“Alexandru Ioan Cuza” University of Iaşi
Faculty of Biology

Physiological and biochemical comparative research on taxa of the genus *Mentha* L.

- doctoral thesis -

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INTRODUCTION

The meaning of the term *mint* was, is and it will be the subject of extensive studies which started sometimes in the Antiquity until nowadays and which fell in various research areas and sectors, trying to cleave or reshape various well-known characteristics of plants. And yet, after centuries of study, debates and discoveries, this species is still a source of study, which aims at revealing the complexity of its characteristics in relation to thei native ecosystems, finally outlining a transition to the artificial reproduction their oil *qualities*, in order to be marketed widely as food additives.

Physiological and biochemical research on taxa of the genus *Mentha* depict a significant image of the essential oil content depending from one

In recent decades researchers have shown a constant concern on the quality of essential oils and substances they are composed of. Essential oil content varies greatly depending on a number of *intra* and interspecific factors. The phenophase in which the plant material is sampled, the temperature and climatic conditions have a decisive role on the amount of oil produced by the plant and especially on its quality (Yamaura et al., 1989).

Using essential oils is a matter of great interest to the food industry because consumers prefer natural rather than synthetic additives. Due to their antimicrobial activity of essential oils can be added to foods in order to extend their validity, but also to improve the taste and smell.

The present doctoral thesis aims at a logical continuation of studies conducted on the genus *Mentha* so far, but brings novel information about the anatomy, physiology, biochemistry and microbiology of some species predominant in Eastern Romania. Also, the research deals with the innovative area of food additives synthesis, with relevance in the food industry.

<u>Objectives</u>:

- Histo-anatomical study of aerial organs, vegetative ones and secretory hairs present on the surface of these organs in the genus *Mentha* species studied by applying conventional optical microscopy techniques alongside modern techniques of electronic microscopy (SEM).

- Physiological research on the dynamics of some foliar indicators the at investigated taxa (interpreted in terms of their ontogenetic cycle).

- Determination of chemical composition of essential oils from taxa of the genus *Mentha* (cultivated and from the native flora of Romania) in different stages of the ontogenetic cycle.

- Investigating possible inhibitory effects of essential oils extracted from vegetative and generative organs of taxa analyzed against certain strains of Gram positive and Gram negative test (collection) by "in vitro" microbiological tests.

- Obtaining menthol derivatives by processes of biocatalysis with enzymatic complexes produced strains of *Candida rugosa* in patenting the idea of obtaining specific food additives, compounds with wide practicality in the food industry.

ACKNOWLEDGEMENTS
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CHAPTER ONE

The Actual State of Research

The study and use of medicinal plants to cure various diseases has been a constant preoccupation of man over the years. In this context, mint is among the plants that have often been used for its therapeutic properties. These properties are due to the essential oils synthesized by secretory hairs on surface of it's aerial organs.

Secretion of Essential Oils

Essential oils are products of the secondary metabolism synthesized at the level of idioblasts, osmophors, secretory papillae, glandular hairs, secretory channels etc.

On the surface of strains and leaves of the representatives of the genus *Mentha* one can notice be two types of secretory hairs: peltate and capitate. The difference between them is the number of secretory cells included in the gland: the gland of capitate secretory hairs consists of a single secretory cell and that of the peltate hairs is composed of 8-12 secreting cells (Fahn, 1979, Fahn, 1988 Turner et al., 2000, Maffei, et al., 2006). Secretory hairs are initiated early in ontogenetic development of plants and begin to accumulate these substances when their leaves are about 5 mm long (Gershenzon et al., 1989).

Biosynthesis of Essential Oils

Monoterpenes synthesis takes place in the metileritrol-4-phosphate cycle with biosynthesis at the leucoplasts level (Maffei et al., 2006) and the sesquiterpens takes place in the mevalonate cycle at the cytosol biosynthesis (Newman and Chappell, 1999).

The Antimicrobial Activity of Essential Oils

In recent decades the antimicrobial properties of essential oils and of their compounds became subject of numerous investigations. Plants or plant extracts, including peppermint ones, were used for preservation of food and beverages and for the preparation of pharmaceutical or cosmetic products, many times used in industry due to their antimicrobial and antioxidant properties (Baratta et al. , 1998a, 1998b, Stanley, 2006).

Enzymatic Synthesis of Food Additives Derivatives of Menthol

Due its pleasant flavor, menthol is widely used in the form of esters, in various confectionery, pharmaceutical, cosmetic and oral hygiene products. Menthol has many different isomers but the ones frequently used in the preparation of food additives (esters) are: (l)-menthol, (d)-menthol and racemic mixture (d,l)-menthol (EPA, 2004). The use of essential oils is a matter of great interest to the food industry because consumers prefer natural additives rather than synthetic ones. Thanks to their antimicrobial activity essential oils can be added to foods in order to extend their validity and to improve their taste and smell.

CHAPTER TWO

Taxonomy and Morphology of the Genus *Mentha* L.

Taxonomic Aspects Related to the Genus *Mentha* L.

Belonging to the *Lamiaceae* family, the genus *Mentha* includes herbaceous perennial plants (rarely annual) with a strong characteristic odor due to the essential oil produced by secretory hairs present on the surface of aerial organs.

The first botanical description of the hybrid *Mentha x piperita* belongs to Raius (1696), while a few years later Karl Linnaeus (1751) describes 10 species and 3 varieties of mint (Păun, 1975).

