variation again provides additional evidence for high genetic structuring of populations. Considering two levels, when populations were grouped by hydrographic basin, the proportion of total variance residing within populations was 50%, among populations 44% and among basins 6% (P = 0.001), which indicates that there is only a small proportion of the variation associated with grouping by river basin, even though it is statistically significant (Table 4). The nearly identical Φ_{ST} from the AMOVA analysis (0.469) and the G_{ST} from the POPGENE and from HICHORY analysis provide additional support of the statistics used in this study and robustness of the results.

Table 3. Measures of genetic diversity in each population and the entire data in *Mentha cervina*. PPB, percentage of polymorphic loci (at the 5% level); I, Shanon's Information index; H_e, Nei's gene diversity.

Population	**PPB**		**I**	**He**
	Number	Percentage		
Mc10	101	57.7	0.29	0.193
Mc29	36	20.6	0.12	0.080
Mc32	102	58.3	0.31	0.208
Mc33	112	64.0	0.33	0.222
Mc34	25	14.2	0.08	0.051
Mc35	93	53.1	0.28	0.187
Mc36	57	32.6	0.18	0.124
Mc37	95	54.3	0.24	0.156
Mc38	91	52.0	0.24	0.159
Mc39	66	37.7	0.19	0.128
Mc40	47	26.9	0.14	0.097
Mc41	54	30.9	0.17	0.116
Mc42	63	36.0	0.20	0.131
Mc43	113	64.6	0.33	0.219
Mc44	64	36.6	0.18	0.117
Mc45	85	48.6	0.25	0.169
Mc46	105	60.0	0.29	0.193
Mc47	90	51.4	0.26	0.170
Mean	78	44.4	0.23	0.151
Total	172	98.3	0.23	0.325

Table 4. Analysis of molecular variance (AMOVA) of intersimple sequence repeat (ISSR) data using GENEALEX, to determine the genetic structure for different hierarchical levels of the eighteen *Mentha cervina* populations. d.f., degree of freedom; SSD, sums of squares; MSD, mean square deviations; variance component estimates; percentage variation is the distribution of variation at a given level of hierarchy (among groups/among populations/within populations) and *P* value is the significance of variance after 9999 permutations.

Source of variation	**d.f.**	**SSD**	**MSD**	**Variance component**	**Percentage**	**Θ_{ST}**	***P* value**
Mentha cervina							
Among populations	17	3104,54	182,62	15,671	49%	0,493	<0.001
Within populations	174	2807,174	16,133	16,133	51%		
Four goups: Douro, Tejo, Sado, Guadiana							
Among groups	3	752,34	250,78	1,96	6%	0,061	<0.001
Among populations	14	2352,20	168,01	14,26	44%	0,469	<0.001
Within populations	174	2807,17	16,13	16,13	50%	0,501	<0.001

Genetic Relationships

The STRUCTURE analyses pointed out that the eighteen populations of *M. cervina* in the present study share 7 genetic pools, with migrants and admixed individuals. Mc37 and Mc38 are considered to share the same genetic pool in the two analyses, which is not surprising since they are geographically very close (10 km) and share the same river basin. The populations from the north region constitute another gene pool (Mc32, Mc33, Mc34 and Mc35) and 3 of them grouped together in the UPGMA tree (Fig. 2) (although with weak bootstrap support). The populations from the center-south are from two gene pools, that were also clustered in the UPGMA tree (although only with moderate bootstrap support) and the other 3 gene pools are found in the midland with weak and arbitrary clustering (Figs.2 and 3).

The PCoA (Fig. 4) provided additional evidence for the highly structured populations, and in each population the individuals formed cohese clusters. Overall the populations were grouped in 4 main groups, and the relationships were more or less in agreement to that implied by the Bayesian analysis (STRUCTURE) and the cluster analysis. The first two components, accounted for 48.87% (axis 1 =27.15%; axis 2 = 21.71%) of the total variability. The clearest separation indicated in this analysis was of the southern populations (Mc42, Mc43, Mc44, Mc45, Mc46 and Mc47). The relationship of groups in the center and northern range presented broad overlap of populations (Mc10, Mc29, Mc32, Mc33, Mc34, Mc35, Mc39, Mc41). The spread also indicated likely admixture of gene pools in some populations, namely Mc33, Mc35, and Mc43. This is in agreement with the mixed gene pools inferred for up to 25-30% of these populations in the STRUCTURE analysis (Fig. 1). This admixture and the complex pattern of relationships which does not indicated strong geographical groups are corroborated by the Mantel test which did not find significant correlation between genetic and geographic distance matrices (r = 0.064, P <0.298, 9999 permutations), indicating that the isolation by distance is not an important mechanism for explaining the present *M. cervina* population genetic structure. Also, the analysis of clonality found no matching multilocus genotypes.

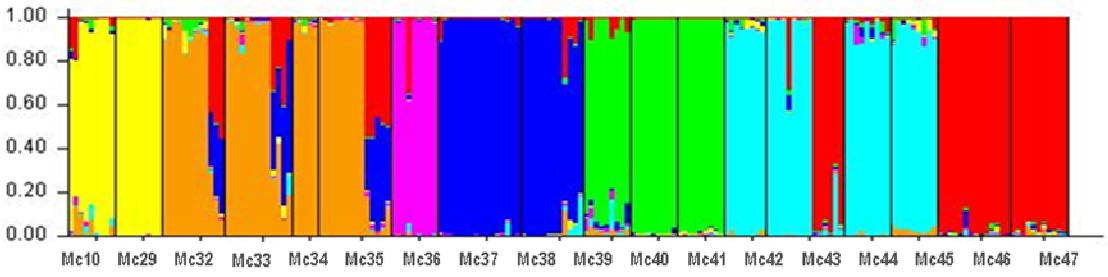

Fig. 1 Bayesian admixture proportions of *M. cervina* (K=7).

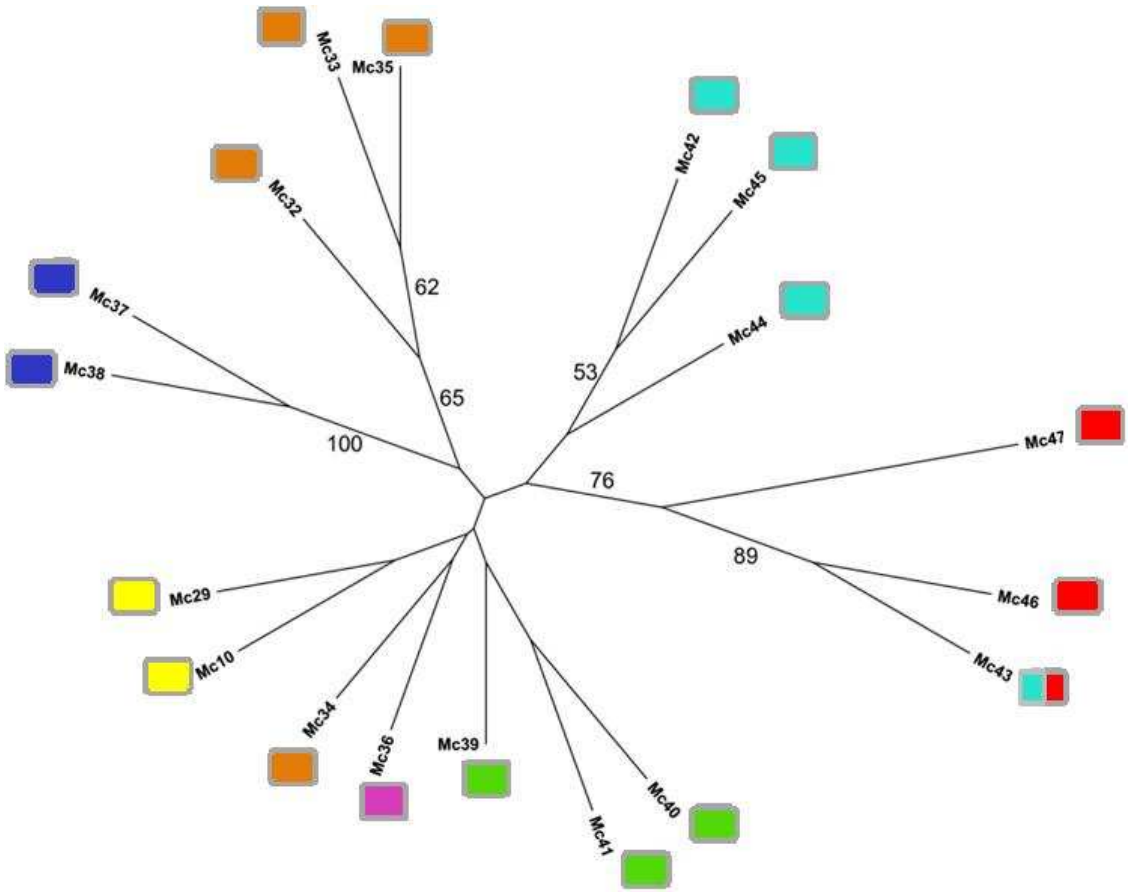

Fig. 2 UPGMA Dendrogram based on Nei's genetic distance matrix for *Mentha cervina* populations. One thousand replicates of bootstrapping analysis were used to assess the statistical support of each branch. Numbers in branchs correspond to the bootstrap analyses (50% or more). Square color blocks correspond to the structuring of populations according to the STRUCTURE analysis (fig.1). See Table 1 for population abreviations.

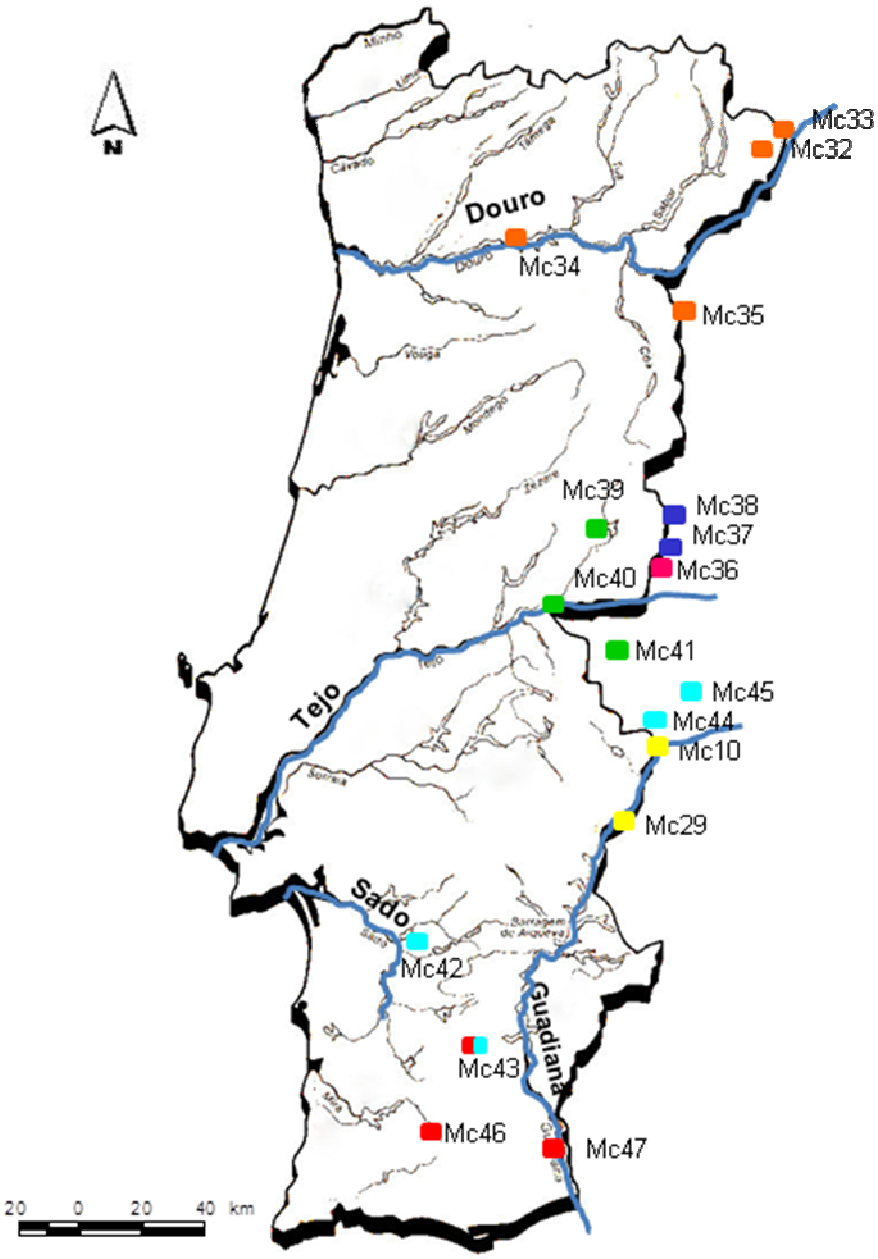

Fig. 3 Map of Portugal with the location of *Mentha cervina* populations analysed. Square color blocks correspond to the structuring of populations according to the STRUCTURE analysis. See Table 1 for population abreviations.

