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Investigation of useful traits in breeding of *Melissa officinalis* L.

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Investigation of useful traits in breeding of *Melissa officinalis* L.

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List of abbreviations:

Acc. No.: Accession number

BD: Becton Dickinson

BLBP: intern coding for Genotypes of the Bavarian state Institute for agriculture

BMEL: German Federal Ministry of Nutrition and Agriculture

BSA: N,O-bis(trimethylsilyl)acetamide

CCD Camara: Charge-coupled device camara

COL: Collection

Ct: Chemotypes

DAPI: 4’, 6-diamidino-2-phenylindole

DNase: deoxyribonuclease

dUTP: Desoxyuridine triphosphat

EDTA: Ethylenediaminetetraacetic acid

EO: Essential oil

FISH: Fluorescent *in situ* hybridization

FITC: Fluorescein isothiocyanate

FNR: Agency Renewable Resources

GAT: Gatersleben

GC: Gas chromatography

HCA: Hierarchical cluster analysis

HPLC: High-performance liquid chromatography

IPK: Leibniz Institute of Plant Genetics and Crop Plant Research

JKI: Julius Kühn Institute

LfL: Bavarian State Institute for Agriculture

MELI: intern coding of the Leibniz Institute of Plant Genetics and Crop Plant Research

MS: Mass spectrometry

ÖPV: JKI-Institute for Ecological Chemistry, Plant Analysis and ´Stored Procuct Protection

PCA: Principal component analysis

PCR: Polymerase chain reaction

Ph. Eur.: European Pharmacopoeia

QLB: Quedlinburg

RA: Rosmarinic acid

RNase: Ribonuclease

Sibth. & Sm.: Sibthorp and Smith

SSC: Saline Sodium Citrate

UPOV: Union internationale pour la protection des obtentions végétales, International Union for the Protection of New Varieties of Plants

VIR: N.I. Vavilov All-Russian Research Institute of Plant Industry

ZG: JKI-Institute for Breeding Research on Horticultural Plants

1 GENERAL INTRODUCTION:

Melissa officinalis L. (*M. officinalis*) is one of the most significant and well-known medicinal and aromatic plants in Europe. The uses of this plant have been known since ancient times and it has been in use since the middle ages and continues to be used today. *M. officinalis* belongs to the botanical family of Lamiaceae. The species has been used for so long that, although it is believed to originate in the Mediterranean region or Western Asia, the exact region of origin is difficult to ascertain (Davis 1982). The crop is perennial and grown in the temperate and subtropical regions of Europe, North America and Asia for production of phytopharmaceuticals and spices (FNR).

Due to its proven spasmolytic, carminative, sedative, virostatic, bacteriostatic, anti-inflammatory, antioxidative and bactericide properties (Forster et al.1980; Hefendehl, 1970; Koch-Heitzmann and Schultze, 1984; Parnham and Kesselring, 1985; Toth et al. 2003; Wagner and Sprinkmeyer, 1973) the dried leaves (Melissae folium), the dried herb (Melissae herba), the essential oil (Melissae aetheroleum), and the extract of dried leaves (Melissae folii extractum siccum) (Hänsel 2013) are accessible for pharmacological use, mainly in watery or alcoholic extracts. Beside essential oil (Aziz and El-Ashry 2009) a wide spectrum of other active components was found in lemon balm like phenolic acid derivatives, flavonoids, and triterpenes (Bomme et al. 2013). According to the European Pharmacopoeia, the dried leaves have to exhibit a lemon-like scent and a minimum content of 1 % rosmarinic acid and 2 % for the leave extract (Ph. Eur. 2014) determined via HPLC. Medicines based on lemon balm are used to treat sleep disturbances and functional gastrointestinal disorders (Aziz and El-Ashry, 2009), nervous agitation, indigestions and infection with herpes simplex virus (Mazzanti et al. 2008; Schilcher, 2016; Wölbling and Leonhardt, 1994). There is also strong evidence that lemon balm has a positive effect on patients who suffer from Alzheimer’s disease (Akhondzadeh et al. 2003; Moradkhani et al. 2010). Those effects are based on the content of essential oil and phenolic carbon acids, such as rosmarinic acid.

The literature identifies two to three subspecies within the species *M. officinalis*. The subspecies are *M. officinalis* L. subsp. *officinalis*, *Melissa officinalis* L. subsp. *altissima* (Sibth. & Sm.) Arcangeli (Tutin et al. 1972) and *Melissa officinalis* L. subsp. *inodora* (Bornm.) (Davis 1982, Hanelt and IPK 2001). These subspecies can be divided by morphological traits like pubescence and structure of blossoms (Hoppe 2013). Furthermore, the composition of essential oil and the resulting scent can be another way to discriminate the subspecies (Moradkhani et al. 2010, Hoppe 2009, Kittler et al. 2015), although the composition varies during the ontogenesis of the plant. Additionally, the set of chromosomes is a way to characterize the present subspecies. All naturally occurring accessions of *Melissa*, which conform to the Pharmacopoeia have a set of 2n = 2x = 32 chromosomes. The tetraploid ssp. *altissima* has 64 chromosomes and the triploid form introduced in Kittler et al. (2015) has 2n = 3x = 48 chromosomes. These accessions contain only traces of lemon-like scented components. Currently there are 23 phytopharmaceuticals and 2 homoepathics registered in Germany. In addition to the pharmacological use in medicine and folk medicine, infusions and alcoholic extracts *M. officinalis* is also used in perfume-, aroma- and cosmetic production and in its traditional role as an aromatic plant for the seasoning of dishes and drinks (Bagdat 2006).

1 GENERAL INTRODUCTION:

1.1 PHARMACEUTICAL RELEVANT INGREDIENTS:

1.1.1 ESSENTIAL OIL:

The essential oil of *M. officinalis* can occur in dried leaves in concentrations of 0.02 – 0.8% (Adzet et al., 1992a). It is a complex mixture of volatile compounds isolated by physical means. The compounds mainly derived from three biosynthetic pathways, the mevalonate pathway, leading to sequiterpenes, the methyl-erithrytol-pathway leading to mono and diterpenes and the shikimic acid pathway leading to phenylpropenes (Baser, 2010). The essential oil of *M. officinalis* contains mainly six substances in larger amounts: the monoterpenes citronellal, (*Z*)-citral and (*E*)-citral (Krüger et al. 2010), and the sesquiterpenes gemacrene, ß-caryophyllene and ß-caryophyllene oxide (Figure 1). In most cases it is a mixture of this mono- and sequiterpenes with up to additional 56 components. The monoterpenes (*E*)-Citral and (*Z*)-Citral, as well as citronellal causes the lemon-like scent. The proportions of the different substances to the composition of the essential oil can vary substantially (Hoppe 2009, Hänsel 2013, Adzet et al. 1992a, Adzet et al. 1992b, Kittler et al. 2017, Kittler et al. 2018). Other occurring monoterpenes are: Linalool, geraniol, geranyl acetate, methyl citronellate, trans-ß-ocimene and methylhepton.

Occurring sequiterpenes are: β-caryophyllene, β-caryophyllene-oxide and germacrene D, alpha-copaene, alpha-cubebene, humulene und ∂-cadinene (Hänsel 2013, Kittler et al. 2017). The variation in the composition of the essential oil can be strongly influenced by origin of the crop, climatic conditions, harvesting time, as well as the age of the plant (Hoppe 2009, Moradkhani et al. 2010, Kittler et al. 2018).

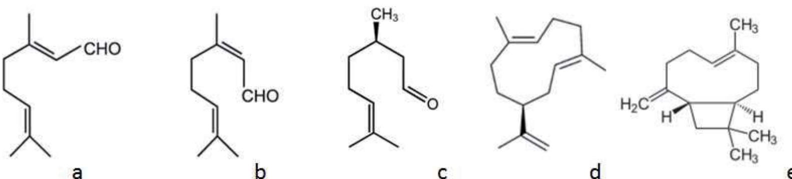

Figure 1: Structural formula of the main components of the essential oil of *M. officinalis*. (*E*)-citral (a), (*Z*)-citral (b), citronellal (c), germancrene D (d) and β-Caryophyllene oxid (e) (Hoppe 2013).