Throughout time they over 3,000 names for taxa of this genus have circulated, most of them being synonymous (Tucker and Naczi, 2006). The genus *Mentha* has a complex taxonomy which makes it difficult to identify its taxa because of their phenotypic plasticity, genetic variability and the fact that most species are capable of producing cross hybrids (Harley, 1972).

Recent studies conducted by Tucker and Naczi (2006) include 18 species and 11 hybrids in the genus *Mentha*.

Generalities on the Morphology of Taxa from the Genus *Mentha* L.

Most species belonging to this genus have erect rhizomes with quadratic cross section (Guşuleac, 1961), but there are taxa where can be observed stolons: underground ones at *Mentha arvensis* and typical aerial ones at *Mentha aquatica* (Tucker and Naczi, 2006).

Aerial stems are quadric edged, erect, oblique or recumbent, simple or branched. The leaves are arranged opposite with elliptical form more or less broad ovate, lanceolate or rounded; the edge of leaf is often full (Guşuleac, 1961). Plants can be hairless or may have tectorial multicellular hairs, (Tucker and Naczi, 2006), but all species have secretory multicellular hairs (Guşuleac, 1961). The flowers are hermaphrodite or rarely unisexual, arranged on the same plant or on different plants, usually grouped in inflorescences (Guşuleac, 1961).

For Romanian flora were described 6 species of *Mentha* (with varieties) and 6 hybrids (Beldie, 1979; Ciocârlan, 2009).

CHAPTER THREE

Materials and Methods

1. The Vegetal Material

Research were conducted were on fresh plant material collected in three different stages from 7 taxa of the genus *Mentha*, both from the spontaneous flora of Romania and cultivated ones.

There were three species from the native flora harvested from locations with a low degree of human intervention: *Mentha aquatica* L. and *Mentha pulegium* L. were collected from Caraorman (Tulcea county), while the species *Mentha longifolia* (L.) Huds. was taken from the town of Negreşti (Vaslui county) and the village Cioatele (Vaslui).

The cultivated plants were taken from the experimental field of the Centre for Biological Research "Stejarul" Piatra Neamt (*Mentha spicata* L., *Mentha x piperita* var. *black*) and from a private gardens located in the town of Vaslui (*Mentha* × *piperita* var. *columna*) and in Bucharest (*Mentha* x *rotundifolia*).

The plant material was determined by Prof. Dr. Nicolae Stefan and Lector Dr. Ciprian Mânzu, both taxonomists within the Faculty of Biology, "Al. I. Cuza " University of Iași.

The Plants were collected on sunny days from June to September of 2010 and 2011, covering all the stages of the ontogenetic cycle.

2. Materials and Methods used for Anatomical Research

2.1. Photonic Microscopy Analysis of Plant Material

Anatomical investigations were carried out on plant material preserved alcohol 70%. Sections were made with microtome and botanical razor, further analyzed with Novex - Holland binocular optical microscope.

2.2. Analysis of Plant Material with Electronic Microscope

The investigated plant material was analyzed by scanning with electronic microscope in order to observe hairs secreting essential oils. Analysis of samples was performed using Tescan Vega II SBH. Anatomical measurements were performed in the Laboratory of Electronic Microscopy of the Faculty of Biology ("Alexandru Ioan Cuza" University, Iași).

3. Methods Used for Physiological Research

3.1. Method for Determining Humidity and Dry Material

Also known as the gravimetric method, it consists in measuring the loss of water from the mass of plant product investigated.

3.2. Method for Determining the Content of Assimilating Pigments

Assimilating pigments were identified by the method Mayer - Bertenrath, with additions made by Ştirban and Fărcuş.

Physiological research analyzes were performed in the Laboratory of Plant Physiology, Faculty of Biology, University "Alexandru Ioan Cuza", Iași.

4. Methods Used for Biochemical Research

4. 1. Method of Essential Oil Extraction

Separation of volatile oil from **fresh plant material** is based on the property of components being entrained with water vapors. Essential oils were extracted by steam distillation of water using the Neo-Clevenger device.

The essential oil extractions were performed in the laboratory of Plant Biology within the Faculty of Biology, "Alexandru Ioan Cuza" University of Iași.

4. 2. Gas Chromatography Analysis of Essential Oils

In the qualitative analysis of essential oils the gas chromatography method (GC) coupled with mass spectrometry (MS) were used.

Separated components are identified by comparing their mass spectra with the Wiley library spectra, in the database of the device. The validation of identifications is performed using Kovats indices (Salimpour et al., 2011).

Essential oils were analyzed qualitatively in the Research Center for the Study of Quality of Horticultural Products within the Faculty of Horticulture at the University of Agricultural Sciences and Veterinary Medicine of Bucharest and at the Center for Biological Research "Stejarul" Piatra Neamt.

4.3. Enzymatic Synthesis of Food Additives Derivatives of Menthol: Butyrate of menthyl

Butyrate of menthyl synthesis consists of:

4.3.1. Immobilization of lipase extracted from *Candida rugosa*

4.3.2. Testing of enzymatic activity

4.3.3. Enzymatic synthesis of (l) - and (d)-butyrate of menthyl

4.3.4. HPLC analysis (high performance liquid chromatography).

Investigations were carried out in the Laboratory of Food Microbiology and biocatalysis of the Institute of Food Research, Autonomous University of Madrid.

5. Methods used for microbiological research

To highlight inhibitory effects of essential oils the it was used the diffusion method on nutrient agar (Lorian, 2005), modified.