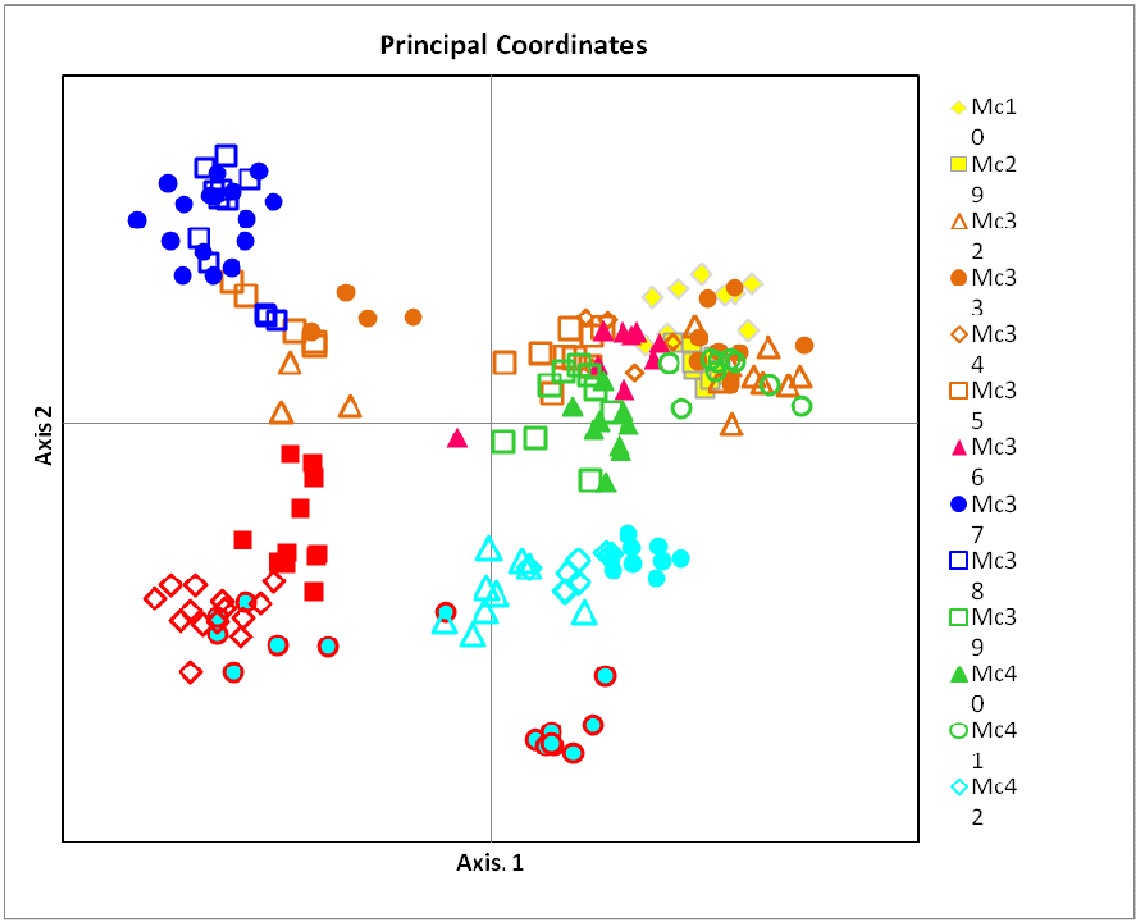

Fig. 4 Representation of the scores on the first two axes of the principal coordinate analysis (PCoA) from the matrix of genetic distances of 192 individuals from 18 populations of *M. cervina*, based on 175 ISSR loci. Percentage of variance accumulated on the first two axes = 48.87% (axis 1 = 27.15%, axis 2 = 21.71%). For population names see Table 1.

Discussion

Genetic Diversity and Differentiation

Geographic range is usually regarded as an approximate measure of the total number of individuals of a species, so we can expect species with a wider distribution to tend to have higher genetic diversity than rare and endangered species (Karron, 1987; Sheeja et al., 2009; Xiao et al., 2004;). However, many studies have shown that endangered or endemic species can also maintain high genetic diversity (Ellis et al., 2006; González-Astorga and Castillo-Campos, 2004, Luan et al., 2006; Zhang et al., 2010). *M. cervina* has a large extent of occurrence (from Italy to North Africa), but a restricted area of occupancy (approximately 600 km^2) (Rhazi and Grillas, 2010). In Portugal, the same pattern is observed, and populations can be found from north to south, but in each location the area of occupancy is also very restricted, and most of the populations are found growing near our in river banks, with very limited number of individuals.

The overall genetic diversity of *M. cervina*, based on ISSR analysis, is relatively high (H_e = 0.323; I = 0.226; PPB = 98.3%) at the species level, however, is relatively low at the population level. The PPB values ranged from 14.19 to 64.57%, averaging 44.4%, and the parameters H_e (ranging from 0.0802-0.222, with an average of 0.151) and *I* (ranging from 0.076-0.332, with an average of 0.2264) display a similar trend. It is clear from data in table 5, that the two indices of genetic diversity (PPB and H_e) have the highest value for *M. cervina* at the species level in comparison with all other species. However, at the population level, the values are lower than the genetic diversity reported for other populations of Lamiaceae species and are consistent with other populations of endangered species (Table 5 and references there in). The high overall genetic diversity displayed by the species itself can be explained mostly by differences among populations due to the high genetic structure.

Table 5. Genetic diversity measurements in *Mentha cervina*, in other Lamiaceaes and in endangered species. PPB percentage of polymorphic loci, H_e Nei's gene diversity: Numbers between () correspond to population values, and the other number to the mean value of the populations; G_{st} coefficient of gene differentiation.

	PPB		H_e		G_{st}	Methodology	Reference
	species	population	species	population			
Lamiaceae							
Mentha cervina	98.29	44.4 (14.2-64.6)	0.325	0.151 (0.08-0.222)	0.532	ISSR	Present study
Lamiophlomis rotata	96.7	51.8 (37.9-69.2)	0.291	0.291	0.430	ISSR	Liu et al. 2006
Lamiophlomis rotata	93.13	47.5 (23.6-64.4)	0.287	0.287	0.422	RAPD	Liu et al. 2006
Hemigenia exilis	97.00	72.0 (45.5-91.7)	0.27	0.39 (0.355-0.431)		RAPD	Mattner et al. 2002
Phlomis purpurea			0.085	0.085		allozymes	Aparicio et al. 2000
Phlomis composita			0.079	0.079		allozymes	Aparicio et al. 2000
Mentha pulegium		72.0 (60-90)	0.229	0.30 (0.40-0.21)		allozymes	Ben and Boussaid 2004
Endangered species							
Cycas guizhouensis	38.9	14.2 (8.9-20.5)	0.1082	0.0597 (0.036-0.088)		ISSR	Xiao et al. 2004
Camellia nitidissina	75.24	42.4	0.2302	0.098	0.575	ISSR	Bin et al. 2005
Camellia nitidissina	63.22	18.8 (11,5-24,1)	0.1561	0.083 (0.051-0.105)	0.406	ISSR	Xiao et al. 2008
Sinocalycanthus chinensis	73.08	23.65	0,1987	0.084	0.578	ISSR	Jin and Li 2007
Emmenopterys henryi	56.05	22.56	0.191	0.071 (0.053-0.105)		ISSR	Li and Jin 2008
Gynostemma pentaphyllum	96.4	8.9 (1-25.3)	0.2624	0.026 (0,004-0,084)	0.889	ISSR	Wang et al. 2008
Saruma henryi	73.7	22.8 (10.3-36.6)	0.260	0.086 (0.045-0.124)	0.690	ISSR	Zhou et al. 2010
Vellozia gigantea	88.8	56.6 (47.2-68.5)	0.256	0.183 (0.140-0.225)	0.280	ISSR	Lousada et al. 2011
Astragalus nitidiflorus	51.3	31.8 (28.2-37.2)	0.171	0.129 (0.109-0.146)	0.242	ISSR	Vicente et al. 2011

The present diversity pattern could not be explained by the isolation by- distance model, as revealed by Mantel test (r = 0.064). Although evolutionary divergence could be associated with rivers, because they are believed to be major geographical barriers that might largely hinder gene flow via seed and pollen dispersal among populations (Pfeifer and Jetschke, 2006), and also can act as dispersal routes for species that grow nearby or in the water, the AMOVA analysis revealed a weak partitioning of variation associated with the share of the river basin of populations, suggesting that each population analyzed is genetically defined and structured as a distinct genetic pool. Therefore, although highly structured, the populations of *M. cervina* are structured without a strong geographical pattern and in a more or less stochastic fashion, indicating a likely predominance of stochastic processes to shape genetic variation.

M. cervina was usually observed in severely fragmented habitats and with small population sizes (from 10 to 1000 individuals), which make this species extremely vulnerable to fluctuations in climate and habitat disturbance (Travis et al., 1996). Indeed, populations with small population size and severe human disturbance (MC29, MC34, MC36 and Mc40), showed the lowest genetic diversity, while population MC33 and MC43, with relatively large population size and limited human disturbance, showed higher genetic diversity. It seems that populations in fragmented habitats and small effective size, are more subjected to stochastic events, genetic drift and inbreeding (Hartl and Clark, 1997), leading to a low genetic diversity and the high genetic structure pattern observed.

Another effect of habitat fragmentation and other human disturbance is being reported on plant breeding systems, with an increase in self-fertilization (Aguilar et al., 2008; Eckert et al., 2009). In plants, breeding system can significantly affect genetic diversity and it's partitioning within and among populations (Hamrick and Godt, 1996; Nybom, 2004). Selfing species are expected to have reduced effective population sizes (Ingvarsson, 2002), to have lower genetic diversity within populations and a partition of diversity of about 50%, whereas outcrossing species partition on average 20% among populations (Evans et al.,2000; Hamrick and Godt, 1989; Nybom and Bartish, 2000; Tarayre and Thompson, 1996). Although there are no reports on the breeding system of *M. cervina*, it is likely an outcrossing species based on the breeding features of its closely related species (Judd et al., 1999), and so, most of the genetic diversity of *M. cervina* was expected to be partitioned within populations (Hamrick and Godt, 1996),

instead, the intra- and inter-population genetic partitioning were on average 51% and 49%, respectively.

M. cervina exhibits vigorous vegetative growth by rhizomes and an almost absent seed production (personal observations). Furthermore, seed dispersal is not likely to be very efficient in *M. cervina* because seeds are very small and light and lack any apparent dispersal structures.

Based on the ISSR data and on our field survey of habitats, the low levels of variability within populations and the high genetic structuring among populations probably resulted from 1) genetic drift and inbreeding dictated by fragmented habitat and small population sizes and 2) a low seed setting/germination/dispersion. In this context, the genetic diversity within population is mostly dependent on the first colonizing plants, and works in a very stochastic manner.