1.1.2 ROSMARINIC ACID:

Rosmarinic acid (RA) is an ester consisting of caffeine acid and 3-(3,4 - dihydroxyphenyl) lactic acid (figure 1). This acid is the main component of the existing phenolic carbon acids and a codetermining active pharmaceutical ingredient in lemon balm (Hoppe, 2009; Schilcher, 2016). Dried lemon balm

leaves (Melissae folium) from Central European cultivation have a content of up to 6 % rosmarinic acid measured as sum of hydroxycinnamic acid derivatives according to Parnham and Kesselring (1985) and 7.3 to 14.8 % to Bomme et al. (2008). The main activities of rosmarinic acid are adstringent, antioxidative, anti-inflammatory, antimutagen, antibacterial and antiviral (Petersen and Simmons, 2003). In 1958, rosmarinic acid was successfully isolated from rosemary (*Rosmarinus officinalis* L.) for the first time by Scarpati and Oriente (1958) and gets its name from that plant. In the year 1991, the chemical synthesis was developed (Rao et al. 1990; Zinsmeister et al. 1991). In the biosynthesis of rosmarinic acid eight enzymes are involved. All are characterised (Petersen and Simonds, 2003; Weizel, 2009). The synthesis of rosmarinic acid starts in the Shikimic acid pathway (Gao et al. 2015).The content of rosmarinic acid can be confirmed for mono- and dicotyledonous, as well as hornwort species Anthocerotaceae (Takeda et al. 1990, Petersen and Simmonds 2003, Vogelsang et al. 2006) and ferns (Blechnaceae) (Harborne 1966, Bohm 1968, Häusler et al. 1992) In Lamiaceae rosmarinic acid can be detected in *Rosmarinus officinalis* (rosemary), *Thymus vulgaris* (thyme), *Plectranthus scutellarioides* (coleus), *Satureja sp*. (savory), *Salvia officinalis* (sage) and *Origanum vulgaris* (oregano). Pharmacological examination on histamine release from mast cell (Makino et al. 2000), COX-2 expression (Youn et al. 2003), the C3b factor in the complement cascade and viral replication (Sahu et al. 1999) prove these anti-inflammatory, anticarcinogen and antiviral impacts of RA (Weitzel and Petersen 2010). Until 2008, rosmarinic acid had to be calculated by a photometric method as the sum of all hydroxycinnamic acid derivatives and had to reach minimal 4 % (Ph. Eur. 6., 2008). Since 2009, the Pharmacopoeia Europaea changed considerably to calculate specific rosmarinic acid content by using high performance liquid chromatography (HPLC) and to reach 1 % (Ph. Eur. 6. 2009). The correlation coefficients of R^2 = 0.74 and R^2 = 0.73 between both methods are insufficient. A correct transformation of values is impossible (Krüger et al. 2010).

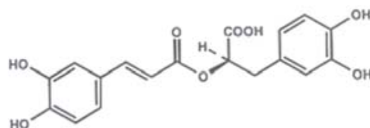

Figure 2: Structural formula of Rosmarinic acid (Hoppe 2013).

1.2 BREEDING OF LEMON BALM:

Due to the limited acreage of this crop (approximately 250 ha in Germany and 5000 ha in Poland (Seidler-Łożykowska et al. 2013)) *Melissa* is a niche culture and did not receive much attention of plant breeders in Germany. It is noteworthy that published results about breeding or established new varieties are rare. Usually these were so called industrial lines, which were not freely accessible or tradable. Most seed producers practised conservation of already established varieties, so that those could develop

different characteristics based on their individual origin. The two cultivars "Quedlinburger Niederliegende" and "Erfurter Aufrechte" are the most distributed varieties.

However, in the 1990s, the breeding activities started to grow again. In a breeding trial by Adzet et al. (1992) genetic variability and essential oil content were evaluated over 5 years. During this trial, the average essential oil content of 0.3 % could be raised to 0.5 %, with individual results of up to 0.68 %. Phenotypic characteristics, such as the number of branches as well as height of the plants were examined. To improve the essential oil content from generation to generation, the morphologically favourable cultivars with the highest essential oil content were taken as basis for the next generation. Hybridisation was carried out by means of inbreeding and crossbreeding of the inbred lines. Most of the resulting lines showed higher content of essential oil compared to the improved lines. Fast growing plants, characterised by a branched phenotype, tended to show lower essential oil contents in contrast to slowly growing ones. Furthermore, a correlation between content of essential oil and yield of essential oil could be determined. Therefore, the content of essential oil can be used as a selective criterion for profitable essential oil-biotypes (Wolf et al. 1999.

1.3 THE PROJECT UNDERLYING THIS THESIS:

The project "Development of generative propagatable high yielding lines of lemon balm (*Melissa officinalis* L.) via conventional generating of homozygote lines as a prereqisite for synthetic and hybrid variaties" (original title in German: „Entwicklung generativ vermehrbarer Hochleistungslinien von Zitronenmelisse (*Melissa officinalis* L.) durch konventionelle Erzeugung homozygoter Linien als Voraussetzung für Synthetiks oder Hybridsorten") is a sub-project of the demonstration project "Improving of the international position of the German production of medical herbs and spices, by taking the example of the breeding and cultivation of valerian, camomile and lemon balm" (original title in German: „Verbesserung der internationalen Wettbewerbsposition des deutschen Arznei- und Gewürzpflanzenanbaus am Beispiel der züchterischen und anbautechnologischen Optimierung von Kamille, Baldrian und Zitronenmelisse") and was funded by Fachagentur Nachwachsende Rohstoffe (FNR). During the first of the three planned project phases, the basis for modern breeding of *Melissa officinalis* should be established in co-operation with the projects of N.L. Chrestensen GmbH and Dr. Junhghanns GmbH. Therefore, capabilities of different *Melissa officinalis* accessions concerning winter hardiness, leaf yield, content of essential oil and rosmarinic acid were evaluated. Moreover, strategies for self-fertilisation for the conventionial production of homozygous species should be established. For the production of new breeding material, strategies for cross breedings had to be established. In context of the related project "Development of a procedure for lemon balm (*Melissa officinalis* L.) to generate doubled haploid plants and finding elements for a system to regulate fertilisation based on male sterility." (original title in German: „Entwicklung eines Verfahrens für die Zitronenmelisse (*Melissa officinalis* L.) zur Erzeugung von Doppelhaploiden und Suche nach Elementen für die Schaffung eines Systems zur Befruchtungsregulierung auf der Grundlage männlicher Sterilität") the fundamental basis for the *vitro* culture were laid. Additionally, investigations on the presence of natural male sterility were performed.

In this project ovular-, anther- and microsporeculture and different hormon and stress treatments were used to establish a method for the development of doubled haploid plants (Kästner et al. 2016).

1.4 BASIS OF BREEDING IN *MELISSA OFFICINALIS*:

The publications which form part of this dissertation “Chromosome number and ploidy level of balm (*Melissa officinalis*)”, “Evaluation of 28 balm and lemon balm (*Melissa officinalis*) accessions for content and composition of essential oil and content of rosmarinic acid” and “Content and composition of essential oil and content of rosmarinic acid in lemon balm and balm genotypes (*Melissa officinalis*)“ are parts of the fundamental investigation on *Melissa* in context of the main project “Improving of the international position of the German production of medical herbs and spices, by taking the example of the breeding and cultivation of valerian, camomile and lemon balm“ (original title in German: „Verbesserung der internationalen Wettbewerbsposition des deutschen Arznei- und Gewürzpflanzenanbaus am Beispiel der züchterischen und anbautechnologischen Optimierung von Kamille, Baldrian und Zitronenmelisse“). Knowledge about the genetic background and potential of various breeding lines was needed for the selection of appropriate breeding material and methods. Various and partly conflicting information about chromosome number, ploidy and the presence of subspecies of *Melissa officinalis* were found in the primary literature research. However, during the project work, the level of ploidy of different accessions could be determined. Three levels of ploidy were identified in the collections. The predominant part of the accessions (101 of 120) was found to be diploid with $2n = 2x = 32$ chromosomes. 13 accessions were found to be tetraploid with $2n = 4x = 64$ and 6 were triploid with $2n = 3x = 48$. Moreover, the intensive investigation of valuable ingredients was another important part of the project. High variations in proportion of these ingredients were found. The evaluations in publication two and three depict genotypes, which can be useful for breeding new varieties with high content of rosmarinic acid and essential oil. The data of rosmarinic acid evaluation in connection with ploidy level and amount and composition of essential oil can contribute to taxonomical studies inside the species *M. officinalis*. The analysis of the composition of essential oils discovered three different Chemotypes: citral, germacrene D and β-caryophyllene-oxide.