Testing the inhibitory effects of essential oils extracted from *Mentha* samples was performed on six test bacterial strains: Gram-negative (*Escherichia coli* (DH5α), *Morganella morganii* (CECT 8860), *Pseudomonas putida* (ATCC 13430) and *Seratia liquefaciens* (IFI 65)) and Gram-positive (*Enterococcus faecium* (MC1116) and *Lactobacillus plantarum* (CECT 4180)).

The culture medium used for the development of Gram-negative bacterial strains was LB (Luria Bertani) and for the development of Gram-positive MRS (Man, Rogosa, Sharpe).

Measurements were performed in the Laboratory of Food Microbiology and biocatalysts in the Food Research Institute of the Autonomous University of Madrid.

CHAPTER FOUR

Structure of the Aerial Vegetative Organs at Species of the Genus *Mentha* L.

There were histo-anatomically investigated mature plants belonging to the genus *Mentha*, represented by three cultivated taxa (*M. spicata*, *M.* × *piperita* var. *black*, *M.* ×*piperita* var. *columna*) and three of the native flora (*M. pulegium, M. aquatica, M. longifolia*).

During investigations were noticed:

At Stem Level

All species have a prismatic strain in cross section, with the ribs more or less prominent and rounded.

The stomata protrude visible above external level of epidermic cells at *M.longifolia*, *M. aquatica* and *M. pulegium* Tectorial hairs are numerous, long, multicellular, uni-seriated, with a sharp point at *M. longifolia* and extremly rare at *M. piperita* and *M. spicata*, while secretory hairs, always multicellular, (more often with unicellular gland and rarely with octo-cellular gland), with differing frequency per unit area.

The cortex is collenchymatous in the ribs and parenchymatic assimilatory elsewhere, with both meatus and aerial gaps between cells at *M. aquatica*.

Conductive tissues form large libero-ligneous bundles, open collateral type in the 4 ribs, and between these there are 2, 4 or more small or intermediary bundles.

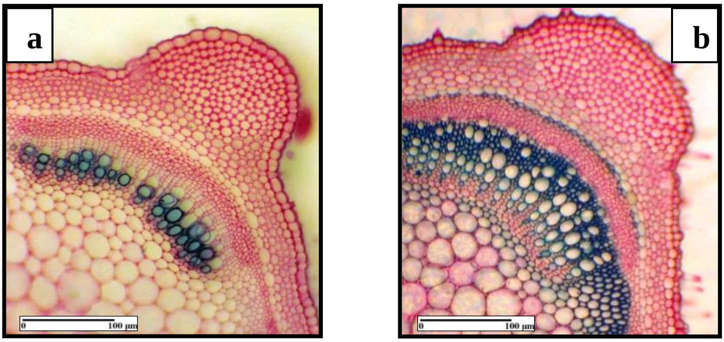

Fig. IV. 1. Cross section through the superior stem level of : a- *M. spicata* ; b- *M. longifolia*

<u>At Leaf Level</u>:

The median vein, always very proeminent on the bottom of the lamina comprises one libero-xylematous bundle; an exception is *M.* × *piperita* var. *columna*, with two bundles.

On the surface of the leaf there are tectorial hairs (numerous per unit area at native species and very rare at cultivated ones) and secretory hairs (extremly rare at native species and numerous at cultivated ones).

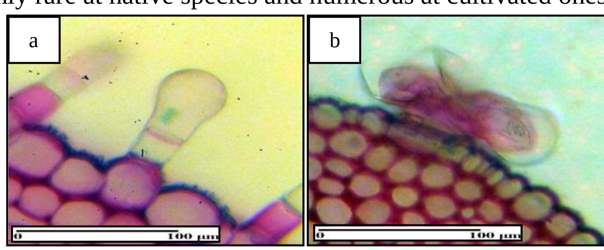

Fig. IV. 2. Secertory ahirs with unicellular (a) multicellular gland (b) at *M. spicata*

The stomata are located in the inferior epidermis, the lamina being hypostomatous. One exception is the species *M. pulegium*, where stomata are present on both epidermises (amphystomatous lamina).

The mesophyll is palisade like on the superior layer and lacunous type in the inferior layer, so lamina has a bifacial upper-inferior structure (dorso-ventral), the palisadic tissue being unistratified. The exception is *M.* × *piperita* var. *columna*, where the mesophyll is lacunous and the lamina bifacial isofacial.

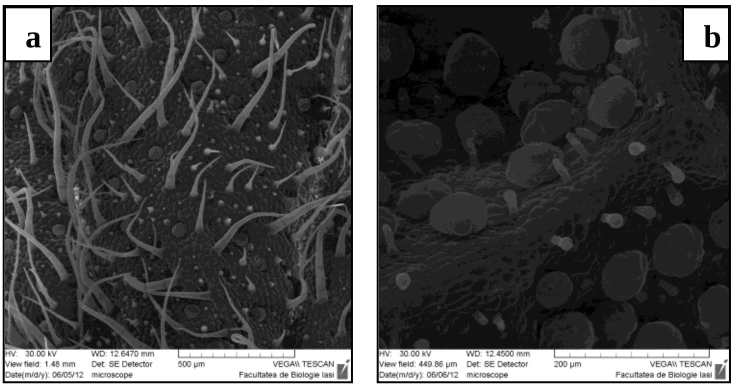

Fig. IV. 3. Scanning electron microscopy image of the leaf lower epidermis in: a-*M. aquatic*a; b-*M.* × *piperita* var. *black.*

CHAPTER FIVE
Physiological Research on Taxa of the Genus *Mentha* L.