Although ISSRs markers are considered to be neutral and thus to provide no direct assessment of fitness, the diminished genetic diversity found within these populations, despite the high genetic diversity at the species level, might explain the lack of phytochemical diversity at the essential oil composition reported in Rodrigues et al. (2008).

Nevertheless, to better understand the patterns of genetic diversity and structure further studies on the quantitative genetic differentiation, species breeding system and the effects of habitat fragmentation and other human disturbance on plant diversity will be needed.

Implications for Conservation

The main goal of current conservation plans is mainly focus in maintaining species diversity, in detriment of the intraspecific genetic diversity (Margules and Pressey, 2000). Nevertheless, the intraspecific genetic diversity is the primary source of diversity and has suffered extinction rates three to eight times higher than species extinction rates (Hughes et al., 1997). In *M. cervina*, the high genetic diversity at the species level is coupled with significant structuring and very low diversity at the population level. For this reason for satisfactory conservation of genetic diversity it should be considered the intraspecific genetic diversity together with the habitat preservation in an integrative approach to species conservation.

In situ conservation is usually the preferred strategy for most wild plant species because allows populations to continue to be exposed to evolutionary processes in its natural habitat enabling the perpetuation and integration of co-adapted gene complexes, especially in producing new resistances to stresses (pests, diseases, climate changes) (Aga et al., 2005; Vinceti et al., 2004). Because *M. cervina* populations currently face the problem of conservation link to the disappearance of the species habitat (general problem of wetland conservation) and the harvest pressure on wild populations, preserving and expanding the habitat at each site to allow natural expansion of populations would be a good strategy for its conservation before populations become too small to persist naturally. According to the field survey, the construction of a hydroelectric dam has flooded the habitat of population Mc29, and so this population is already lost. Given that Mc10 is genetically more close to Mc29, the survival of this population should be assigned priority for the conservation plan.

In conservation biology genetic diversity is recognized as an important criterion to consider when prioritizing populations for protection, but conservation and management measures in a long-term perspective should ensure that the vast majority of the diversity is preserved for upcoming adaptations (i.e. the highest neutral genetic diversity that may be the future target of natural selection) (McKay and Latta, 2002), and so preserve the populations that together maximize the species genetic diversity should be applied in opposition to the traditional approach of targeting populations that are the most diverse individually. Considering the different genetic pools found it would be worthy to

prioritize populations in a matter as to represent all the genetic pools, and within these the most diverse populations. Populations Mc10, Mc33, Mc36, Mc37, Mc39 and Mc45 can be good representatives.

In order to increase the genetic diversity of *M. cervina* populations, the transfer of individuals between populations should also be considered. Nevertheless, one should take into consideration that when populations are genetically highly structured, outbreeding depression might be a potential genetic threat for already weakened populations (Sagvik et al., 2005). And so, it may be wiser to protect a network of populations that exchange genetic material and are able to reinforce each other. Given the genetic clusters found, we can suggest that only individuals within these clusters should be exchanged.

Moreover, considering that the samples collected in this study provide a snapshot of the species distribution area as a whole in Portugal, it would be also wise to preserve populations in different regions in order to limit population declines caused by large-scale environmental catastrophes and also to harbour possible local adaptative variation.

Commercial harvesting of *M. cervina* for essential oil extraction is one of the major forms of its disturbance because its composition determines that the most favorable harvesting season is before seed dispersion. To meet the commercial demand for this herb and reduce harvesting pressure in *M. cervina* wild populations, cultivation by seed and tissue culture, should be carried out as soon as possible as an alternative source of raw materials for trade.

Not only in Portugal, but also throughout its range, the populations of *M. cervina* are suffering severe and rapid declines and are therefore classified as Near Threatened in the IUCN Red List of Threatened Species (Rhazi and Grillas, 2010). The localities once known in Italy (Abruzzi) are presumed extinct. In France, it is known in six departments and is considered as vulnerable (one level upward of the Near Threatened, according to the IUCN nomenclature) and in North Africa it is considered rare (Rhazi and Grillas, 2010). Taking in to account the levels of diversity of Portuguese *M. cervina* populations, its threatened habitat status and the high harvesting pressures, it is also suggested to consider *M. cervina* as Endangered Species in Portugal in one of the forthcoming volumes of the IUCN Red List of Threatened Species.

Enlarge sampling to represent the full distribution range of *M. cervina* and complete the genetic landscape picture of this species is needed for the effective conservation management of this medicinal and aromatic species.

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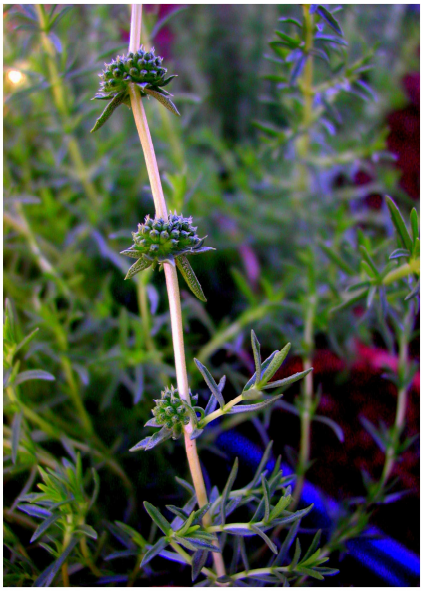
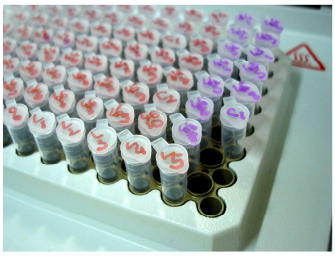
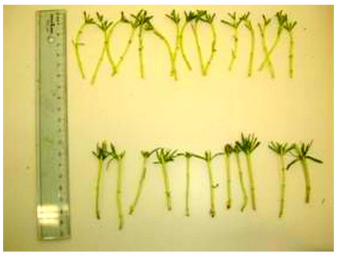
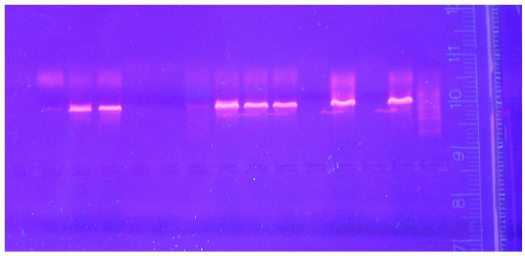

CHAPTER VI

Study of genetic diversity in *Mentha cervina* (L.) Opiz based on morphological traits, essential oils profile and ISSRs markers

Submited *(*2012b)

Study of genetic diversity in Mentha cervina (L.) Opiz based on morphological traits, Essential oils profile and ISSRs markers

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Abstract

Morphological, phytochemical and genetic differences were studied to evaluate the level and distribution of diversity in twelve populations of the Portuguese endangered medicinal plant *Mentha cervina* (L.) Opiz. Eleven morphological traits were scored for each population. Morphological variation was correlated with ecological conditions at

the site of origin. Pulegone was the major essential oils compound in all of the populations (68-83%) collected at full flowering, in different growing conditions (51-82%), and for all the developmental stages studied (47-82%). Although clusters were defined in the base of isomenthone relative amounts, the analysis revealed a high chemical correlation among all populations ($S_{corr} \geq 0.95\%$). Inter-simple sequence repeats (ISSRs) markers were used to assess the population structure and genetic variation. The *M. cervina* populations exhibited a relatively low genetic diversity (percentage of polymorphic loci *PPB* = 14.3-64.6%, Nei's genetic diversity H_e = 0.051-0.222, Shannon's information index *I* = 0.076-0.332), with high structuring between them (G_{ST} = 0.51). However, the genetic diversity at species level was relatively high (*PPB* = 97.7%; H_e= 0.320). The levels and patterns of genetic diversity in *M. cervina* populations were assumed to result largely from a combination of evolutionary history and its unique biological traits, such as breeding system, clonal growth, low capacity of dispersion and habitat fragmentation. Evidence for differentiation in morphological and genetic traits was found, but patterns of differentiation of morphological traits did not completely correlate with ISSRs differentiation. The relatively low genetic diversity in the populations analyzed indicates that the maintenance of their evolutionary potential is at risk if population sizes are maintained and if there is no protection of the habitats.

Keywords: *Mentha cervina*, Morphological traits, Essential oils, Genetic diversity, ISSRs, Conservation genetics.

Introduction

Genetic variation is fundamentally involved in the ability of a species to adapt to biotic and abiotic changes and in its evolution. Recognition of the levels and distribution of genetic variation within and among populations of a species is the base for development and selection of plant genotypes in breeding programs and increases the understanding of the historical processes underlying the genetic diversity providing information for the management and preservation of endangered and geographically restricted species (Escudero et al., 2003; Shah et al., 2008).

Plants belonging to the genus *Mentha* L. (Lamiaceae) have evolved in nature through natural hybridization and selection, showing substantial variation in terms of their

natural habitats, growth characteristics, and aromas (Tutin, 1972; Franco, 1984). They have a substantial importance in the botanical economy and to the pharmaceutical industry, mainly because of the essential oils produced and their antimicrobial properties, used since ancient times for the treatment of many digestive tract diseases and in culinary (İşcan et al., 2002).

Mentha cervina (L.) Opiz (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, preventing different gastric disorders and inflammations of the respiratory tract (Monteiro et al., 2007; Póvoa et al., 2006; Rodrigues et al., 2008). It has a western steno-Mediterranean distribution, found in France, Portugal, Spain, Morocco, Algeria and it is presumed extinct in Italy (Rhazi & Grillas, 2010). In Portugal it occurs, mainly in river banks and other damp and wet places, which require a longer flooded period that is characteristic of the priority habitat Mediterranean temporary ponds (3170) (Silva et al., 2009). The growth of commercial demands in recent years, the excessive harvesting from the wild, overgrazing and the unfavourable conservation status of their habitats, has shrunk the natural resource of *M. cervina* to a narrow distribution (Póvoa et al., 2006). Nowadays, it is considered to be decreasing in number and classified as Near Threatened in the IUCN Red List of Threatened Species (Rhazi and Grillas, 2010).

Morphological, molecular and biochemical markers are complementary in determining the genetic similarity of inter- and intra-species and the relationship between the populations (Chahal and Gosal, 2002, Kohler and Friedt, 1999). Given the well-known genus chemical variability, the essential oil composition from cultivated *M. cervina* populations cultivated (Alentejo Region, Portugal) was recently examined (Rodrigues et al., 2008). This study showed no essential oil chemical polymorphism despite the cultivated population's different provenance. A low genetic diversity associated with high differentiation among populations was also observed when *M. cervina* genetic diversity was assessed by Inter Simple Sequence Repeats (ISSRs) (Rodrigues et al., submitted 2012a).

Given the medicinal and aromatic potential of this species and its current threatened situation, the present study aims at assessing *M. cervina* genetic diversity level in Portugal based on the combination of molecular, phytochemical and morphological traits and also to provide guidelines for the conservation and sustainable use of this medicinal species.

Materials and Methods

Plant Material

A total of 12 populations of *M. cervina* with different geographic origins were included in the analysis. Geographic distances between populations vary from 9 km (between Mc32 and Mc33) to 450 km (between Mc33 and Mc43). Vouchers for each population were deposited in the LISI Herbarium (Table 1).

Morphological Study

In this study, the 12 populations of *M. cervina* were kept in the same culture conditions, in the essay field at the Elvas Agrarian School (Alentejo), Portugal (Table 1). For each population, 24 plants were employed, in three lanes 1 m apart. The soil was soft and well drained. Dripping wings for irrigation and fertilization were placed among the lanes throughout their length. The cultural operations, until harvesting, consisted of manual elimination of weeds. For each population, 15 plants (5 plants per lane) were observed. Because no morphological descriptor list was developed yet for *Mentha*, morphological variables observed were adapted from the morphological descriptor list developed for *Coriander* by Diederichsen (1996). In a first stage (2 years observation), 35 morphological variables were scored. Analyses of correlation coefficients between all pairs of morphological variables, cluster analyses and principal components analysis allowed elimination of 24 that had or low discrimination value. In total, 11 morphological variables were scored in this study (Table 2)

.