1.5 OBJECTIVES:

The aim of this thesis is to build a foundation for modern breeding attempts in lemon balm and related medicinal and aromatic plants. With the aid of the detailed and extensive evaluation data sets and the determination of the genetic properties of the numerous accessions and genotypes this thesis is an important work to depict the genetic variability of lemon balm. The results of the three incorporated scientific papers can be split in two groups.

1 Establishing basic cytologic knowledge: To use modern breeding systems and strategies, fundamental data like the number of chromosomes, the chromosome number of a haploid nucleus, the possible level of ploidy is necessary for the adaptation of cytologic and molecularbiologic methods (Chapter 2).

2 Evaluation of a wide variety of accessions and genotypes of balm and lemon Balm: The intensive evaluation of numerous accessions and genotypes revealed the agronomical and economical value of lemon balm and its sub-species. Characteristic traits such as content and composition of essential oil and content of rosmarinic acid were examined and add to the essential information needed for further breeding activities in lemon balm (Chapter 3 and 4).

2 CHROMOSOME NUMBER AND PLOIDY LEVEL OF BALM (MELISSA OFFICINALIS):

Kittler *et al. Molecular Cytogenetics* (2015) 8:61
DOI 10.1186/s13039-015-0166-z

RESEARCH Open Access

Chromosome number and ploidy level of balm (*Melissa officinalis*)

J. Kittler, O. Schrader, U. Kästner and F. Marthe*

Abstract

Background: Lemon balm (*Melissa officinalis* L.) is of increasing importance resulting in rising growth area. Improved knowledge on the genome structure, number of chromosomes in connection with the taxonomical structure of balm is indispensable for improved new varieties.

Results: A collection of 40 balm accessions (*M. officinalis*) was characterized by flow cytometry and FISH (18/25S and 5S rDNA) to determine the chromosome number and ploidy level. Three different types were found: diploid genotypes with $2n = 2x = 32$ chromosomes; tetraploid $2n = 4x = 64$ chromosomes and triploid $2n = 3x = 48$ chromosomes. A haploid base number of $x = 16$ chromosomes is likely. First time described triploid accessions are sterile but cytologically and morphologically stable for many years. Triploids express better winter hardiness and regeneration after harvesting cuts as well as bigger leaves and internodes.

Conclusions: A basic chromosome number of $x = 16$ is reported for the first time for the species *M. officinalis*.

Abstract

Die wachsende Bedeutung von Zitronenmelisse (*Melissa officinalis* L.) führt zur Ausdehnung des hierfür erforderlichen Anbauumfanges. Ein verbesserter Kenntnisstand der Genomstruktur, der Chromosomenzahl und der hiermit in Zusammenhang stehenden taxonomischen Struktur der Melisse sind unerlässliche Voraussetzungen für verbesserte, neue Sorten.
Eine Kollektion von 40 Melisseherkünften (*M. officinalis*) wurde durchflusszytometrisch und durch FISH (18/25S and 5S rDNA) untersucht, um den Ploidiegrad und die Chromosomenzahl zu ermitteln. Drei unterschiedliche Typen wurden konnten bestimmt werden: diploide Genotypen mit $2n = 2x = 32$ Chromosomen; tetraploide mit $2n = 4x = 64$ Chromosomen und triploide mit $2n = 3x = 48$ Chromosomen. Die haploide Chromosomenzahl ist mit $x = 16$ anzunehmen. Die erstmalig beschriebenen triploiden Herkünfte sind steril aber zytologisch und morphologisch über viele Jahre stabil. Sie zeigen eine bessere Winterhärte und einen schnelleren Wiederaufwuchs nach Ernteschnitten, wie auch größere Blätter und Internodien.
Die Basischromosomenzahl von $x = 16$ wird erstmalig für die Art *M. officinalis* beschrieben.

Background

Lemon balm (*Melissa officinalis* L.) is an old crop and is used for phytopharmaceuticals, as an aromatic plant and in traditional folk medicine. A wide spectrum of secondary metabolites exists in lemon balm. For the medicinal use, the active ingredients essential oil with lemon fragrance and rosmarinic acid are necessary [6, 2, 3, 13].

M. officinalis belongs to the family Labiatae (syn. Lamiaceae). This crop plant is grown worldwide but its origin is not well-defined, however the Mediterranean Region or western Asia is considered as the area of origin [9]. The subspecies *M. officinalis* L. ssp. *officinalis* and ssp. *altissima* (Sibth. & Sm.) Arcangeli are distinguishable especially by the shape of calyx and density of different types of hairs [5, 20]. The middle tooth of the three upper lip teeth of fruiting calyx is broadly triangular for ssp. *officinalis* whereas it is inconspicuous, truncate or

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emarginated for ssp. *altissima*. Pignatti [17] classified ssp. *altissima* as species *M. romana*.

The chromosome base number in Labiatae ranges from x = 5 to 11, but also x = 13, 15, 17 and 19 occur [10]. For higher numbers, polyploidy is assumed followed by structural rearrangements. Chromosome numbers of 32 and 64 were reported for *M. officinalis* ssp. *officinalis* and ssp. *altissima*, respectively [20]. Ssp. *altissima* has been suggested as an ancestor of the cultivated diploid ssp. *officinalis* despite being tetraploid [9]. Davis [5] separated the ssp. *inodora* (Bornm.) Bornm. based on the long patent hairs on stems and fairly distinct, triangular middle tooth of upper calyx lip and mentioned intermediates between all three subspecies. Pignatti [17] described beside *M. officinalis* L. (2n = 32) the species *M. romana* Miller as synonymous with ssp. *altissima* because of the absence of any offspring (2n = 64). Darlington and Wylie [4] presented the haploid chromosome number for *M. officinalis* with x = 8.

Lemon balm is of increasing importance, resulting in rising growth area and investigations for improved new varieties. Better winter hardiness, higher content of essential oil and higher yield of *M. folium* (lemon balm leaves) are of special interest in these programmes. Adaptation of technologies for acceleration of the breeding process using doubled haploid lines has been initiated. Therefore, the knowledge on the genome structure and number of chromosomes in connection with the taxonomical structure of *M. officinalis* is indispensable. In order to determine the ploidy level of 40 accessions and the chromosome number of the haploid *M. officinalis* genome, we employed flow cytometry and FISH using rDNA-specific probes. Beside di- and tetraploid accessions, first time triploid accessions (2n = 3× = 48) have been identified for *M. officinalis*.

Results and discussion

Genome size determination by flow cytometry of *M. officinalis* revealed for 23 accessions a diploid, four accessions a triploid and 13 accessions a tetraploid ploidy level (Table 1).

To confirm the ploidy predictions, chromosome numbers were determined and multicolour FISH was performed with 18/25S- and 5S rDNA-specific probes (Table 2). In *M. officinalis* 5S and 18/25S rDNA were localized on different chromosomes. Unlike this result in some other genera e.g. *Helianthus, Brassica* and *Alstroemeria* [18, 19, 1] localization of 5S and 18/25S rDNA on the same chromosome was found. Analysis of six selected accessions revealed for the putative diploid genotypes a chromosome number of 32 and two chromosome pairs exhibiting either 18/25S rDNA- or 5S rDNA-specific signals (Fig. 1a, b). Putative triploid accessions contained 48 chromosomes and revealed six distinct hybridization signals: three 18/25S rDNA and three 5S rDNA (Fig. 1c, d). The last category exhibited 64 chromosomes and eight signals: four 18/25S rDNA and four 5S rDNA sites (Fig. 1e, f).

In accessions showing six or eight hybridization signals, the intensity of rDNA signals varied. Metaphases of accessions showing six signals displayed one strong and two weak 18/25S rDNA signals (Fig. 1c). The same was true for 5S rRNA sites. Metaphases of genotypes showing eight rDNA signals for each marker, one chromosome pair displayed a strong and one pair a weak hybridization signal (Fig. 1e). Hence accessions with 32 chromosomes and one pair of 18/25S rDNA and 5S rDNA signals are diploid (2n = 2× = 32). Accessions with 48 chromosomes and three rDNA signals are triploid (2n = 3× = 48), and accessions with 64 chromosomes and two pair of rDNA signals are tetraploid (2n = 4× = 48). Therefore, a chromosome basic number of x = 16 in the genus *Melissa* is likely. In contrast, Darlington and Wylie [4] postulated a basic chromosome number of x = 8 and a somatic number of 2n = 32, without giving any information about investigated subspecies. Later on, Tutin et al. and Pignatti [20, 17] reported a chromosome number of 2n = 32 for *M. officinalis* ssp. *officinalis. M. officinalis* ssp. *altissima* and *M. romana* has 2n = 64 chromosomes [17, 20]. The reports of Heidari et al. [11] and Murin [15] of 2n = 32 chromosomes and Löve [14] of 2n = 64 chromosomes for *M. officinalis* provide no information about the analysed subspecies.