Physiological research aimed at determining water content, dry matter and assimilating pigments. The analyzes performed were conducted on four species (*Mentha pulegium*, *Mentha aquatica*, *Mentha longifolia*, *Mentha spicata*) and two varieties of hybrid *Mentha* × *piperita* (*black* and *columna*).

V.1. Variation of Water and Dry Matter Content

Water content: from investigations conducted was noticed in the studied taxa, that in the phenophase of vegetation was recorded the highest water content, which gradually decreased to senescence. The highest value was recorded at *M. spicata*, and the lowest was the at species *M. longifolia* (Cioatele), content sufficient to carry out physiological processes in normal parameters.

Dry matter accumulates during the development of the plant, its highest value occurring in senescence stage at *M. longifolia* collected from Cioatele.

Our results indicate that the investigated plants have a higher metabolic rhythm during vegetative stage, which gradually decreases towards senescence.

V.2. Variation of Assimilating Pigments Content

On the taxa taken into study one may note an upward trend of assimilatory pigments in plant during the growing season. The assimilating pigments content in cultivated taxa was obviously higher than those of spontaneous flora.

It was recorded a significant quantitative increase of chlorophyll a from the vegetative phenophase to senescence in the case of *M. aquatica* and *M. pulegium* taxa. At three of the taxa studied (*M. longifolia,* (Negreşti), *M. x piperita* var. *black*, *M. spicata* the maximum amount of chlorophyll a was recorded at flowering and at the other two (*M. longifolia* (Cioatele), *M.* × *piperita* var. *columna* the maximum amount of chlorophyll a was recorded in vegetative phenophase.

In the case of chlorophyll b was found a similar dynamic, the values recorded being obvious lower. The variations recorded for chlorophyll a and chlorophyll b change the relationship between the two fractions of chlorophyll. We see therefore that the ratio of 3/1 expressed in specialty literature (Zamfirache *et al*., 1995; Zamfirache *et al*., 1997; Burzo *et al*., 1999; Stratu 2002; Masarovičová and Král'ová, 2005) for many species is not recorded constantly throughout the period analyzed.

Carotenoid pigments are found in small quantities, 0,0001 to 0,0006 mg/g fresh matter, compared with chlorophylls a and b. The maximum value for carotenoid pigments was recorded during the flowering period for *M. spicata* taxon.

The intensity of photosynthesis varies throughout the year. In spring, when the leaves are still small, photostnthesis is reduced. With the increase in leaf size the number of chloroplasts and the amount of chlorophyll also increases, the photosynthesis process becoming more intense (Bădulescu, 2009).

CHAPTER SIX
Composition of Essential Oils from *Mentha* L.

Essential oils of the genus *Mentha* species were analyzed by GC-MS (Agilent). The results showed qualitative differences depending on the organ, species, phenophase and studied population.

Variation of Essential Oil Composition Depending on the Organ Analyzed

Tests on the three essential oils extracted from the hybrid *Mentha* × *piperita* var. *columna* in flowering phenophase have revealed the presence of 25 chemical compounds, 16 of which are common to all three samples. We note that the chemical components of essential oils of *M.* × *piperita* var. *columna* varied depending on the organ analyzed. In plant stems were recorded maximum values for mentone, in leaves for menthol and β - caryophyllene, and in flowers for isomenthone and pulegone.

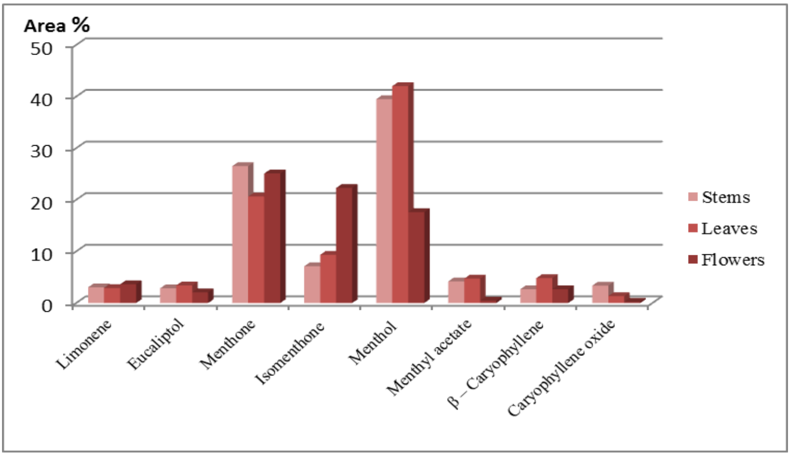

Fig. VI.1. The main chemical compounds in essential oils from aerial organs of the hybrid *M.* × *piperita* var. *columna*

Variation in the Chemical Composition of The Essential Oil According to Species and Phenophase

The chemical composition of the essential oil from *Mentha* undergoes changes during the ontogenetic cycle depending on phenophase analyses are performed. The data show that the volatile oil extracted from these plants contains between 14 (*M. pulegium*) up to 47 compounds (*M.* × *piperita* var. *columna*).

In the samples of essential oil extracted from plants of the genus Mentha in vegetative phenophase there were identified 81 chemical compounds, while in the samples from the flowering phenophase occurred 73 chemical compounds, and in those obtained at senescence were 95 chemical compounds.

The chemical composition of the essential oil varied depending on the species, each having a main substance and 2-4 substances in relatively high amount.