Table 1. Data on collection site and sample type of *Mentha cervina* populations studied.

Populations	Sample Type	Specific sample collection sites					Voucher
		Location	Altitude (m)	Latitude	Longitude	Hydrographic basin	
Mc10	M/G/W/C/DS	Ouguela, Campo Maior	207	39° 4'54.96"N	7°0'4.33"W	Guadiana	532/2005
Mc32	M/G/W	Vilar Seco, Miranda do Douro	725	41°31'25.48"N	6°24'5.56"W	Douro	759/2008
Mc33	M/G/W	Póvoa, Miranda do Douro	750	41°34'22.71"N	6°19'17.53"W	Douro	760/2008
Mc34	M/G/W	Bagaúste, Peso da Régua	50	41°9'0.41"N	7°45'2.24"W	Douro	761/2008
Mc35	M/G/W	Escarigo, Figueira de Castelo Rodrigo	560	40°50'34.73"N	6°49'33.62"W	Douro	762/2008
Mc36	M/G/W/C/DS	Segura, Idanha-a-Nova	235	39°49'11.06"N	6°58'52.99"W	Tejo	763/2008
Mc39	M/G/W/C/DS	Oledo, Idanha-a-Nova	335	39°58'10.64"N	7°18'27.85"W	-	764/2008
Mc41	M/G/W/C/DS	Valência de Alcântara	313	39°28'1.17"N	7°12'24.16"W	Tejo	766/2008
Mc42	M/G/W	Torrão, Alcácer do Sal	50	38°17'0.32"N	8°13'57.81"W	Sado	767/2008
Mc43	M/G/W	Entradas, Castro Verde	154	37°44'36.51"N	7°58'44.60"W	Guadiana	768/2008
Mc44	M/G/W/C/DS	La Codosera	298	39°16'48.08"N	6°52'20.89"W	Guadiana	769/2008
Mc45	M/G/W/C/DS	Alburquerque	234	39°11'0.69"N	7° 1'59.03"W	Guadiana	770/2008

Morphological study (M); Genetic study (G); and Phytochemical study from wild grown plants (W), from Cultivated *vs* wild growing conditions (C), and at Developmental stages (DS).

Table 2. Morphological variables examined.

Morphological variables	Abreviation and units
Plant height	Alt (cm)
First basal leaf length	comp1fba (cm)
Stem length	comC_tt (cm)
First inflorescence leaf length	c_foflor (cm)
Stem diameter at the plant base	diam_ba (cm)
Stem diameter at the first inflorescence	diam_inf (cm)
First basal leaf width	lrg_foba (cm)
First inflorescence leaf width	lrg_foi (cm)
Number of nodes until first inflorescence	nos_cauP
Number of flowers in the first inflorescence	n_flor
Number of flowered verticillaster at full-flowering	nv_flor

Data analysis. Morphological variables discriminant analysis was used to assess the degree of separation of the populations by multivariate measurements and to test the impact of individual variables on the discrimination (Sokal and Rohlf, 1995). The cluster analysis was performed using the unweighted pair-group arithmetic average method (UPGMA) and the euclidian distance as the similarity coefficient, in the STATISTICA software (StatSoft). In the discriminant analysis, 15 measurements were used for each population, and in the Cluster analyses the score for each character was the mean value of the 15 measurements.

Phytochemical Study

The essential oils were isolated by hydrodistillation and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) as reported by Rodrigues et al., (2008). Three populations, from the 20 populations analyzed in Rodrigues et al., (2008) in addition to other 9 populations were included in the present study. To characterize the essential oil composition and identify possible chemotypes, the 12 populations were collected, during the flowering phase, from natural habitats. In order to understand the evolution in essential oil composition and yield along the plant life cycle, and compare cultivated with wild growing conditions, a time-course study was undertaken. In this study, 6 of these populations (15 plants per

population), where 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 m^2 plots, and drip irrigated periodically (each 7–10 days). Samples from the 6 populations, in the wild and in the cultivated essay field, were harvested at the vegetative, pre-flowering and full flowering phases.

Data analysis. The essential oils composition 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) as reported by Rodrigues et al., (2008).

Genetic Study

In this study, DNA extraction and amplification of the 121 individuals (corresponding to the 12 populations) with 10 ISSRs markers, and data analysis was performed as reported by Rodrigues et al., (submitted 2012a).

To test the correlation between Nei's genetic distance (*D*), morphological distance matrix, essential oil composition distance matrix and geographic distances (in km) between populations, Mantel tests (Mantel, 1967) were performed using GenAlex 6 program (Peakall and Smouse, 2006). All matrices were transformed to zero mean and unit variance before performing Mantel tests.

Results and Discussion

Morphological study

The results of assessment between the 11 morphological variables, using statistical analyses, showed that the plant height, stem length and the number of flowers in the first inflorescence variables had maximum coefficient of variance, respectively. The stem diameter at the first inflorescence, the first basal leaf width and length had minimum diversity variance coefficient (Table 3). Discriminant analysis revealed that the three first functions represented 91% of the total variation in the data set (Table 3,

Fig. 1). The first discriminant function accounted for 70% of the total variance, and separated cluster II from the others (Fig. 1). The standardized coefficients of function 1 were highest for plant height and stem length parameters. Function 2 represented another 16% of the total variance and roughly separated cluster I and cluster III from cluster IV (Fig. 1). This function was related to the number of flowers in the basal inflorescence and stem length. Function 3 accounted for 6% of the total variation and was a number of flowered verticillaster at full-flowering and number of nodes until 1st inflorescence function.

Cluster analysis was used to investigate further the inter-relationships of these populations (Fig. 2). The clusters formed were similar to the relationships observed in the discriminant analysis. The results allowed the discrimination of the populations from the north (Cluster II) in both, the cluster and the discriminant analysis.

The multivariate taxonomic distance matrix for all traits showed no significant association with geographic distance (P = 0.25, r = 0.198).

Table 3. Summary table obtained by stepwise discriminant analysis and standardized coefficients for the first three discriminant functions based on quantitative values of morphometric plant characters of 12 *Mentha cervina* populations

	Stepwise discriminant analysis summary				**Standardized Function Coefficients**		
	N	F statistics	R^2	Wilks'lambda	Function 1	Function 2	Function 3
Plant Traits							
Alt	1	65.5	0.14	$6\ 10^{-3}$	-0.798		
comC_tt	2	39.9	0.71	$5\ 10^{-3}$	-1.094	0.552	
n_flor	3	31.2	0.20	$3\ 10^{-3}$		-0.543	
c_foflor	4	26.0	0.27	$3\ 10^{-3}$			
nv_flor	5	22.0	0.60	$3\ 10^{-3}$			0.93
nos_cauP	6	19.1	0.23	$3\ 10^{-3}$			0.569
diam_ba	7	16.9	0.11	$3\ 10^{-3}$			
lrg_foi	8	15.4	0.15	$3\ 10^{-3}$			
diam_inf	9	14.1	0.30	$3\ 10^{-3}$			0.535
comp1fba	10	12.8	0.14	$3\ 10^{-3}$	-0.209		
lrg_foba	11	11.9	0.16	$3\ 10^{-3}$			
cumulative variation					70.2	85.7	91.2

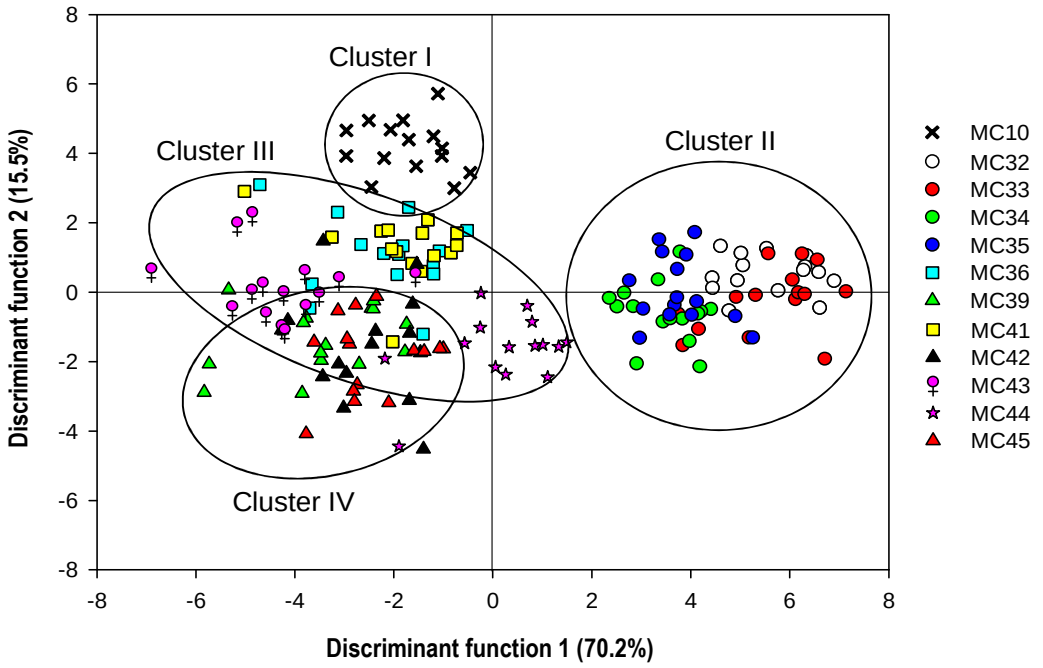

Fig. 1. Discriminant analysis based on the morphological trait in the 12 *Mentha cervina* populations. For samples grouped on each of the clusters, see Fig. 2.

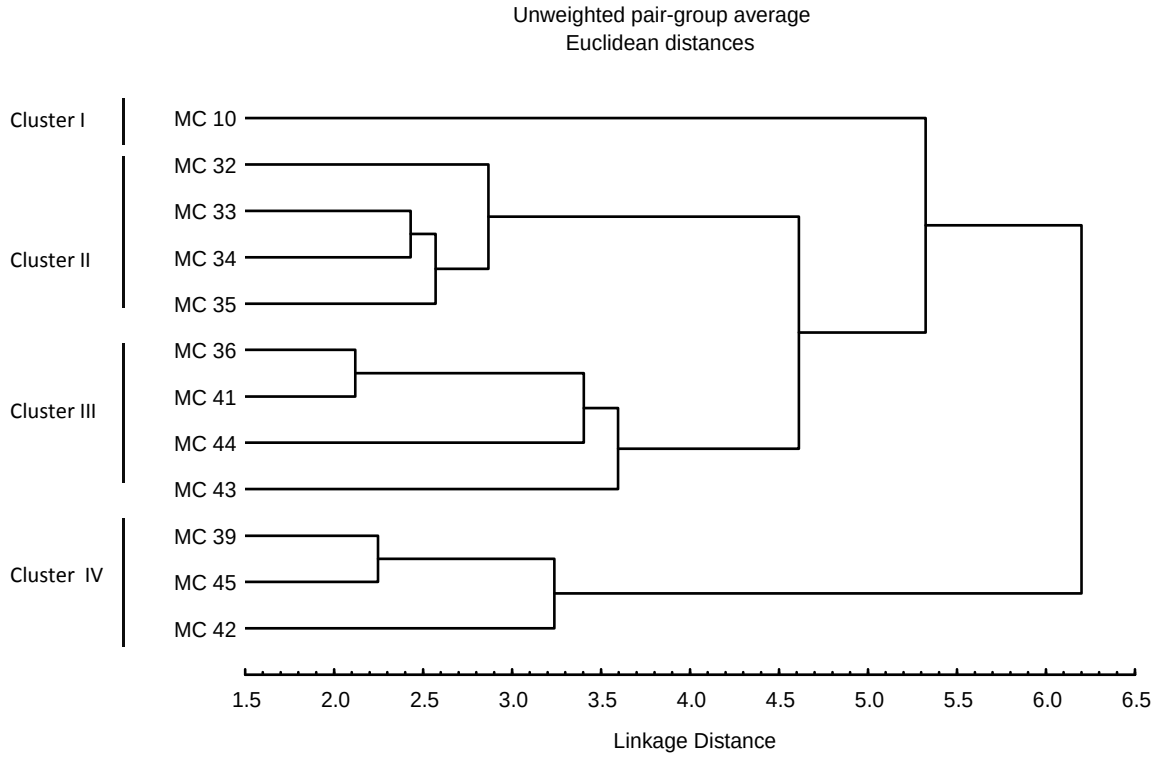

Fig. 2. Hierarchical cluster analysis dendrogram of euclidean similarity distance showing the relationship among 12 *M. cervina* populations based on average values of morphological variables.