Two scenarios regarding the origin of triploid balm can be postulated: an unreduced gamete of a diploid plant formed triploid offspring after fertilization with a haploid gamete. Alternatively, a tetraploid parent hybridized with diploid parent and formed triploid offspring. The different signal intensity of either 5S or 18/25S rDNA sites in triploid balm could be explained by the different copy number of parental rDNA repeats.

Phenotype characterization of triploid balm

The plant phenotype of the four triploid accessions was characterized. Stems of the specimen BLBP 78, BLBP 88, BLBP 111 and BLBP 113 reached 120 to 140 cm (ssp. *officinalis* 50 to 80 cm, [17]) with tendency of laying down and entangling. The size of leaves (7.6 cm length, standard deviation s 0.59 and 5.4 cm width, s 0.56) was bigger than diploid ssp. *officinalis* type leaves (6.9 cm length, standard deviation s 0.51 and 4.5 cm width, s 0.42). The internodes are longer (9.4 cm, standard deviation s 0.99) in comparison with diploid ssp. *officinalis* (6.5 cm, s 0.78) accessions (Table 3). Triploid accessions had very good cold resistance and regenerated faster after winter and harvesting cuts (results not shown). The colour of leaves

Table 1 Determined ploidy level of balm accessions (*Melissa officinalis*) from Leibniz institute for plant genetics and crop plant research at Gatersleben, Germany (IPK) and bavarian state institute for agriculture at Freising, Germany (LfL) based on flow cytometry

Accession No.	COL[a]	Taxonomical group of *Melissa officinalis* L.[b]	Origin[b]	Ploidy level
BLBP 5	LfL	*M. officinalis* L.	southern France	diploid
BLBP 8	LfL	*M. officinalis* L.	Spain	diploid
BLBP 19	LfL	*M. officinalis* L.	Germany	diploid
BLBP 27	LfL	*M. officinalis* L.	Germany	diploid
BLBP 33	LfL	*M. officinalis* L.	botanical garden Halle, Germany	diploid
BLBP 48	LfL	*M. officinalis* L.	Germany	diploid
BLBP 49	LfL	*M. officinalis* L.	Unknown	diploid
BLBP 50	LfL	*M. officinalis* L.	Unknown	diploid
BLBP 78	LfL	*M. officinalis* L.	France	triploid
BLBP 87	LfL	*M. officinalis* L.	Georgia, landrace	diploid
BLBP 88	LfL	*M. officinalis* L.	botanical garden Liege, France	triploid
BLBP 111	LfL	*M. officinalis* L.	Hungary	triploid
BLBP 113	LfL	*M. officinalis* L.	France	triploid
MELI 1	IPK	*M. officinalis* L.	Unknown	diploid
MELI 2	IPK	*M. officinalis* L.	Unknown	diploid
MELI 4	IPK	*M. officinalis* L.	East Germany	diploid
MELI 5	IPK	*M. officinalis* L. 'Erfurter Aufrechte'	East Germany	diploid
MELI 6	IPK	*M. officinalis* L.	Germany	diploid
MELI 7	IPK	*M. officinalis* L. 'Citra'	Unknown	tetraploid
MELI 8	IPK	*M. officinalis* L.	Georgia	diploid
MELI 10	IPK	*M. officinalis* L.	France	diploid
MELI 11	IPK	*M. officinalis* L. 'ital. Melissa, Cedronella'	Italy	diploid
MELI 12	IPK	*M. officinalis* L.	Italy	tetraploid
MELI 13	IPK	*M. officinalis* L.	Georgia	diploid
MELI 14	IPK	*M. officinalis* L. subsp. *altissima* (Sibth. & Sm.) Arcang.	Italy	tetraploid
MELI 15	IPK	*M. officinalis* L. subsp. *altissima* (Sibth. & Sm.) Arcang.	Italy	tetraploid
MELI 16	IPK	*M. officinalis* L. subsp. *officinalis*	Unknown	diploid
MELI 17	IPK	*M. officinalis* L.	Greece	tetraploid
MELI 18	IPK	*M. officinalis* L. subsp. *altissima* (Sibith. & Sm.) Arcang.	Unknown	tetraploid
MELI 19	IPK	*M. officinalis* L.	Italy	tetraploid
MELI 20	IPK	*M. officinalis* L.	Italy	tetraploid
MELI 21	IPK	*M. officinalis* L. subsp. *altissima* (Sibth. & Sm.) Arcang.	Albania	tetraploid
MELI 22	IPK	*M. officinalis* L.	Turkey	tetraploid
MELI 23	IPK	*M. officinalis* L. subsp. *altissima* (Sibth. & Sm.) Arcang.	Italy	tetraploid
MELI 24	IPK	*M. officinalis* L. subsp. *altissima* (Sibth. & Sm.) Arcang.	Italy	tetraploid
MELI 25	IPK	*M. officinalis* L. 'Zitronenmelisse'	Unknown	diploid
MELI 26	IPK	*M. officinalis* L.	Armenia	diploid
MELI 27	IPK	*M.* sp.	Italy	diploid
MELI 28	IPK	*M. officinalis* L.	Italy	tetraploid
D9597	IPK	*M.* sp. 'Zitronenmelisse'	Unknown	diploid

[a]COL: collection
[b]taxonomical classification according to information of collection holders

Table 2 Level of ploidy, number of chromosomes and signals resulting from FISH with 18/25S rDNA and 5S rDNA probes in accessions of balm (*Melissa officinalis*)

Accession	Number of chromosomes	Signals of 18/25S rDNA	Signals of 5S rDNA	Ploidy
BLBP 48	32	2	2	diploid
MELI 1	32	2	2	diploid
BLBP 78	48	3	3	triploid
BLBP 113	48	3	3	triploid
MELI 14	64	4	4	tetraploid
MELI 22	64	4	4	tetraploid

was bluish to greyish green in comparison to green leaves of ssp. *officinalis* (Fig. 2). Young leaves of triploid accessions have indumenta similar to ssp. *altissima* (Fig. 2) whereas according to Tutin et al. [20], leaves of diploid ssp. *officinalis* are glabrescent or sparsely hairy above, glandular-puberulent and more or less sparsely hairy beneath. Adult leaves of triploid accessions are more similar to ssp. *officinalis*. Stems of triploid accessions are greyish- or whitish-tomentose beneath with similarity to ssp. *altissima*. The triploid accessions are not lemon-scented. They had a soap-like, nauseating scent. These plants had an inconspicuous formation of flowers but do not produce any seed, neither under conditions of isolation nor by open pollination, likely due to meiotic problems. These first time described triploid accessions were propagated by cuttings and are cytologically and morphologically stable for at least six years.

Conclusions

The basic chromosome number of x = 16 is reported for the first time for the species *M. officinalis* and for family Labiatae.

This is the first characterization of triploids in *M. officinalis*. These triploid accessions are sterile but cytologically and morphologically stable. The length and width of the leaves and the length of internodes exceeded the comparable data for diploid accessions but are not significant for all characters.

For exact origin analysis of triploids as well as the characterization of allotetraploid or autotetraploid character of tetraploids analysis of meiotic chromosome pairing is necessary. Chromosome specific molecular markers would open the chance to ascertain the level of similarity of homoeologous groups of chromosomes. This is a prerequisite for better characterization of phylogenetic distance of *M. officinalis* ssp. *altissima* in comparison to ssp. *officinalis*.

Material and methods

Plant materials

A set of 40 accessions of *M. officinalis* have been characterized, of which 27 and 13 were provided from the Federal *ex-situ* Collection of Agricultural and Horticultural Plants of the Leibniz Institute for Plant Genetics and Crop Plant Research at Gatersleben, Germany (IPK) and the Bavarian State Institute for Agriculture at Freising, Germany (LfL) respectively. LfL collection contained old varieties and breeding material from middle and western Europe, the IPK collection includes mainly landraces and wild types from eastern and middle Europe (Table 1).