***Mentha pulegium* L.:** pulegone, mentone and menthol.

***Mentha aquatica* L.:** menthophuran, limonene and trans-β-ocimene.

***Mentha longifolia* (L.) Huds.:**

Populația Negreşti:

<u>Vegetative phenophase</u>: piperiton-oxide, limonene, β-cubebene, mircene and trans-β-ocimene.

<u>Flowering phenophase</u>: linalool, carvone, limonene and mircene.

<u>Senescence</u>: linaool, carvone, β-caryophyllene and cis-d-dihydrocarveole.

***Mentha spicata* L.:** carvone, limonene, menthol and menthone.

***Mentha* × *piperita* var. *black*:** menthone, menthol, neoisomenthol, pulegone and eucaliptol.

***Mentha* × *piperita* var. *columna*:** menthone, menthol, menthophuran, pulegone and eucaliptol.

***Mentha* × *rotundifolia*:** menthone, menthol, menthophuran and pulegone.

Variations recorded for chemical compounds of essential oils extracted during the three stages may be caused by changes in environmental factors. The phenophase in which plant material is collected, temperature and environmental factors play a decisive role on the quality of volatile oil (Yamaura *et al*, 1989).

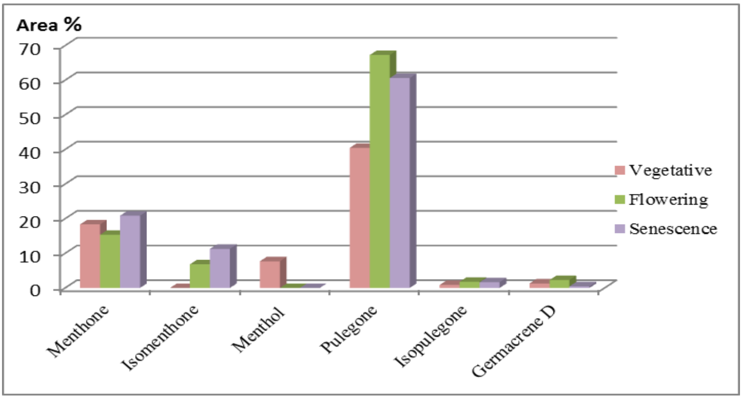

Fig. VI.2. Evoultion of the main chemical compounds in essential oils of *M. pulegium* during the ontogenetic cycle

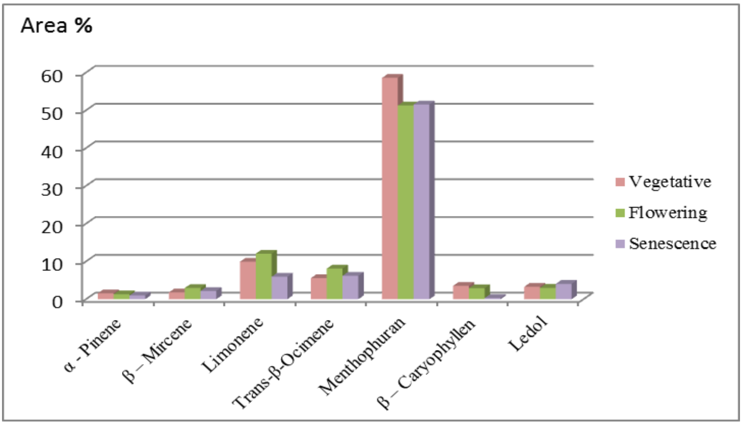

Fig. VI.3. Evoultion of the main chemical compounds in essential oils of *M. aquatica* during the ontogenetic cycle

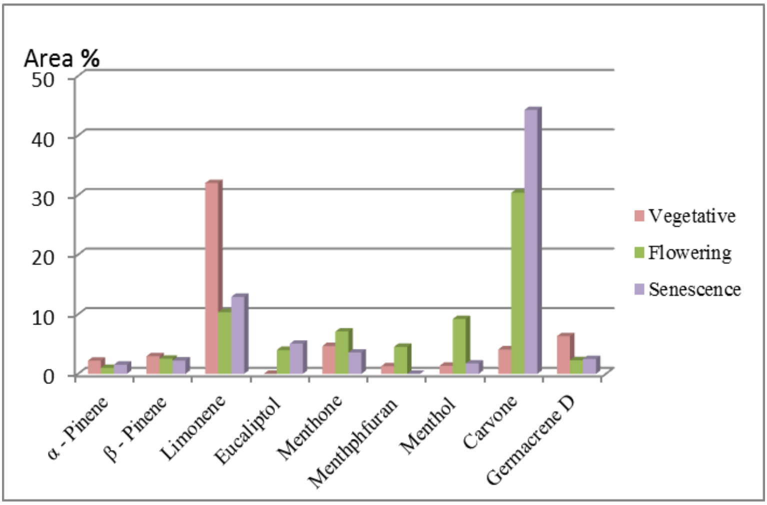

Fig. VI.5. Evoultion of the main chemical compounds in essential oils of *M. spicata* during the ontogenetic cycle