In this study, multivariate analyses revealed a structure in which the populations from the north region (Mc32, Mc33, Mc34 and Mc35) were differentiated from those in the more central-south localities. Nevertheless, the observed trend of morphological variation seems not to be associated with the interpopulation distance. Several studies have indicated that morphological variation is apparently the result of an adaptive response to the environment. For example, variation of some traits is associated with a latitudinal and altitudinal range (Kleinschmit, 1993). In the present study, the morphological variation pattern, linked with the short plant height and stem length, suggests adaptation to the contrasting climatic conditions.

Phytochemical Study

The essential oil yield, in the 12 wild populations of *M. cervina,* collected at full flowering ranged from 0.4% to 1.6% (w/d.w.), averaging 1.0% (w/d.w) (Table 4). These values are in accordance with the reported oil yield study by Vázquez Vicente (1981) and with some reported oil yields at full flowering for wild *Mentha* (*M. pulegium* 1.2%, Hassanpouraghdam *et al*., 2011; *M. arvensis* 1.7%, *M. piperita* 1.2%,, *M. spicata* 1.2%, and *M. longifolia* 1.0%, Hussain et al., 2010). In the cultivated populations the essential oil yield at full-flowering ranged between 0.3 and 1.1% (w/w.d.), less than half the yield found (2.4% to 4.0% w/d.w.) for cultivated populations in the Alentejo region in the study by Rodrigues et al. (2008). According to Voirin et al. (2004), the oil yield is favoured with higher temperatures, water deficit and higher summer sunshine, which is the case in the Alentejo Region, but not so much in the Lisbon Region, which may explain the difference in the yields found.

Thirty three components were identified, ranging from 92-100% 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 (Fig. 3), 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 (80-97%) were dominant in all oils, Table 4. Pulegone was the major compound in all of the populations (68-83%) at full flowering, followed by isomenthone (0.1-22%), limonene (3-9%), and menthone (1-

2%). Cluster analysis (Fig. 3), confirmed a high chemical correlation among all populations (S_{corr}≥0.95%). Even though, two clusters were defined in the bases of isomenthone relative amounts. In Cluster I, which included 11 out of the 12 samples, isomenthone ranged from 0.1-15%, whereas the one sample Cluster II showed a higher percentage (22%). No correlation was detected between the clusters and the geographical collection site. Sub-cluster a has a relative amount between 0.4 and 7%, sub-cluster b between 9 and 15% and sub-cluster c (Mc34) has the lower relative amount (0.1%). No significant correlation was detected between the clusters and the geographical collection site (*P*=0.070, *r*=0.301).

Table 4. Minimum and maximum percentage range of components identified in the essential oil, isolated from the aerial parts of 12 *Mentha cervina* wild populations collected at full-flowering phase. For samples grouped on each of the clusters, see Fig. 3.

Components	**RI**	**Cluster I**					**Cluster II**
		a		**b**		**c**	
		Min	**Max**	**Min**	**Max**	**Mc34**	**Mc36**
3-Methyl cyclohexanone	914	T	t	t	t	t	t
α-Thujene	924	T	t	t	t	t	t
α-Pinene	930	0.2	0.6	0.3	0.5	0.1	0.3
Camphene	938	T	t	t	t	t	t
Sabinene	958	T	0.4	0.1	0.2	0.2	0.1
3-Octanone	961	T	t	t	t	t	t
β-Pinene	963	0.2	1.1	0.3	0.4	0.3	0.3
2,5-Dimethyl-1-hexene*	970	T	t	t	t	t	t
3-Octanol	974	0.7	1.6	1.2	1.5	1.2	1.0
β-Myrcene	975	0.4	1.1	0.8	1.0	0.8	0.7
p-Cymene	1003	T	t	t	t	t	t
1,8-Cineole	1005	T	t	t	t	t	t
Limonene	1009	2.6	6.7	2.8	4.8	8.6	3.1
cis-β-Ocimene	1017	T	t	t	t	t	t
trans-β-Ocimene	1027	T	t	t	t	t	t
γ-Terpinene	1035	T	t	t	t	t	t
n-Octanol	1045	T	t	t	t	t	t
cis-Linalol oxide	1045	T	t	t	t	t	t

Components	RI	Cluster I					Cluster II
		a		b		c	
		Min	Max	Min	Max	Mc34	Mc36
trans-Limonene oxide	1112	T	t	t	t	t	t
Menthone	1120	0.6	1.7	1.0	1.6	1.2	1.8
Isomenthone	1126	0.4	6.7	9.4	15.4	0.1	21.8
Menthofuran	1134	T	0.2	t	t	t	t
cis-Isopulegone	1134	0.5	1.6	0.3	1.1	1.0	0.9
Terpinen-4-ol	1148	T	t	t	t	t	t
Verbenone	1164	T	t	t	t	t	t
Myrtenol	1168	T	t	t	t	t	t
Pulegone	1210	78.4	83.4	71.9	75.7	73.7	68.1
Piperitone	1211	T	t	t	t	t	t
Carvotanacetone*	1222	T	t	t	t	t	t
Piperitenone	1289	0.3	3.9	0.3	0.9	9.2	0.4
β-Caryophyllene	1414	T	0.1	t	0.1	0.2	t
β-Caryophyllene oxide	1561	T	0.3	t	0.4	0.2	t
Humulene epoxide	1579	0.2	1.3	0.5	1.7	0.5	0.2
% Identification		97.4	99.5	98.0	99.3	92.1	98.9
Grouped components							
Monoterpene hydrocarbons		3.5	9.9	4.4	6.9	10.1	4.6
Oxygen-containing monoterpenes		80.3	97.4	82.9	94.6	85.2	93.0
Sesquiterpene hydrocarbons		T	0.1	t	0.1	0.2	t
Oxygen-containing sesquiterpenes		0.2	1.6	0.5	2.1	0.7	0.2
Others**		0.7	1.6	1.2	1.5	1.2	1.0
Oil Yield (v/w)		0.9	1.5	0.7	1.6	0.4	1.1

RI: Retention index relatite to C_9-C_{16} *n*-alkanes on the DB-1 column; t: traces (<0.05%). * Identification based on mass spectra only. ** Components that do not fit on the classification of terpene or phenylpropanoid and which are mainly non-aromatic alcohols, ketones and alkenes.

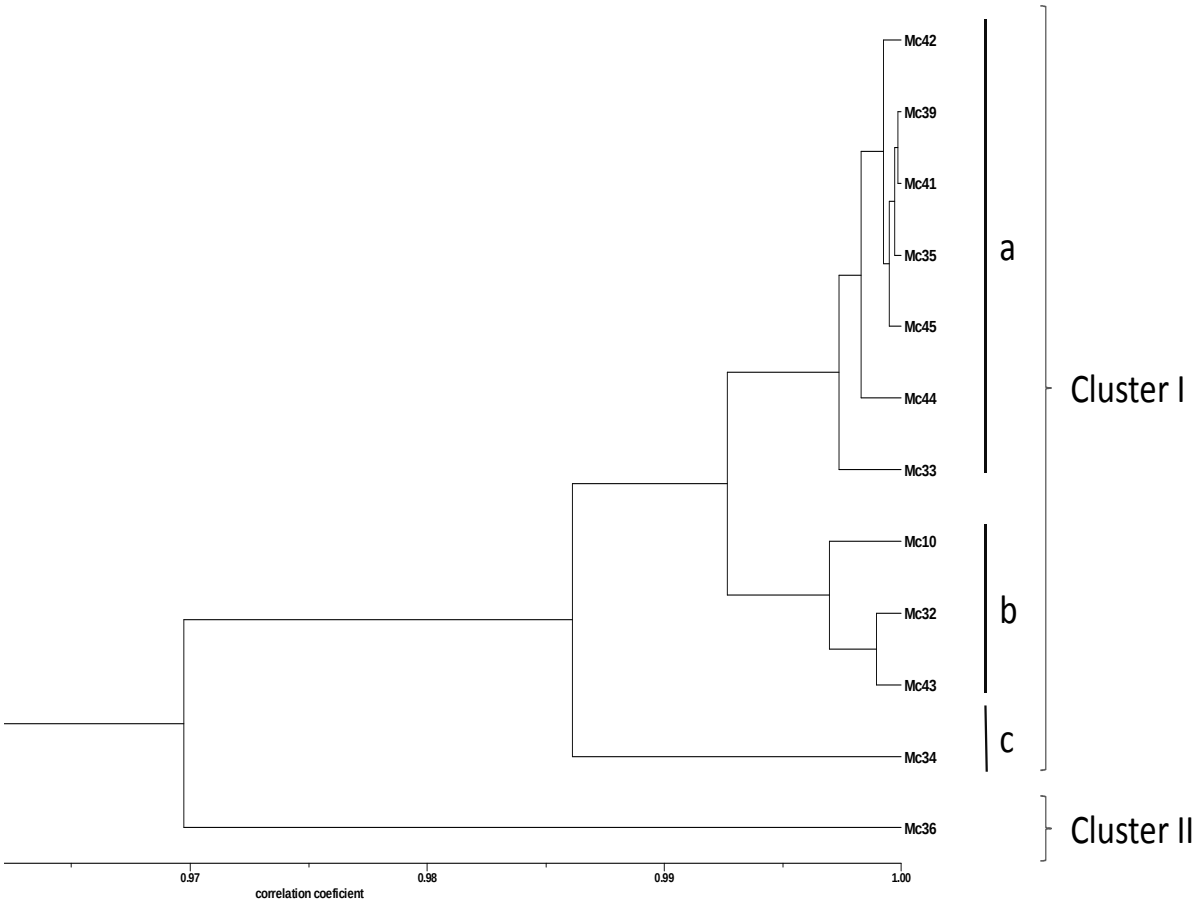

Fig. 3. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the *Mentha cervina* samples examined, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA). For abbreviations, see Table 1.

All the essential oils studied belonged to the pulegone chemotype, in accordance with the results of Rodrigues et al. (2008) on cultivated Portuguese populations collected during the flowering phase. *M. cervina* essential oils studied until now showed high uniformity, which is not usual in *Mentha* species and hybrids that are known to have different chemotypes (Kokkini and Vokou, 1989).

In plant developmental terms, the essential oil yield had a different behaviour according to the growing conditions. The yield of the essential oils isolated from wild growing populations increased rapidly from the vegetative stage (mean value 0.4% w/d.w.) until full flowering, June and July (mean value 1.1% w/d.w). In the cultivated ones, the essential oil yield increased from the vegetative until the pre-flowering phase (1.2% w/d.w) and then it started to decrease towards the flowering stage (0.7% w/d.w).

The analysis of the main essential oil 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).