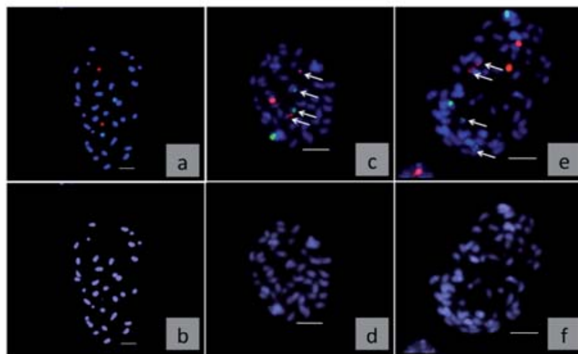

Fig. 1 a-f: Mitotic metaphase chromosomes of balm (*Melissa officinalis*) after FISH with 18/25S rRNA and 5S rRNA-specific probes. Above (**a**, **c**, **e**): FISH, red 18/25S rDNA, green 5S rDNA, arrows mark weak signals, below (**b**, **d**, **f**): DAPI stained chromosomes; a: diploid MELI 1, 2n = 2x = 32; c: triploid BLBP 78, 2n = 3x = 48, e: tetraploid MELI 22, 2n = 4x = 64. The size bars equals 5 μm

Table 3 Length and width of second leaf and length of second internode from base of stem for balm (*Melissa officinalis*) of different ploidy level

ploidy	leaf length [cm]				leaf width [cm]				internodes [cm]				n^b
level	Min	mean	max	s^a	min	mean	max	s^a	min	mean	max	s^a	
diploid	6.2	6.9	7.5	0.51	4.0	4.5	5.1	0.42	5.8	6.5	8.3	0.78	9
triploid	6.9	7.6	8.1	0.59	4.7	5.4	6.0	0.56	8.4	9.4	10.5	0.99	4

s^a standard deviation
n^b number of accessions; For each accession at least ten plants were measured

All IPK accessions were grown from seeds whereas all LfL accessions were propagated by cuttings starting with a single plant. Radish (*Raphanus sativus* L.) was used as genome size marker in flow cytometry.

Evaluation of ploidy level by flow cytometry
Measuring of relative DNA amount of nuclei occurred by flow cytometry (Facs calibur, Becton Dickinson, BD) with a red fluorescence laser as basis for detection of ploidy level. For each probe, leaf material was chopped with razor blades in 500 μl nuclei extraction buffer (CyStain PI absolute P, Sysmex) and stained with the corresponding staining buffer, containing 5 % polyvinyl-pyrrolidone 25 (Serva) and 0.6 % propidium iodide (Serva). Immediately after staining, the nuclei suspension was filtered using a 5 ml polystyrene round-button tube with a cell-strainer cap (BD). For reference, radish was measured in separate sample after five samples of balm.

Chromosome preparation
M. officinalis seeds were germinated and the cell division synchronized with 1.25 mM hydroxyurea for 17 h. For vegetative multiplied accessions (LfL), root tips from potted plants were used. In contrast to Pan et al. [16], the recovery time after hydroxyurea treatment was 24 h at 6 °C. Root tips were fixed in ethanol-acetic acid (3:1, 24 h) and stored in 70 % ethanol at −20 °C. After washing with aqua dest. root tips were digested with an enzyme mixture (4 % cellulase, 'Onozuka R-10', Serva and 1 % pectlyase Y-23 (Seishin Pharmaceutical)) in 75 mM KCl and 7.5 mM Na-EDTA, (pH 4.0 for 36 min. at 37 °C, [12]). Softened root tips were squashed in 45 % acetic acid. After removal of the coverslip by freezing (−84 °C) the slides were air dried overnight at 24 °C and stored at −20 °C.

Fluorescence *in situ* hybridization
The 18S-5.8S-25S rDNA loci were detected with a 220 bp-long 25S repeat-specific probe labelled with biotin-16-dUTP (Boehringer Mannheim) during polymerase chain reaction (PCR) amplification of the genomic DNA of *Allium ampeloprasum* with primers designed according to the sequence published by Yokota et al. [21]. For the localization of 5S rRNA genes, a 117 bp fragment obtained after PCR amplification from the same genomic DNA with specific primers coding for this region [8] was used. The labelling of this amplified probe was performed with digoxigenin-11-dUTP (Boehringer Mannheim). The hybridization mixture contained 80 ng of each DNA probe (5S and 25S rDNA) and 10 μg of salmon-sperm DNA in 20 μl of hybridization buffer (50 % deioinized formamide, 10 % dextran sulphate, 2 x SSC) per slide [19].

The FISH procedure was performed according to Fuchs and Schubert [7] with the following modifications: prior to hybridization, slides were incubated in 50 ng/μl of DNase-free RNase in 2 x SSC for 1 h at 37 °C, washed three times in 2 x SSC for 5 min and treated with 0.5 ng/μl of proteinase K for 10 min at 37 °C, followed by three times washing in 2 x SSC for 15 min. The slides were then postfixed in 4 % paraformaldehyde for 10 min, washed three times in 2 x SSC for 15 min, dehydrated in a graded ethanol series (70, 80 and 96 %) at −20 °C, and air-dried. The hybridization mixture (probe) was denaturated (80 °C, 7 min), incubated on ice (about 5 min), dropped onto

Fig. 2 leaf colour and density of pubescence in balm (*Melissa officinalis*); **a**: diploid, **b**: triploid and **c**: tetraploid. The size bars equals 1 cm

slides, covered with coverslips, and sealed with rubber cement. Probes and chromosomes were denatured together on a heated desk (7 min, 80 °C). The slides were then incubated overnight at 37 °C in a humidity chamber. After hybridization and removing the coverslips, the slides were washed in 2 x SSC at 37 °C three times for 5 min each, followed by three 5 min stringent washes in 0.3 x SSC at 60 °C and then blocked for 30 min at 37 °C with a solution of 4 x SSC, 3 % BSA and 0.1 % Tween 20. The biotinylated probe was detected with 10 ng/µl of streptavidin-Cy3 (Dianova) and amplified with two steps of 10 ng/µl of biotinylated anti-streptavidin (Vector) and 10 ng/µl strepavidin-Cy3. Together with the first amplification step of the biotin labelled probe, the detection of the digoxigenin labelled probe with 9 ng/µl of anti-digoxigenin-fluorescein (Roche) was done and then amplified with 8 ng/µl anti-sheep-fluorescein (Dianova). Chromosomes were counterstained and embedded in 15 µl of DAPI-VECTASHIELD antifade solution (Vector Laboratories). Images were captured for each fluorescence dye separately with a cooled CCD camera system Axiocam (Zeiss) on a microscope Axioimager Z1 (Zeiss) with the following filter combinations: 02 (DAPI), 10 (FITC) and 20 (Cy3). Pseudocoloration and mergence of images were done with software of the Isis program (Metasystems).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JK carried out the flow cytometry, measurement of leaves and internodes, chromosome preparation and performed the statistical analysis and drafted the manuscript. OS carried out fluorescence *in situ* hybridization. UK participated in the flow cytometry. FM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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3 ABSTRACT, INTRO.: KITTLER *ET AL.* 2017, EVALUATION OF 28 BALM AND LEMON BALM (*MELISSA OFFICINALIS*) ACCESSIONS FOR CONTENT AND COMPOSITION OF ESSENTIAL OIL AND CONTENT OF ROSMARINIC ACID:

3 EVALUATION OF 28 BALM AND LEMON BALM (*MELISSA OFFICINALIS*) ACCESSIONS FOR CONTENT AND COMPOSITION OF ESSENTIAL OIL AND CONTENT OF ROSMARINIC ACID:

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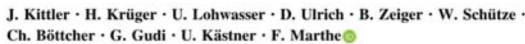
RESEARCH ARTICLE

Evaluation of 28 balm and lemon balm (*Melissa officinalis*) accessions for content and composition of essential oil and content of rosmarinic acid



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Abstract Lemon balm (*Melissa officinalis* L.) is a well-known aromatic and medicinal plant. Because of its proven sedative, spasmolytic and antiviral effects, it is often used in watery or alcoholic extracts for self-medication or pharmaceutical purposes. Its therapeutic effect is due to the content of essential oil and rosmarinic acid. A set of 28 balm accessions was evaluated for the variability of essential oil content and composition and the content of rosmarinic acid. For analysis of secondary metabolites distillation, gas chromatography and high performance liquid chromatography was used. The content of essential oil in the first cut of the years 2009 and 2010 varied in this study in a range between 0.02 and 0.48% and in the second cut between 0.01 and 0.72%. The rosmarinic acid content of the collection ranged in the year 2009 for the first cut from 3.99 to 8.78% and in the second cut from 2.45 to 6.78%. Ploidy was determined for all accessions and two cytotypes were found: diploid 2n = 2x = 32 (15 accessions), tetraploid 2n = 4x = 64 (13 accessions). Via statistical analyses two chemotypes of essential oil were found: chemotype citral and chemotype germacrene D.