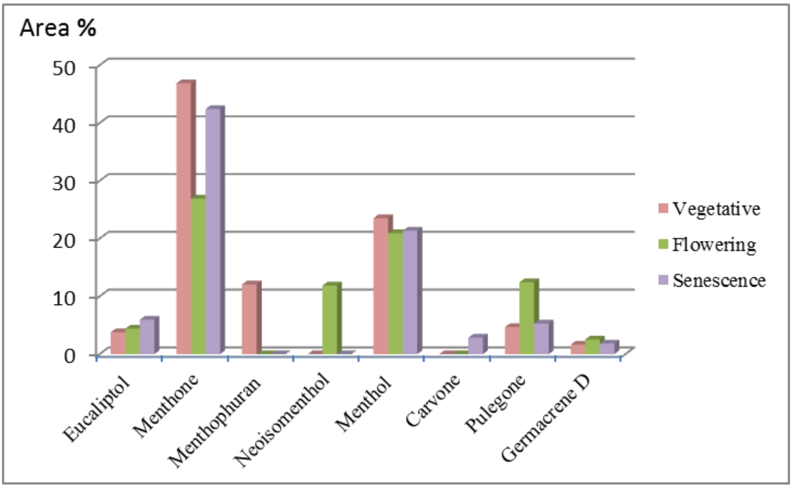

Fig. VI.5. Evoultion of the main chemical compounds in essential oils of *M.* × *piperita* var. *black* during the ontogenetic cycle

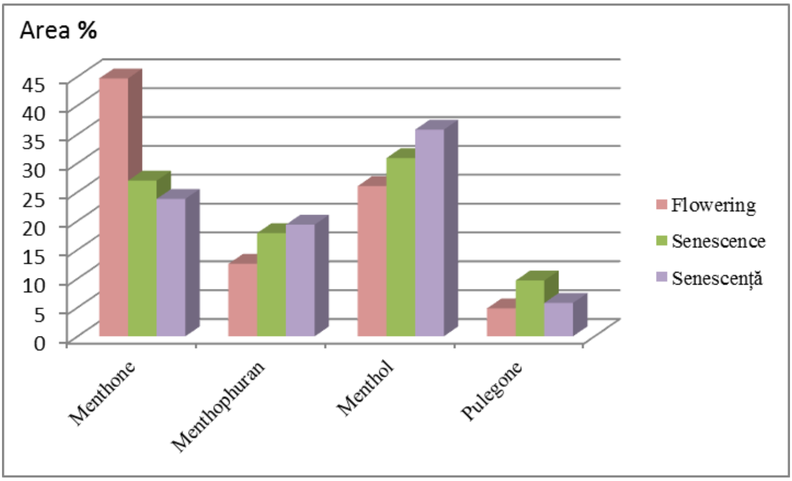

Fig.VI.6. Evoultion of the main chemical compounds in essential oils of *M.* × *rotundifolia* during the ontogenetic cycle *M.* × *rotundifolia*

CHAPTER SEVEN

In Vitro Testing of Antibacterial Effects of Essential Oils from *Mentha* L.

The inhibitory effect of essential oils extracted from species of the genus *Mentha* was tested on six strains of test bacteria (Gram positive and Gram negative).

Dimethylsulfoxide, essential oil solvent (control) showed no inhibitory effect against the six test bacterial strains used.

The antibacterial effect of the 21 samples of *Mentha* oil could be observed both for Gram positive and Gram negative bacteria. In each case, the largest zone of inhibition was recorded for 100% concentration of tested essential oils.

Testing essential oils of *M. pulegium* on the six bacterial strains showed inhibitory effects only for the Gram-negative strains. For *E. coli* strains (ø 7-12 mm), *M. morganii* (ø 7-13 mm) and *S. liquefaciens* (ø 8-14 mm), the inhibitory effect was more pronounced compared to P. putida (ø 7-8 mm).

For samples of essential oil from *M. aquatica* it was noticed an inhibitory activity against the bacterial strain of E. coli. Each of the four concentrations of essential oil tested showed inhibitory effect. None of the three essential oil samples did inhibit the growth and development of Gram positive (absence of inhibition areas).

Essential oils of *M. longifolia*, collected from the town Negreşti, showed inhibitory activity only on Gram-negative bacterial strains, while no such effect was noticed to Gram-positive strains. Essential oils of *M. longifolia* taken from Cioatele area showed inhibitory effects against all strains tested.

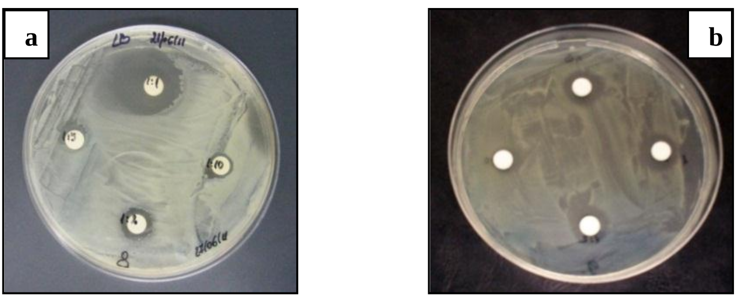

Fig. VII.1. Testing of antimicrobial effect of essential oil from *M. longifolia* (Negreşti) against: a- *E. coli;* b- *S. liquefaciens*

M. spicata essential oils tested showed inhibitory activity against three bacterial strains: *E. coli*, *M. morganii*, *S. liquefaciens* (Gram negative). The same volatile oil samples had no inhibitory activity on Gram-positive bacterial strains.

Volatile oils of two varieties of hybrid *M.* × *piperita* studied, *Columna* variety was remarked by the inhibitory effect both on Gram negative and Gram positive bacteria. Concerning volatile oils from *Black* variety was observed that the inhibitory effect was present for all concentrations applied.