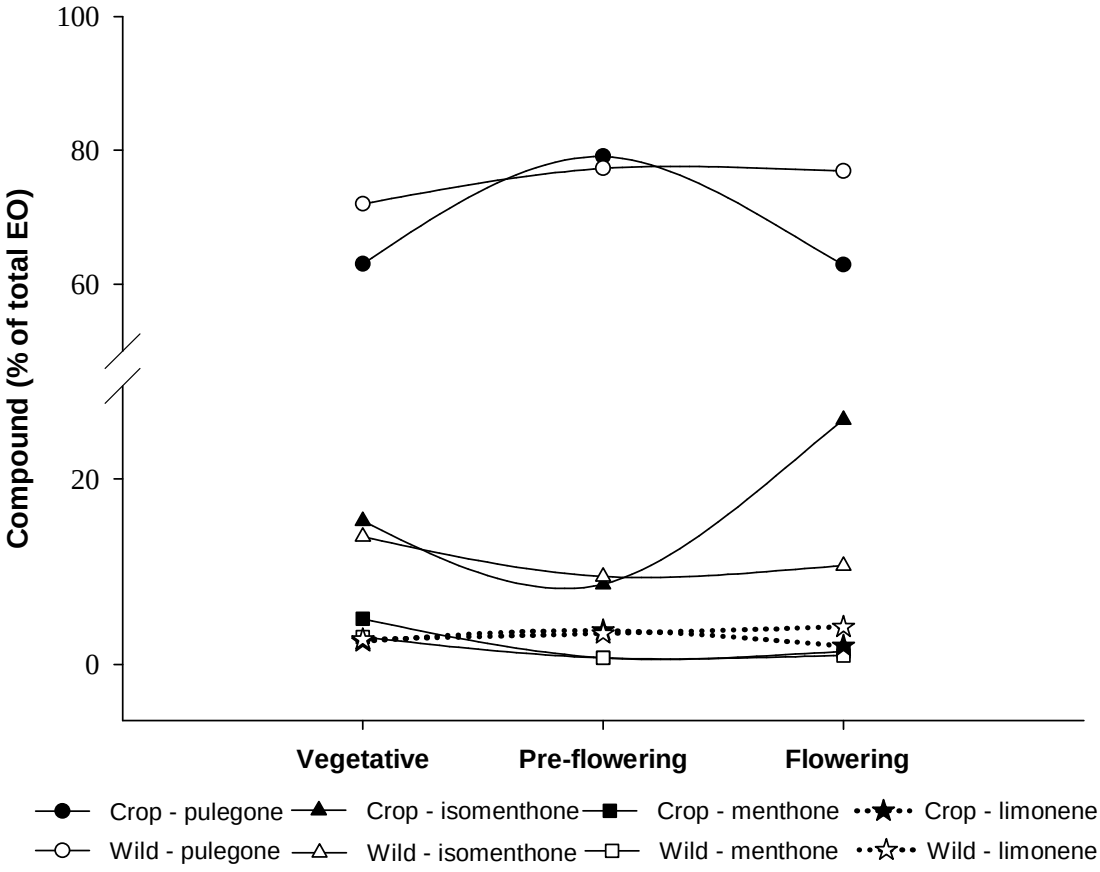

Fig. 4. Time-course study of the main components of the *Mentha cervina* essential oils isolated in wild (open symbols) and cultivated (closed symbols) growing conditions at different developmental stages. The values are the mean values from 6 populations.

In the essential oil isolated from cultivated populations, pulegone relative amount increased from the vegetative until the pre-flowering phase and then it started to decrease towards the full flowering phase. The pulegone relative amount from wild growing populations increased until the vegetative phase and then stabilized toward the end of the plant life cycle. These changes were followed by changes in the main components relative amounts, whenever the pulegone decreased, the isomenthone and menthone tended to increase (Fig. 4), in particular the isomenthone. Physiological variations (i.e. organ and leaf position), environmental conditions (i.e. harvest date and planting time), geographic variations and genetic factors and evolution are known to affect the biosynthesis of the essential oils (Figueiredo et al., 2008). Thus, these type of

variations, that where already seen in *M. pulegium* (Rodrigues et al., submitted 2012b) may be due to the influence of the developmental stage and environmental conditions on the regulation of the essential oil biosynthesis.

Molecular Study

ISSR amplification of the 121 individuals, gave a total of 175 bands that could be scored, corresponding to an average of 82.4 fragments per individual. Of these bands, 171 were polymorphic (97.7%). Genetic diversity estimates from ISSR are summarized in Table 5.

Based on ISSR analysis, the genetic diversity of *M. cervina* at the species level is relatively high (H_e = 0.323; I = 0.226; PPB = 98%), however, in contrast, relatively low genetic diversity occurred at the population level (Table 5). The proportion of polymorphic bands at the population level varied from 14 % to 65%, with a mean of 45%. The least genetically diverse population is Mc34 with 5% gene diversity and the most diverse populations is Mc43 with 22% gene diversity (Table 5). These values are lower than the genetic diversity reported for other Lamiaceae species (Aparício et al. 2000; Ben and Boussaid 2004; Liu et al. 2006; Mattner et al. 2002) and are consistent with other endangered species (Xiao et al. 2004; Bin et al. 2005; Jin and Li 2007; Li and Jin 2008; Wang et al. 2008; Vicente et al. 2011; Zhou et al. 2010).

According to Nei's analysis of gene diversity, the percentages of genetic variation among *M. cervina* populations were 51% (G_{st}) which indicated a high inter-population genetic differentiation. The AMOVA showed that most of the variation was found within populations (52%) provided additional evidence for the genetic structuring of populations. The nearly Φ_{ST} from the AMOVA analysis (0.478) and the G_{st} from the POPGENE software analysis (Yeh et al. 1997) provide additional support for the robustness of ISSR markers used in this study.

The high level of genetic differentiation (G_{st} = 0.532) detected among *M. cervina* populations suggest that each population analyzed is genetically defined and structured as a distinct genetic pool. Also, the values of heterozygosity found for the fragments analyzed (0.051- 0.222) are lower than the average outcrossing-animal species (H_e = 0.260), and for some of the populations also lower than self-pollinating plants (H_e= 0.091), using RAPD markers (Nybom and Bartish 2000), which indicates

inbreeding within *M. cervina* populations. The reason behind this low heterozogosity may be partly attributed to the clonal growth of this species and the low seed setting and dispersion (Rodrigues et al, submitted 2012a). Clonal growth can significantly decrease the effective population size, and hence contribute to the loss of genetic diversity and the genetic differentiation via increased levels of genetic drift and inbreeding (Erickson and Hamrick 2003). Also, *M. cervina* was usually observed in severely fragmented habitats and with small population sizes (from 10 to 1000 individuals), which make this species extremely vulnerable to stochastic events, genetic drift and inbreeding (Hartl and Clark 1997), leading to a low genetic diversity and the high genetic structure pattern observed.

Table 5. Measures of genetic diversity in each population and the entire data in *Mentha cervina*. PPB, percentage of polymorphic loci; I, Shanon's Information index; H_e, Nei's gene diversity.

Population	PPB		I	He
	Number	Percentage		
Mc10	101	57.7	0.290	0.193
Mc32	102	58.3	0.310	0.208
Mc33	112	64.0	0.332	0.222
Mc34	25	14.3	0.076	0.051
Mc35	93	53.1	0.278	0.187
Mc36	57	32.6	0.181	0.124
Mc39	66	37.7	0.191	0.128
Mc41	54	30.9	0.170	0.116
Mc42	63	36.0	0.195	0.131
Mc43	113	64.6	0.328	0.219
Mc44	64	36.6	0.178	0.117
Mc45	85	48.6	0.253	0.169
Mean	78	44.5	0.232	0.155
Total	171	97.7		0.320

To further understand the relationships among populations, a bayesian analysis with the

software STRUCTURE (Pritchard et al. 2000) was used to reveal the number of genetic pools and a cluster analysis (UPGMA) was also used to generate an un-rooted tree based on Nei's genetic distances (Fig.5). The STRUCTURE analyses revealed that the twelve populations of *M. cervina* in the present study share 5 genetic pools, with migrants and admixed individuals. The populations from the north region constitute one gene pool (Mc32, Mc33, Mc34 and Mc35) and grouped together in the UPGMA tree (despite the week bootstrap support). The two populations from the south share the gene pool with two populations from the centre-south and were also clustered in the UPGMA tree (with moderate bootstrap support) and the other 3 gene pools are encountered in the midland with week and arbitrary clustering (Fig. 5).

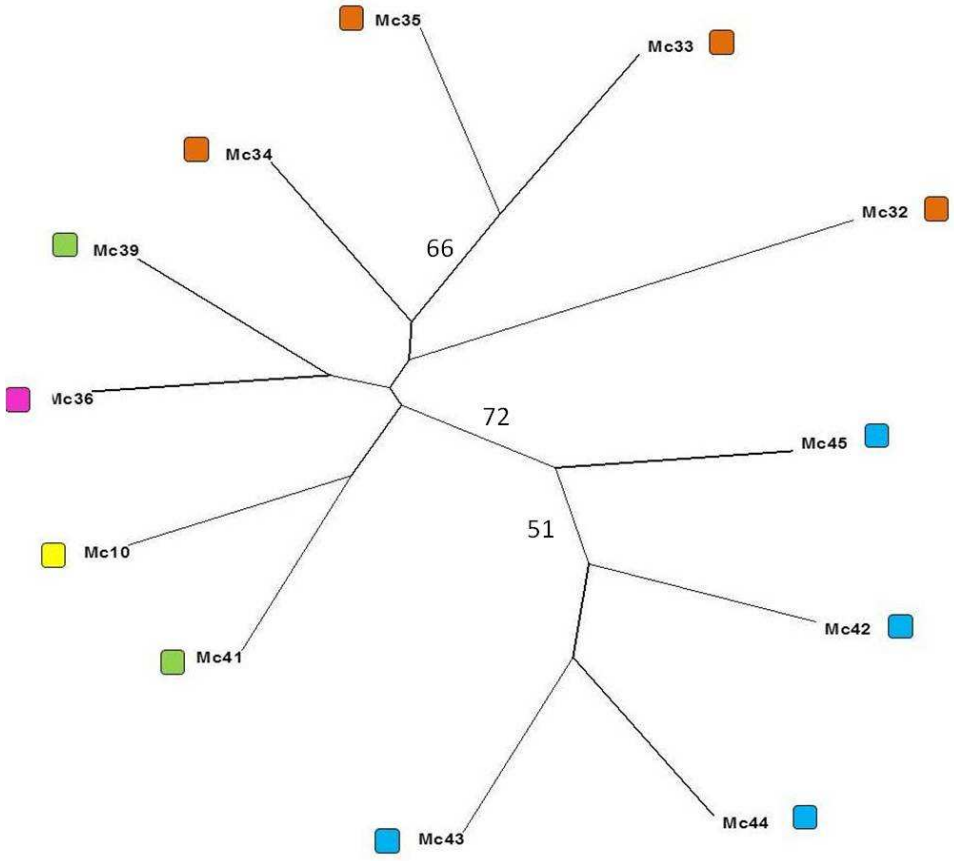

Fig. 5. UPGMA Dendrogram based on Nei's genetic distance matrix for *Mentha cervina* populations. One thousand replicates of bootstrapping analysis were used to assess the statistical support of each branch. Numbers in branches correspond to the bootstrap analyses (50% or more). Square colour blocks represent the different gene pools according to the STRUCTURE analysis. See Table 1 for population abbreviations.

On the other hand, Mantel test did not find significant correlation between genetic and geographic distance matrices (r = 0.031, P <0.5, 999 permutations), indicating that the isolation by distance is not shaping the present *M. cervina* population genetic structure.

Correlation between morphological, phytochemical and molecular traits

The obtained results showed that four populations (Mc32, Mc33, Mc34 and Mc35), out of 12, grouped together, in both the morphological and molecular studies, whereas the essential oils correlation evaluation grouped the populations in an entirely different way (Figs. 2, 3 and 5). Several reasons can determine this different grouping:

ISSRs are considered to be neutral and thus to provide no direct assessment of fitness. The forces that cause differentiation for these markers would be the result of mutation, genetic drift, low gene flow and no selection. Conversely, morphological traits and phytochemical profiles are generally believed to be subject to natural selection, and their expression is partially under the influence of environmental factors (Bruschi et al., 2003). Adding to this, the ISSRs are random markers that show differences in the whole genome, and are not necessarily related to a specific morphological trait or secondary compound. However, previous studies on *Salvia fruticosa* (Skoula et al., 1999), *Ocimum gratissimum* (Vieira et al., 2001) *Tanacetum vulgare* (Keskitalo et al., 2001), *Primula ovalifolia* (Nan et al., 2003) and *Vitex rotundifolia* (Hu et al., 2007) reported that the patterns of relatedness observed in chemical profiles seemed to correspond well with the genetic profiles generated by RAPDs and ISSRs. On the other hand, there are also studies, where no correlation could be found among collection site, chemical and molecular analysis (Trindade et al., 2008 in *Thymus caespititius*). The same pattern of correlations can be observed for the morphological traits. Liu et al. (2007) and Hamza et al. (2004) demonstrated a clear correlation between the morphological traits and the detection of the genetic variability as revealed by RAPD analysis. Conversely, Schut et al. (1997) and Eshraghi et al. (2006) reported a few correlations between the molecular and the morphological traits. Together these studies, suggests that may be there is a genetic basis for the chemical profiles and morphological traits that can be observed with the ISSRs markers, although they are not clear.