Keywords Content and composition of essential oil · Content of rosmarinic acid · Genetic resources · Chemotypes

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Introduction

Lemon balm (*Melissa officinalis* L.) is a perennial plant of the family Lamiaceae (syn. Labiatae). The origin of this crop plant is not well-defined, however the Mediterranean Region or Western Asia is considered as area of origin (Hanelt and IPK 2001). Today lemon balm is grown in subtropical and temperate climate worldwide. Dried lemon balm leaves („Melissae folium) from Central European cultivation have an average essential oil content of 0.05–0.30% (Adzet et al. 1992b) and up to 6% rosmarinic acid (Parnham and Kesselring 1985). It is mainly used for watery or alcoholic extracts. The essential oil is a complex mixture of volatile compounds isolated by physical

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means. The compounds mainly derived from three biosynthetic pathways, the mevalonate pathway, leading to sequiterpenes, the methyl-erithrytol-pathway leading to mono and diterpenes and the shikimic acid pathway leading to phenylpropenes (Baser and Buchbauer 2010). The essential oil of *M. officinalis* contains mainly six substances in larger amounts the monoterpenes citronellal, (*Z*)-citral, (*E*)-citral, which cause the lemon like scent of lemon balm and the sesquiterpenes germacrene D, β-caryophyllene and β-caryophyllene oxide. Citronellol is found only in very small amounts.

Lemon balm is a well-established crop used for phytopharmaceuticals, as an aromatic plant and in traditional folk medicine. A wide spectrum of secondary metabolites exists in lemon balm like essential oil constituents, phenolic acid derivatives, flavones, flavonols and triterpenoids. For medicinal use, appropriate levels of essential oil with lemon fragrance and rosmarinic acid are required (Bomme et al. 2013; DAB 2015; Ph. Eur. 8 2014; Krüger et al. 2010). In this study these accessions are called lemon balm whereas genotypes without lemon fragrance are named balm. *M. officinalis* has proven sedative, carminative, antispasmodic, bacteriostatic, antibacterial, antiinflammatory, antioxidative and antivirus activity (Forster et al. 1980; Hefendehl 1970; Koch Heitzmann and Schultze 1984; Parnham and Kesselring 1985; Toth et al. 2003; Wagner and Sprinkmeyer 1973). Lemon balm is a component of 23 phytopharmaceuticals and two homeopathics, which are currently registered in Germany (FNR 2016). It is used for treatment of nervous agitation, indigestions and infection with herpes simplex (Mazzanti et al. 2008; Wölbling and Leonhardt 1994). There is also strong evidence that lemon balm has a positive effect on patients who suffer from Alzheimer's disease (Akhondzadeh et al. 2003; Moradkhani et al. 2010).

For *M. officinalis* different systems for subclassifying exist (Davis 1982; Hanelt and IPK 2001; Pignatti 1982; Tutin et al. 1972). All grown lemon balm conforming to pharmacopoeia (Ph. Eur. 8 2014) belong to diploid *M. officinalis* subsp. *officinalis* and have 2n = 2x = 32 chromosomes whereas the tetraploid subsp. *altissima* (Sibth. et Sm.) Arcangeli has 2n = 4x = 64 chromosomes and no lemon fragrance. Kittler et al. (2015) also mention triploid accessions (2n = 3x = 48), which are genetically stable but sterile and also without lemon-like scent.

The majority of investigated balm collections were compiled for practical aspects and included only lemon balm (Adzet et al. 1992a, b; Bahtiyarca Bagdat and Cosge 2006; Basker and Putievsky 1978; Bomme et al. 2008; Mrlianova et al. 2002; Seidler-Łożykowska et al. 2013). For the presented results accessions of balm as well as lemon balm were included in the set. This collection was characterised for amount and composition of essential oil and content of rosmarinic acid in connection with ploidy. Besides differences in the amount of essential oil two chemotypes (ct.) were found in the collection: ct. citral and ct. germacrene D. The presented results can be used as a source for new breeding attempts and as a basis for research in taxonomy, yield enhancement and ontogenesis of lemon balm.

Materials and methods

Material and cultivation

Of the 28 accessions obtained from the federal ex situ collection of agricultural and horticultural plants at the Leibniz Institute of Plant Genetics and Crop Plant Research at Gatersleben, Germany (IPK) 16 were assigned to *M. officinalis* subsp. *officinalis*, twelve to *M. officinalis* subsp. *altissima* (Sibth. et Sm.) Arcangeli. The set consisted of collected material from Central Europe and old varieties (Table 1, https://gbis.ipk-gatersleben.de). In the evaluation trials, 20 plants from seeds per plot were planted at two different trial sites, JKI Quedlinburg (Qlb) and IPK Gatersleben (Gat) on 15/05/2008 in a planting scheme of 50 cm × 30 cm. The inner six plants of the plot were harvested as a representative probe. The plants were harvested on 15/06/2009 (first cut) and the 17/09/2009 (second cut), dried and analysed in Quedlinburg. In the year 2010 the plants were harvested on 22/07/2010 (first cut) dried and analysed in Quedlinburg. The experimental station Quedlinburg, Germany is located at the northern foreland of Harz Mountains. Altitude: 140 m, black earth, valuation index of field: 91–97, annual depth of rainfall and average annual temperature 2009: 683 mm, + 9.59 °C 2010: 751.2 mm, + 7.88 °C and 2011: 324 mm, + 10.01 °C. The experimental station Gatersleben, Germany is also located at the northern foreland of Harz Mountains. Altitude: 110.5 m, soil type: black cotton soil,

Table 1 Ploidy level and passport data of IPK lemon balm and balm collection (*Melissa officinalis*)

Accession no.	Scientific name	Origin	Ploidy
MELI 1	*M. officinalis* L. subsp. *officinalis*	Unknown	Diploid
MELI 2	*M. officinalis* L. subsp. *officinalis*	Unknown	Diploid
MELI 4	*M. officinalis* L. subsp. *officinalis*	Germany	Diploid
MELI 5	*M. officinalis* L. subsp. *officinalis*	Germany	Diploid
MELI 6	*M. officinalis* L. subsp. *officinalis*	Germany	Diploid
MELI 7	*M. officinalis* L. subsp. *officinalis*	Unknown	Diploid
MELI 8	*M. officinalis* L. subsp. *officinalis*	Georgia	Diploid
MELI 9	*M. officinalis* L. subsp. *officinalis*	France	Diploid
MELI 10	*M. officinalis* L. subsp. *officinalis*	France	Diploid
MELI 11	*M. officinalis* L. subsp. *officinalis*	Italy	Diploid
MELI 12	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Italy	Tetraploid
MELI 13	*M. officinalis* L. subsp. *officinalis*	Georgia	Diploid
MELI 14	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Italy	Tetraploid
MELI 15	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Italy	Tetraploid
MELI 16	*M. officinalis* L. subsp. *officinalis*	Unknown	Diploid
MELI 17	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Greece	Tetraploid
MELI 18	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Unknown	Tetraploid
MELI 19	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Italy	Tetraploid
MELI 20	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Italy	Tetraploid
MELI 21	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Albania	Tetraploid
MELI 22	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Turkey	Tetraploid
MELI 23	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang	Italy	Tetraploid
MELI 24	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Italy	Tetraploid
MELI 25	*M. officinalis* L. subsp. *officinalis*	Unknown	Diploid
MELI 26	*M. officinalis* L. subsp. *officinalis*	Armenia	Diploid
MELI 27	*M. officinalis* L. subsp. *officinalis*	Italy	Diploid
MELI 28	*M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang.	Italy	Tetraploid
D 9597 (MELI 29)	*M. officinalis* L. subsp. *officinalis*	Unknown	Diploid

valuation index of field: 93–97, annual depth of rainfall and average annual temperature 2009: 583.6 mm, + 9.6 °C 2010: 638.5 mm, + 7.9 °C and 2011: 487.6 mm, + 10.0 °C. For every year and trial, a first and second cut was harvested shortly before full flowering of the accessions. Between the plots no ontogenetic differences could be found which were more extensive than within the plots, so all accessions of one trial were harvested on the same day, due to the mostly homogeneous developmental stage of the plants. The plants were cut 10 cm above the ground and were dried on a bench drying system (Lü-Ku GmbH, Germany, 30 °C, 72 h). Leaves and stems were separated by hand. To ensure a representative sample at least 100 g air-dried leaf material per sample were used.