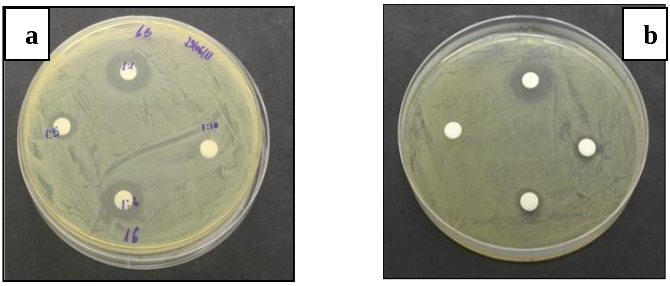

Fig. VII.2. Testing of antimicrobial effect of essential oil from: a-*M. spicata* against *E. coli* ; b- *M.* x *piperita* var. *columna* against *E. coli*

CHAPTER EIGHT

Food Additives Derivatives of Menthol: Butyrate of Menthyl

(*l*)-Menthol and its esters such as butyrate of menthyl are components widely used in preparation of candy, sweets, beverages, toothpaste, cigarettes, local anesthetics, analgesics, medicines, cosmetics and chewing gums (Athawale , et al., 2001).

The butyrate of menthyl can be obtained by synthesis reaction between menthol and methyl butyrate. This reaction was performed in anhydrous medium in the presence of a biocatalyst. The biocatalyst consisted of derived enzymes from *Candida rugosa*. *Candida rugosa* derivatives were obtained by immobilizing a commercial lipases on two solid media (Duolite and Lewatit 1600).

Lipaze de *Candida rugosa* (CRL) au fost imobilizate cu succes în suporturile Duolide (91,45%) şi Lewatit 1600 (84,25%). Derivatele obşinute au avut activitate hidrolitică faţă de metil butirat şi au manifestat specificitate doar faţă de enantiomerul (*l*)-mentol.

The *Candida rugosa* lipases (CRL) were successfully immobilized in Duolide media (91.45%) and Lewatit 1600 (84.25%). Derivatives were had hydrolytic activity towards methyl butyrate and depicted specificity only towards the enantiomer (*l*)-menthol.

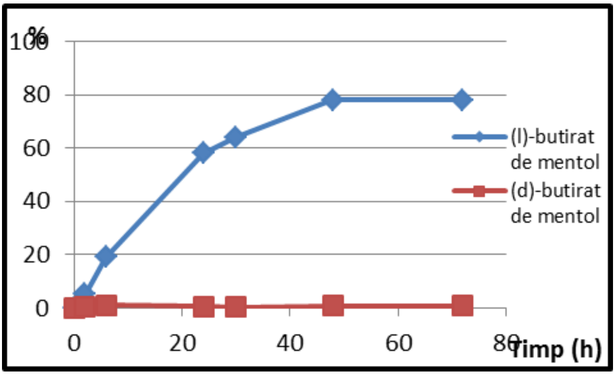

Fig. VIII.1. Quantitative evolution of (*l*) - and (*d*)-menthol butyrate in the reaction catalyzed by CRL Duolite derivate

The butyrate of mentil resulted from the reaction catalyzed by Duolite CRL / CRL Lewatit, showed thermal stability up to 45 °C.

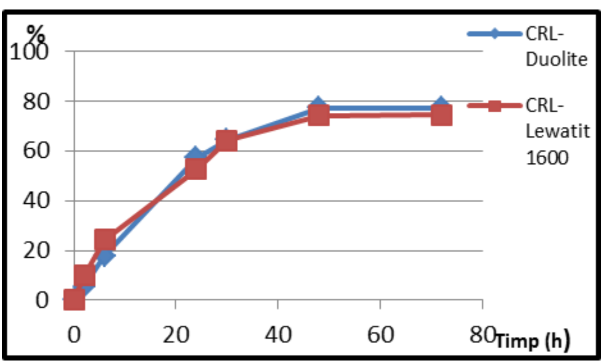

Fig. VIII.2. Thermal testing of (*l*)-butyrate of menthol (45 ºC)

In the future it is proposed the synthesis of (l)-butyrate of mentil (using the same method) from natural menthol in order to obtain “green” food additives and to lower costs due to reactions and especially to satisfy the need for bio products of recent years.

CONCLUSIONS

The doctoral thesis aims to analyze the main features of seven taxa of the genus *Mentha*, three of spontaneous flora (*Mentha pulegium* L., *Mentha longifolia* (L.) Huds) and *Mentha aquatica* L. four cultivated (*Mentha spicata* L., *Mentha x piperita* var. *black* Michal., *Mentha x piperita* var. *columna* L. and *Mentha rotundifolia* (L.) Huds.), in order to highlight certain histo-anatomical, physiological and biochemical features of the biological individuals from the eastern and the southern part of the country, as well as to study the antibacterial effects induced by essential oils produced by vegetative and generative organs of the plants; at the same time, the thesis raises the question of possible synthesis of food additives based on natural products (essential oils) of plants analyzed and their use in the food industry.

- **Histo-anatomical research concluded that:**

➢ All the species from the genus *Mentha* investigated showed a more or less similar histo-anatomical structure, confirming and complementing existing data in the literature

The Stem:

➢ Epidermis presents among cells, stomata and hairs:
  - from species of the native flora (*M. longifolia*, *M. pulegium* and *M. aquatica*) stomata protrude above the epidermis;
  - tectorial hairs are always long, multicellular, uni-seriated, with a sharp point; they are numerous at native species and extremely rare in cultivated ones;
  - Secretory hairs are always multicellular, short, mostly with unicellular gland and rarely multicellular

➢ Conductive tissues form large libero-ligneous bundles, open collateral type in the 4 ribs, and between these there are 2 (*M. piperita*), 4 (*M. longifolia*) or more small or intermediary bundles.