At last, although essential oils may evolve more rapidly than morphological traits, the rather unusual uniformity found in the essential oils composition in populations with

different geographic provenances, at different developmental stages and in different growing conditions, may explain why the morphological traits were more correlated with the genetic variation, than the phytochemical ones.

From a conservation perspective, the low genetic and phytochemical diversity observed, within the populations tested is symptomatic and a signal that ecological management of *M. cervina* habitats is necessary to prevent the consequent decline in population size that could increase the risk of extinction due to demographic and environmental stochasticity.

Acknowledgements

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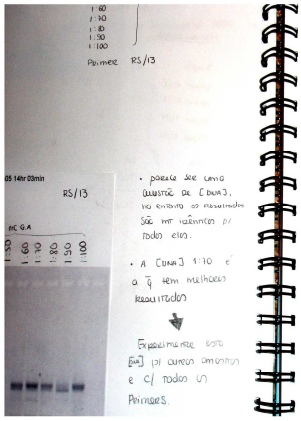
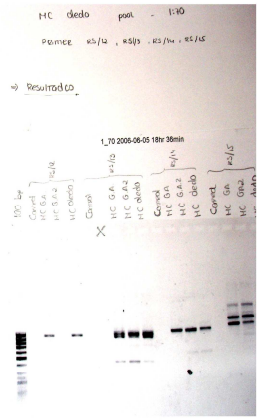
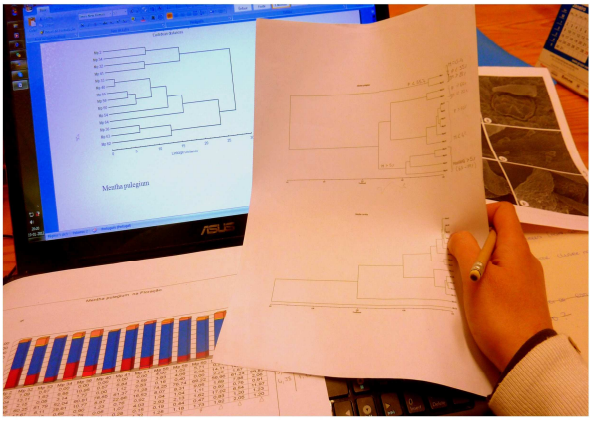
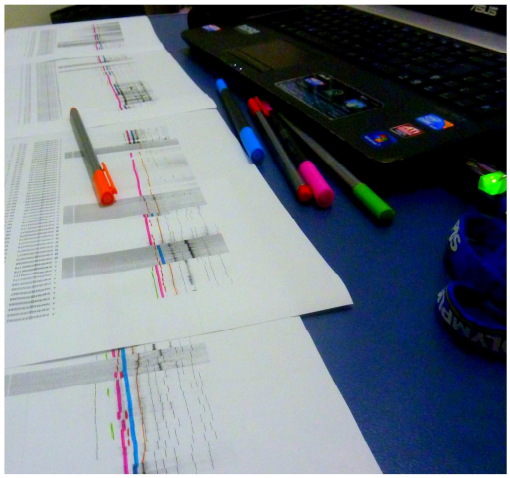


CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

General Discussion and Conclusions

In mainland Portugal, namely in Alentejo, the aerial parts of *Mentha cervina* (L.) Opiz and *Mentha pulegium* L. and the preparations derived from them, have been traditionally used for seasoning of typical recipes, as well as for its medicinal properties (Póvoa *et al*., 2006). The recent interest in these plants has lead to their cultivation and commercialization either as a living plant (potted plants) or as dried material (aerial parts). Nevertheless, their use is still based primarily on plants collected from the wild. Both species are found in the Mediterranean area, typically in wetlands, damp areas and water banks, yet they have a quite different story to tell. *M. pulegium* grows wild throughout Portugal and studies on its morphology and on the composition of their essential oils where already reported from other parts of the world. These studies have made clear that there is essential oil diversity (chemotypes) in this species and also that there are several factors affecting the quality and composition of the essential oil. *M. cervina*, on the other hand, was barely unknown, not just because it is difficult to find growing spontaneously in the natural habitat, but also, because there are few studies about the species. The use and commercialization of both species requires a better knowledge on its essential oil composition and diversity as well as of the genetic diversity to allow better conservation and management of these vulnerable resources.

The results obtained with the present work contributed to a more complete knowledge of secondary metabolites diversity, with particular focus on the essential oil components, their evolution along the lyfe cicle and of the secretory structures involved in their production, in these two species. And also, of the genetic diversity and structure of *Mentha cervina* populations.

Micromorphology of the Essential Oil Secretory Structures

The glandular trichomes of *Mentha cervina* and *Mentha pulegium* belong to two morphologically different types, peltate and capitate, which are similar to the two main types occurring in the *Lamiaceae* family (Werker *et al*., 1993). For many *Lamiaceae* species, the head of the peltate trichomes consists of two more-or-less distinct circles of cells, four in the middle, and a variable number of cells surrounding them. According to Turner *et al*. (2000a) 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. In both species, two circles of cells was the most common arrangement, usually 4 in the inner circle and 4-8 in the outer. This number can reach up to 16 cells in the petals peltate trichomes, usually 4 in the inner circle and 8-12 in the outer.

Estimates of overall peltate gland densities showed a distribution, with the greatest abundance on the abaxial surface, of about twice the number of peltate glandular trichomes, pattern reported for several *Lamiaceae* species (Ascensão *et al*., 1995, 1998; Gavalas *et al*., 1998; Martins, 2002). Nevertheless, the densities were the lowest compared to other results in mints (Turner *et al*., 2000a,b; Martins, 2002), even though they are species with a very strong aroma.

The peltate trichomes are seen all over both leaf surfaces, although dominant on the abaxial surface, on the stem and on the reproductive organs. The presence on the stamens, between the two anther lobes, was a noteworthy finding for both species, although the presence was already reported for other species of *Lamiaceae* (Ascensão *et al*., 1995). Morphologically well developed peltate glandular trichomes, were also observed on cotyledons. 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 growth and dependent of the organ in which it is present. The head dimensions of peltate trichomes are variable, but bigger on the reproductive structures, followed for the abaxial and adaxial leaf surface.

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*. (1985).

Phytochemical Diversity of essential oils in *Mentha cervina* and *Mentha pulegium*

The average essential oil yield, in the wild populations, at the flowering stage was 1.1% w/d.w for *M. pulegium* and 1.0% for *M. cervina*. These values are 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). For both species, the populations under cultivation showed an oil yield, in general, higher than the wild ones. Whereas *M. cervina* cultivated populations from the Alentejo region showed almost twice the oil yield (2.4% to 4.0% w/d.w.), the difference was not so pronounced in the oil yield from populations cultivated in Lisbon (0.3-1.1% w/w.d.). According to Voirin *et al.* (2004), the oil yield is favoured with higher temperatures, water deficit and higher summer sunshine, which is the case in the Alentejo Region, but not so much in the Lisbon Region, which may explain the difference in the yields found.

No chemical polymorphism was found in the essential oils obtained from populations neither with different provenances, in cultivated our in wild growing conditions. The same pattern was observed for the different developmental stages, for both growing wild or cultivated conditions. The oils studied belonged to the pulegone chemotype, the most abundant chemotype in *M. pulegium* and the unique chemotype reported for *M. cervina* (Vidaurreta *et al*., 1992; Gonçalves *et al.*, 2007). The uniformity found in the essential oil contents is unexpected, since almost all the studies involving mints reported the existence of different chemotypes for most of the *Mentha* species and hybrids (Kokkini & Vokou, 1989), including *M. pulegium*.

Our results showed that cultivation, in both species, only seems to affect the essential oil yield, increasing its content, not affecting the essential oil composition that seems to be very stable and uniform in both mints. These are features that turn these species into interesting products for cultivation and commercialization.

Bioactivity of *Mentha cervina* Essential Oils

Although a previous study addressed *M. cervina* essential oil antifungal activity against *Candida*, *Aspergillus* and dermatophyte strains by Gonçalves *et al.* (2007) with relative success, no study had targeted the antibacterial activity. Viewing to test the potential antibacterial use of *M. cervina* essential oils, in the present studies assays were conducted to validate the traditional uses of this plant in folk medicine, for treatment of different gastric disorders and inflammations of the respiratory tract.

The antibacterial activity of three essential oils profiles, differing in the main components relative amounts, was compared to that of the pure standards, using 23 bacterial strains, including multiresistant strains, some of them responsible for digestive and respiratory human diseases. The most effective antibacterial activity was against the

Gram-negative bacteria, *Escherichia coli* and *Acinetobacter baumanni*, with MIC values of 1 mg/mL, providing an explanation for the reported traditional use of this plant. The essential oils complex mixtures were more active than the individual pure standards supporting the hypothesis that the essential oils antibacterial activity is a function of the synergistic effect of their different monoterpene constituents.

The extraction of active compounds in single or combined forms, from this plant, may lead to their use as food preservatives as well as in pharmaceutical and natural therapies for the treatment of infectious diseases.

Genetic Diversity in *Mentha cervina* and Consequences for Conservation

The growth of commercial demands in recent years, the excessive harvesting from the wild, overgrazing and the unfavourable conservation status of the habitats has shrunk the natural resource of *M. cervina* to a narrow distribution (Póvoa *et al*. 2006). Adding to this, the chemical uniformity of the essential oils found (Rodrigues *et al*., 2008), suggested a lack of genetic variation. The use of molecular markers (ISSRs) in the present studies allowed the assessment of levels of variation and differentiation among populations of *M. cervina*.

At the species level the genetic diversity was high; however *M. cervina* exhibited a relatively low genetic diversity at population level. The greater part of the variability was distributed within populations (50%) and among populations (44%), with a weak partitioning associated with the share river basin. In addition, there was no correlation between genetic and geographic distance indicating that the isolation by distance is not shaping the present genetic structure. These results indicate that the current threats (over-harvesting, livestock grazing and habitat fragmentation and loss) have significantly affected this species in a short term. Given that the distances between populations are not large, the levels and patterns of genetic diversity within and among populations of *M. cervina* were assumed to result from a combination of evolutionary history and its unique biological traits, such as clonal propagation, low capacity of seed setting/dispersion and genetic drift and inbreeding dictated by fragmented habitat and small population sizes. In this context, the genetic diversity within population is mostly dependent on the first colonizing plants, and works in a very stochastic manner.

Because *M. cervina* currently retains a low level of genetic variation among individuals within populations, preserving and expanding the habitat at each site to allow natural expansion of populations would be a good strategy for its *in situ* conservation. The over-exploitation of natural populations and the extensive loss of habitats have seriously threatened the survival of populations Mc10 and Mc29, which should be assigned priority for the conservation plan. Also, the transfer of germplasm between populations to increase the genetic diversity and the adaptation capacity is considered. Taking into account the different genetic pools found it would be worthy to prioritize populations in a matter as to represent all the genetic pools, and within these the most diverse populations. Populations Mc10, Mc33, Mc36, Mc37, Mc39 and Mc45 can be good representatives.

Cultivation and tissue culture should be promoted as an alternative source of raw materials, so as to meet the commercial demand for this aromatic plant and reduce harvesting pressure in *M. cervina* wild populations.