Methods

Determination of ploidy level

Measuring of relative DNA amount of nuclei occurred by flow cytometry. The measurement followed the procedure described in Kittler et al. (2015).

Determination of essential oil content

The content of essential oil is displayed in % (v/m), which conforms ml/100 g air-dried leaf material. Distillation was performed for the collection in 2009 and 2010 at ÖPV-JKI using a classical Clevenger-type apparatus described in the European Pharmacopoeia (Ph. Eur. 6.0, 2008). After hydro-distilling of 25 g air-

Table 2 Summary of 6 essential oil components and rosmarinic acid of lemon balm (*Melissa officinalis*)

#	Substance	CAS	Substance group	Odour	Co-el
1	Citronellal	106-23-0	Monoterpene aldehyde	Sweet, floral, rose	1
2	(*Z*)-Citral (neral)	106-26-3	Monoterpene aldehyde	Sweet, citrus, lemon	1
3	(*E*)-Citral (geranial)	141-27-5	Monoterpene aldehyde	Citrus, lemon	1
4	β-Caryophyllene	87-44-5	Sesquiterpene, bicyclic	Spicy, woody, citrus	1
5	Germacrene D	37839-63-7	Sesquiterpene, monocyclic	–	0
6	β-Caryophyllene oxide	1139-30-6	Sesquiterpene oxide	Woody, spicy	0
7	Rosmarinic acid	20283-92-5	Caffeic acid ester	–	1

CAS, Chemical Abstracts Service Registry Number; co-el, coelution of authentic reference substances. All compounds were purchased by Sigma-Aldrich, Taufkirchen, Germany or Carl Roth, Karlsruhe, Germany. Number 1–6 were semi-quantified by GC-FID. Number 7 was analyzed by HPLC

dried leaf material (120 min, 200 mL aqua dest.) essential oil content was estimated. All accessions were distilled once for every harvested sample. A sample consisted of the leaves from six plants.

Determination of essential oil composition

The composition of the essential oil was analysed by gas chromatography (GC, 6890 N, Agilent Technologies, US) equipped with flame ionisation detector and HP-5 capillary column 50 m × 0.32 mm ID × 0.52 µm film thickness. Injector temperature was 250 °C and detector temperature was 280 °C. The oven temperature started at 60 °C and then programmed to 120 °C at a rate of 3 °C/min, then to 250 °C at a rate of 8 °C/min and held at final temperature for 10 min. The carrier gas was hydrogen at 2 mL/min constant flow. The sample volume injected was 1 µL and the split rate was 1:20. The essential oil obtained by distillation was diluted in isooctane 1:1000 before injection. The relative amounts (norm-%) of individual components are based on the peak areas. The components of essential oil were determinated by MS identification, retention time and co-elution of authentic references (Table 2). All essential oil probes were analysed with two analytical replications. The targeted semi-quantification of essential oil components was focussed on the six compounds mentioned in Table 2.

Determination of rosmarinic acid content

Air-dried and crushed balm leaves (approx. 2 g) were powdered for 7 min at 80 s^{-1} using a mixer mill (MM2, Retsch) and a steel ball of 8 mm diameter. Ground leaf material (50 ± 1 mg) was weighed into a 2-mL polypropylene tube and 1.5 mL 50% (v/v) aqueous ethanol was added. The mixture was thoroughly vortex-mixed for 20 s and sonicated for 10 min at 50 °C. After centrifugation (10 min, 12.000×*g*, 22 °C), the supernatant was transferred to a 10 mL volumetric flask. The remaining residue was extracted twice again with 1.5 mL 50% (v/v) aqueous ethanol as described above. The resulting extracts were combined and their volume adjusted to 10 mL using 50% (v/v) aqueous ethanol. An aliquot of the resulting solution was filtered into a vial using a syringe filter of 0.45 µm pore size and stored at 6 °C until analysis. Rosmarinic acid analyses were performed on an AGILENT 1100 Series HPLC system comprising a degasser (G1322A), binary pump (G1312A), autosampler (G1329A), autosampler thermostat (G1330A), column compartment (G1316A) and diode array detector (G1315A). Extracts (injection volume 5 µL) were separated on an Accucore C18 column (3 × 150 mm, 2.6 µm particle size, Thermo Scientific) using 0.1% (v/v) aqueous formic acid and methanol as eluent A and B, respectively. The following binary gradient programme at a flow rate of 500 µL/min was used: 0–20 min, linear from 20 to

100% B; 20–24, isocratic, 20% B. The column temperature was maintained at 40 °C. Rosmarinic acid was detected at 320 nm with a spectral bandwidth of 4 nm. ChemStation software (version B.03.02) was applied for controlling the instrument, data acquisition and quantitative analysis. Rosmarinic acid was quantified using an external standard calibration (calibration range 10–5000 ng on column). A linear calibration model was used resulting in $R^2 > 0.999$.

Statistical analysis

The statistical software package Statistica 7.1 from StatSoft (Tulsa, USA) was used for the calculation of the principal component analysis (PCA) and the cluster analysis (agglomerative method, single linkage, euclidic distances). The heat map was created with the Multi Experiment Viewer TM4 from the MEV Development Team (www.tm4.org). Descriptive stastistic was carried out with SPSS 16.0.

Results

Evaluation of ploidy level by flow cytometry

All 28 accessions were measured for their relative amount of DNA and ploidy level was deduced (Table 1). All 16 diploid (2n = 2x = 32) accessions belong to *M. officinalis* L. subsp. *officinalis*. From the 12 tetraploid (2n = 4x = 64) accessions 11 belong to *M. officinalis* L. subsp. *altissima* (Sibth. et Sm.) Arcang. and one (MELI 23) to *M. officinalis* L. subsp. *officinalis*. Based on the ploidy value this could be corrected into subsp. *altissima*.

Hierarchical cluster analysis and heat map

A hierarchical cluster analysis was carried out with the content and relative amount of the essential oil components: citronellal, (*Z*)-citral, (*E*)-citral, β-caryophyllene, germacrene D, β-caryophyllene oxide and non identified substances as sum of remaining, mostly unknown minor substances ("sum of unknown substances"). Citronellol was excluded because of the very low contents in all accessions. For calculation analytic data from hydro-distillations of the second cut 2009 (Electronical Supplement Table 2) were used. In the resulting dendrogram accessions are clearly divided into two groups (Fig. 1). The first group included 15 accessions, which were all diploid. The second group consisted of 13 accessions. In this group 12 were tetraploid and one (MELI 9) was diploid (Fig. 1; Table 1).

The relative concentrations of essential oil components were standardised and used to generate a heat map (Fig. 2). All accessions with high amount of citronellal and/or (*Z*)-citral and (*E*)-citral are clustered in a group of 15 accessions, identical to the bigger group of dendrogram. These accessions had low amounts of β-caryophyllene, germacrene D and β-caryophyllene oxide.

The accessions with very low or without citronellal, (*Z*)-citral and (*E*)-citral had germacrene D as main component and clustered also. These 12 accessions had also higher amounts of β-caryophyllene and β-caryophyllene oxide. They conform to the smaller group of the dendrogram.

This clear dividing of the accessions leads us to postulate two chemotypes (ct.): ct. citral and ct. germacrene D.

Content of essential oil

The content of essential oil varied for the evaluated material in a range between 0.01 and 0.72%. Differences were found between accessions, the first and second cut of a year, the trial sites and experimental years.

In the collection (Electronical Supplement Table 1) the essential oil content in 2009 ranged for the first cut between MIN = 0.02%, MEDIAN = 0.04% and MAX = 0.16%, the second cut MIN = 0.01%, MEDIAN = 0.08% and MAX = 0.72% and in first cut 2010 between MIN = 0.03%, MEDIAN = 0.06% and MAX = 0.48% (Fig. 3). In both years and on both trial sites, the accession MELI 10 generated the highest amount of essential oil. Of the 28 accessions 15 had a lemon-like scent and 13 had a different, scarcely lemon-like odour. The accessions with lemon-like scents are identical to the bigger groups of dendrogram and heat map (Figs. 1, 2).