The leaf:

➢ At most taxa it is hypostomatic (exception *M. pulegium,* with amphystomatous lamina).

➢ Tectorial hairs are uni-, bi-, multicellular and extremly rare at cultivated species and numerous at native ones.

- Secretory hairs are relatively rare, especially the ones with unicellular gland, more numerous at cultivated taxa compared to the ones in the native flora.
- Is bifacial-heterofacial (dorso-ventral), with unistratified palisadic tissue on the superior side and lacunous tissue on the inferior one (exception is *M.* × *piperita* var. *columna*, where the mesophyll is lacunous and the lamina bifacial isofacial).
- The median rib is poreminent on the bottom with a libero-ligneous bundle at most taxa investigated (exeception *M. piperita* var. *columna*, with two bundles).

Physiological research concluded that:

- **The water content** is reduced through the ontogenetic cycle at all investigated taxa, allowing by the values achieved, the development of normal physiological processes in all phenophases (minimum values in the stage of senescence do not fsiall below the vital minimum).
- **The dry matter** content increases with the aging of the test plant due to the accumulation of newly synthesized substances at the mesophilic level and its aging, a phenomenon that induces modifications to the cellular permeability for constitution water.
- The biosynthesis and accumulation processes of **assimilating pigments** are correlated with the analyzed taxon, as well as with moment of harvest (plant age) ; thus, our data confirms the specialty literature (Masarovičová and Král'ová, 2005).
  - **chlorophyll a** records a significant increase from the vegetative phenophase to senescence in the case of *M.* aquatica and *M. pulegium* taxa; for *M. longifolia* (Negreşti), *M. piperita* var. *black* and *M. spicata* the maximum amount of chlorophyll a was recorded at flowering, and for *M. longifolia (Cioatele*) şi *M. piperita* var. *columna* in the vegetative phenophase;
    - **chlorophyll b** was found a similar dynamic, the values recorded being obvious lower (minimum at senescence and maximum at flowering);
    - The variations recorded for chlorophyll a and chlorophyll b change the relationship between the two fractions of chlorophyll compared with the results from specialty literature (3/1) during the analyzed interval;

- **Carotenoid pigments** are found in larger amounts at cultivated species compared with the native ones.

- **<u>Biochemical research concluded that:</u>**

- Essential oil composition varies qualitatively depending on the organ, species, population, plant material and according to phenophase at the time of measurements.
- The data show that the volatile oil extracted from these plants contains between 14 (*M. pulegium*) up to 47 compounds (*M.* × *piperita* var. *columna*).
- Each essential oil sample analyzed contains one main substance characteristic to the species, two up to four substances in relatively large quantities and substances that are found in smaller quantities, but which contribute to the character of aromatic plants.
- In the case of the species *M. longifolia* (L.) Huds. in the essential oil obtained from the stems of the plants were identified a number of 19 compounds; in oil from their leaves 24 compounds were identified, and from the flowers were identified 23 compounds. Strains had a higher content of linalool compared with other organs examined, while leaves had a higher content of β-caryophyllene and germacren D, while flowers had a higher content of carvone and limonene.
- Tests on essential oils extracted from the organs of hybrid *Mentha* × *piperita* var. *columna* indicate the presence of: 21 compounds in the essential oil sample extracted from the leaves, 20 compounds in the volatile oil sample obtained from flowers and 19 compounds in the sample obtained from the stems; in terms of quality, mentone prevailed in strains, menthol and β - caryophyllene in the leaves and at flower level pulegone and isomenthone.
- Menthol, an alcohol typical to the taxa of the genus *Mentha*, which offers them a characteristic odor was not found at species from the native flora except for *M. pulegium* in vegetative phenophase; however, in our investigation this compound occurs in high concentrations in essential oil samples obtained from cultivated species.

- **<u>Microbiological analyses concluded that:</u>**

- The antibacterial effect of essential oils extracted from investigated taxa of the genus *Mentha* against the strains of Gram positive and Gram negative test, it was more or less obvious, depending on the source of essential oil (vegetal taxon).
- Essential oils produced by *M. longifolia* (Cioatele area) showed inhibitory effect on all tested bacterial strains, while *M. pulegium* oils inhibited only Gram-negative strains, and the oils produced by *M.* × *piperita var*. *columna* differently inhibited bacterial strains (three strains of Gram negative and one Gram positive), and oils of *M. spicata* and *M.* × *piperita* var. *black* inhibited only three Gram-negative strains with no inhibitory effect on Gram-positive strains.
- The maximum zone of inhibition of bacterial test strains was obtained for all taxa analyzed at a concentration of 100% of the essential oil.

- **<u>On the synthesis of butyrate of menthyl by biocatalysis reactions research conclude that:</u>**

- Derivates of *Candida rugosa* showed specificity towards the isomer (*l*)-menthol and showed no specificity to the (*d*)-menthol.
- Synthesis reactions catalyzed by CRL Duolite derivate and Lewatit CRL 1600 derivate had a slightly equal performance, convenient to a future industrial-scale synthesis of this product.
- Reducing the concentration of (*l*)-menthol in the reaction led to a dramatic decrease of (l)-butyrate of menthyl synthesis. Due to the results obtained (data in laboratory conditions) the reaction is of no interest for the synthesis of the additive (l) – butyrate of menthyl.
- The Butyrate of menthyl resulted from the synthesis reaction catalyzed with Duolite CRL / CRL Lewatit, displayed thermal stability up to 45 °C.

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