The inclusion of *M. cervina* in a forthcoming volume of the IUCN Red List of Threatened Species is also advised, given its threatened status and high harvesting pressures.

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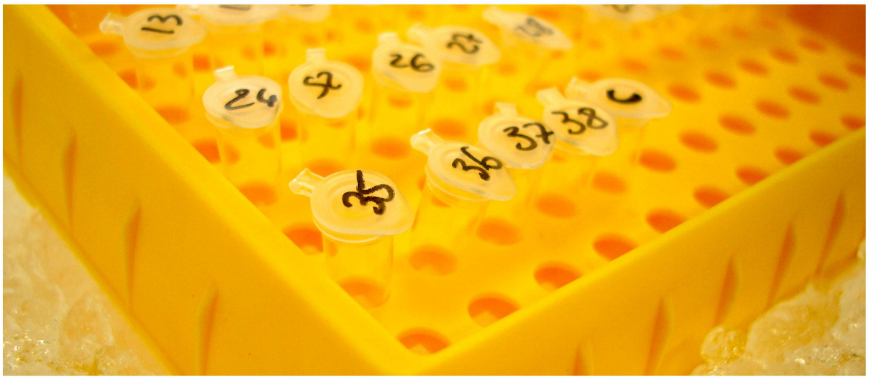

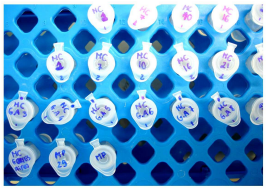

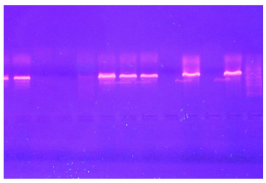

CHAPTER VIII

FUTURE PERSPECTIVES

Future Perspectives

Population Genetic Studies in the *Mentha* Genus

The assessment of genetic diversity may be investigated using a variety of different DNA technologies, that vary in the way that they resolve genetic differences, in the type of data they generate and in the taxonomic levels at which they can be most appropriately applied. Low assay cost, affordable hardware, throughput, convenience and ease of assay development and automation are important factors when choosing a technology. The ISSRs markers were used in this PhD as a tool for assessment of genetic diversity, essentially because of its simplicity, low cost, rapid, use of arbitrary primers, no need of initial genetic or genomic information, and the requirement of only tiny quantities of target DNA. Although the ISSRs are undoubtedly valuable for addressing population genetics and plant breeding issues (Arif *et al*., 2010), they present some disadvantages; are dominant type and they lack of a prior knowledge on the identity of the amplification products which creates problems with reproducibility and co-migration (Lowe *et al*., 1996). With that in mind, microsatellite markers for *M. cervina* were developed, as they are another powerful technique for studying diversity. Microsatellite markers are hypervariable regions of the genome comprised of tandem repeated simple sequences. These repeats vary in number (and, hence, length) and are, therefore, generally called VNTRs (variable number of tandem repeats), although the terms microsatellite (or simple sequence repeat, SSR) are used where the basic repeat unit is around two to six base pairs in length (Queller *et al*, 1993). The great advantage of microsatellite analysis is the large number of polymorphisms that the method reveals. The ability of the method to differentiate individuals when a combination of loci is examined makes the technique very useful for gene-flow experiments, cultivar identification and paternity analyses (Hokanson *et al*., 1998) and has recently been used to establish conservation strategies of endangered plants like *Calystegia soldanella* (Noda *et al*., 2009), *Tricyrtis ishiiana* (Setoguchi *et al*., 2009) and *Galium catalinense* ssp. *acrispum* (Mcglaughlin *et al*., 2009). Microsatellites have proved to be versatile molecular markers, particularly for population analysis, but they are not without limitations, their major problem being the initial screening of an organism for microsatellite library creation, that can be tedious and a costly process (Arif *et al*., 2010). In herewith reported PhD studies an inter simple

sequence repeat (ISSR)-based technique (Provan & Wilson, 2006) and an enrichment procedure based on Kandpal *et al*. (1994) were used to develop microsatellite markers for *M. cervina.* From these libraries, 500 clones were sequenced out of which 56 contained microsatellite sequences, with the possibility to design flanking primers to amplify the repeated regions. Because this is a time consuming technique that was developed in the end of the PhD study, further work is needed to test the amplification and polymorphism of the sequences amplified by the primers designed. Also, the cross-species amplification needs to be tested for the other species of the genus *Mentha*, including *M. pulegium*, because microsatellites are developed for particular species and the percentage of loci that successfully amplify may decrease with increasing genetic distance in closely related species (Jarne & Lagoda, 1996). Further work should be pursued to use these markers to perform population genetic studies in this genus, by assessing the diversity patterns, detecting interspecific hybridization, and understanding population structure.

Reproductive Mode in *Mentha cervina*

To better understand the genetic structure and diversity of *M. cervina* populations and perform effective conservation of the species, it is crucial to recognize how their mode of reproduction is involved in the generation of diversity.

Taxonomy of the *Mentha* Genus

The characterization of mints is often problematic because *Mentha* is a taxonomically complex genus. Several classifications varying in the number of recognized species have been proposed in the past (Harley 1972; Harley & Brighton 1977; Rosch *et al.*, 2002; Tucker & Naczi, 2007). Adding to these, the systematics of section *Mentha* is especially difficult because of frequent hybridization occurring both in wild populations and in cultivation (Harley & Brighton, 1977), complicated by concomitant polyploidy and stabilization of novel forms by vegetative propagation (Tucker *et al*., 1980). Within section *Mentha* it has been suggested that the five basic species *M. arvensis* L., *M. aquatica* L., *M. spicata* L., *M. longifolia* (L.) Huds, and *M. suaveolens* Ehrh. have given rise to 11 naturally occurring and named hybrids (Tucker & Naczi, 2007). However, *M. spicata* and possibly *M. longifolia* are also of hybrid origin and incongruence of nuclear

and plastid DNA based phylogenies indicated that all species of this section may have experienced some extend of reticulate gene flow during their evolution (Gobert *et al*., 2006). Despite intensive morphological, molecular, and cytological studies, the phylogenetic relationships within the genus *Mentha* remain unresolved. The integrated approaches, such as the phytochemical diversity and population genetic studies applied to the genus *Menha*, combined with phylogenetics and biogeography may be used to address this question in the future.

Use of Essential Oils in the Control of Pests and Diseases in Agriculture and Forest

For several years, farmers and scientists have relied on synthetic chemicals to prevent, control, or eradicate menaces, such as insects, plant diseases, and weeds that incur in substantial yield losses. Although effective, their continued or repeated applications may lead to the development of resistant pathogens, toxicity to non-target organisms and environmental problems. The decrease in efficacy and the enhanced concern on the adverse effects on the environment and on the human health caused by synthetic chemicals triggered the development of alternative methods for pest and disease control in agriculture and forest. Plant essential oils may provide potential alternatives to currently used synthetic chemicals, since they are not only considered natural, safe and biodegradable, but also have been reported to have bioactivities such as antifungal, insecticidal or nematicidal.

M. cervina essential oils have already proven to have to some extent, antifungal (Gonçalves *et al*., 2008) and antibacterial (Rodrigues *et al*., 2011) activity against human pathogens, and *M. pulegium* essential oils also showed antibacterial activity (Mahboubi & Haghi, 2008). As future perspectives, it seems interesting to explore the potential economic use of the essential oils by evaluating their biological activity for pests and diseases control in agriculture and forest, which may be used in phytopharmaceutical and biopesticides industries.

M. pulegium essential oils were screened for their nematicidal activity against the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Barbosa *et al*., 2010), and *M. cervina* essential oils were also assessed against the same nematode (Faria *et al*., 2011). *M. cervina* essential oils were also evaluated as insecticide against ants (Belchior, 2009) and as acaricide against *Varroa destructor*, (Silva, 2010) an external parasitic mite that

attacks honey bees. Nevertheless, further research is required to evaluate the practical value of both species essential oils and forms of application. The bioactivity evaluation as fungicide for crop diseases protection, herbicide in weed control, insecticide against pests that often cause extensive loss in crops and stored food grains and as antibacterial and antifungal in food preservatives are also lines of possible future investigations.

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Apendix I – Scientific Outputs

Scientific Outputs

Pappers in International Publications with Referee

Rodrigues L, Póvoa O, Teixeira G, Figueiredo AC, Moldão M, & Monteiro A (2013). Trichomes micromorphology and essential oil variation at different developmental stages of cultivated and wild growing *Mentha pulegium* L. populations from Portugal. Industrial Crops and Products 43: 692–700

Rodrigues L, Duarte A, Figueiredo AC, Brito L, Teixeira G, Moldão M & Monteiro A (2012) Chemical composition and antibacterial activity of the essential oils from the medicinal plant *Mentha cervina* L. grown in Portugal. Medicinal Chemical research 21: 3485-3490

Rodrigues L, Monteiro P, Póvoa O, Teixeira G, Moldão M, Figueiredo A & Monteiro A (2008a) Morphology of secretory structures and essential oil composition in *Mentha cervina* L. from Portugal. Flavour Fragr. J. 23: 340-347.

Oral Comunications

Rodrigues L, Póvoa O, Monteiro P, Monteiro A, Teixeira G, Moldão-Martins M & Figueiredo AC (2007). Quantificação e Caracterização do óleo essencial de *Mentha cervina* L. II Colóquio Nacional de Plantas Aromáticas e Medicinais, 28-29 Setembro, Gerês, Portugal.

Pappers in Actas/Proceedings with Referee

Rodrigues L, Duarte A, Monteiro A, Brito L, Figueiredo AC, Moldão M & Póvoa O (2010) Antibacterial and antifungal activity of *Mentha cervina* essential oils and their main components. Planta Medica, 76 (12):1307-1307

Rodrigues L, Monteiro P, Póvoa O, Teixeira G, Moldão M, Figueiredo A & Monteiro A (2008) Chemodiversity studies on *Mentha cervina* L. populations from Portugal. Planta Medica 74: 1199

Pappers in National Publications

Monteiro A, Rodrigues L, Póvoa O & Teixeira G 2008 Os poejos, saberes e sabores. Revista da APH (Associação Portuguesa de Horticultura), 94: 31-35.

Posters in International Conferences

Rodrigues L, Duarte A, Monteiro A, Brito L, Figueiredo AC, Moldão M & Póvoa O (2010) Antibacterial and antifungal activity of *Mentha cervina* essential oils and their main components. In the 58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA 2010). Berlim, Alemanha

Rodrigues L, Monteiro P, Póvoa O, Teixeira G, Moldão M, Figueiredo AC & Monteiro A (2009) Micromorphology of trichomes and composition of the essential oil of *Mentha pulegium* L. from Portugal. In III International Simposium In Medicinal and Aromatic Plants, 26-29 Março, Djerba, Tunísia

Rodrigues L, Monteiro P, Póvoa O, Teixeira G, Moldão M, Figueiredo AC & Monteiro A (2008) Chemodiversity studies on *Mentha cervina* L. populations from Portugal. 7th Joint Meeting of AFERP, ASP, GA, PSE & SIF – Natural Products with Pharmaceutical, Nutraceutical, Cosmetic and Agrochemical Interest. 3-8 Agosto. Atenas, Grécia

Books

Monteiro A, Póvoa O, Marinho S, Rodrigues L & Monteiro P (2008) *Mentha pulegium* e *Mentha cervina*, Os Poejos na boa Cozinha Portuguesa. ISA*Press*. Lisboa.101pp.

De tudo ficaram três coisas:

A certeza de que estamos começando...

A certeza de que é preciso continuar...

A certeza de que podemos ser interrompidos

antes de terminar...

Façamos da interrupção um caminho novo...

Da queda, um passo de dança...

Do medo, uma escada...

Do sonho, uma ponte...

Da procura, um encontro!

E assim terá valido a pena existir.

Fernando Sabino

João Cutileiro
Caneta sobre papel

Vitor π
Lápis sobre papel

Sara Belchior
Aguarela sobre papel

Carlos Vieira
Caneta e carvão sobre papel