Composition of essential oil for chemotype citral and chemotype germacrene D

For characterisation of chemotypes, two main groups were found by cluster analysis. The ranges, median

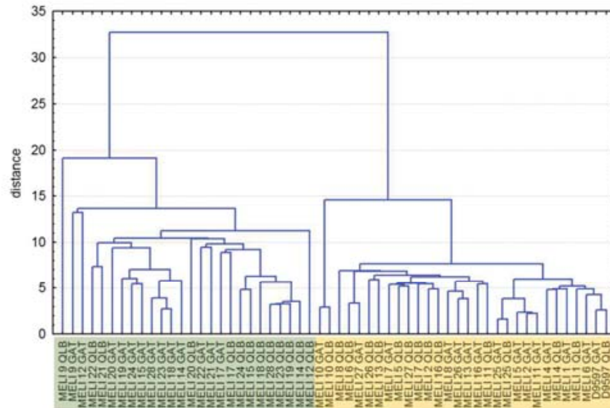

Fig. 1 Cluster analysis for content and relative amount of essential oil components [citronellal, (*Z*)-citral, (*E*)-citral, β-caryophyllene, germacrene D, β-caryophyllene oxide and non identified substances] of 28 *M. officinalis* accessions. Citronellol was excluded. Data of second cut 2009. For each accession two data sets were classified separately at trial site JKI Quedlinburg (QLB) and IPK Gatersleben (GAT). Orange: bigger group of 15 accessions, green: smaller group of 13accessions

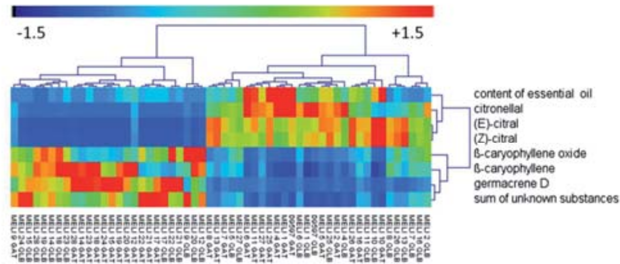

Fig. 2 Heat map for amount and relative concentration of essential oil components [citronellal, (*Z*)-citral, (*E*)-citral, β-caryophyllene, germacrene D, β-caryophyllene oxide and non identified substances, without citronello] of 28 *M. officinalis* accessions. The relative concentrations were standardised before hierarchical cluster analysis (HCL). Color code: deep blue (low) −1.5 and red (high) +1.5

values and standard variations of essential oil components for chemotype citral and chemotype germacrene D are given in Table 3. The attribution of accessions to the chemotypes follows the results of the cluster analysis. In relation of all components of essential oil the ct. citral had highest values as sum of citronellal, (*Z*)-citral and (*E*)-citral whereas in ct. germacrene D this component has highest values.

Substances with a concentration $< 1\%$ were defined as minor components. The number of these mostly non-identified substances and their concentration differed but always ranged as minor components. There were up to 30 such substances and they were grouped in Fig. 2 as "sum of unknown substances". The group of unknown substances (6.8–58.27%) reached high percentages only in the accessions

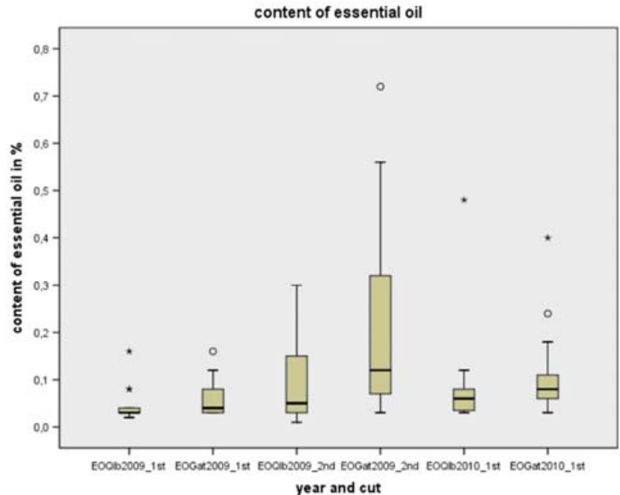

Fig. 3 Box plots for content of essential oil (EO) of 28 *M. officinalis* accessions for the years 2009 and 2010 for both trial sits at JKI Quedlinburg (QLB) and IPK Gatersleben (GAT). Boxes: interquartile range, including 50% of the values; bar: median; whiskers: maximum and minimum value, excluding outliers; circle: aberration, star: extreme value

having very low amounts of essential oil and belonged to ct. germacrene D.

The most common type is chemotype citral [MELI 1, MELI 2, MELI 4, MELI 5, MELI 6, MELI 7, MELI 8, MELI 10, MELI 11, MELI 13, MELI 16, MELI 25, MELI 26, MELI 27, D 9597 (MELI 29)]. The most frequent components in the first cuts had a range of citronellal (7.74–26.1%), (*Z*)-citral (15.17–32.36%), (*E*)-citral (23.58–45.33%), β-caryophyllene (10.74–23.18%), germacrene D (5.47–16.27%), β-caryophyllene oxide (12.98–28.75%) and the second cut citronellal (4.67–45.21%), (*Z*)-citral (13.83–32.96%), (*E*)-citral (25.14–49.66%), β-caryophyllene (3.28–15.47%), germacrene D (0–4.95%), β-caryophyllene oxide (0.59–8.87%) (Fig. 4A, B and Electronical Supplement Table 2). These accessions showed a pronounced difference between the compositions of essential oil in the first and second cut. The essential oil of the first cut was often a mixture of the main components and contained a lot of minor components. In the second cut the ratio between the components strongly shifted towards citral-components, which could represent up to 85% of the essential oil. 15 of 28 accessions were defined as chemotype citral, had a fresh lemon-like scent and a medium to high content of essential oil.

In chemotype germacrene D (MELI 14, MELI 15, MELI 17, MELI 18, MELI 19, MELI 21, MELI 22, MELI 23, MELI 24, MELI 28) the accessions had germacrene D (5.05–59.11%), β-caryophyllene oxide (3.94–38.39%) and β-caryophyllene (2.44–25.71%) as most frequent components in the first cuts (Fig. 4C, D). In these accessions no or very low concentrations of citral components [citronellal, (*Z*)-citral, (*E*)-citral] occurred in the first and second cuts (Fig. 4C, D, Electronical Supplement Table 2). In the second cut the main components were germacrene D (28.12–51.64%), β-caryophyllene (7.9–29.92%), β-caryophyllene oxide (2.81–15.64%) (Fig. 4C, D). Ten of 28 accessions were defined as chemotype germacrene D, had no lemon-like scent and a low content of essential oil.

In three accessions, MELI 9, MELI 12 and MELI 20 the level of germacrene D and β-caryophyllene as well as β-caryophyllene oxide was nearly equal. In these accessions low concentrations of citronellal, (*Z*)-citral or (*E*)-citral occurred in the first cut and second cut, which made them distinguishable from ct. germacrene D. Because of the lack of an obvious abundant essential oil component they could not be assigned to a chemotype. The essential oils contained in the first cut 2009: citronellal (0–1.59%), (*Z*)-citral

Table 3 Ranges, medians and standard deviations s^2 of content of rosmarinic acid and content and composition of essential oil for 28 accessions of *Melissa officinalis* grouped for chemotypes (ct.): ct. citral, ct. germacrene D and not assigned accessions. First cuts 2009 and 2010 and second cut 2009

Chemotype	Content of rosmarinic acid	Content of essential oil (in %)	Citronellal (in %)	(*Z*)-citral (in %)	(*E*)-citral (in %)	β-Caryophyllene (in %)	Germacrene D (in %)	β-Caryophyllene-oxide (in %)	Sum of unknown substances (in %)
Ct. citral, 15 accessions, first cuts 2009 and 2010									
Minimum	3.28	0.02	1.13	5.17	8.22	3.78	0.66	2.37	10.16
Maximum	8.78	0.48	26.10	32.36	45.33	23.18	16.27	28.75	42.72
Median	5.43	0.08	6.27	14.67	23.26	9.34	3.92	11.71	24.17
s^2	1.52	0.08	5.34	5.81	7.93	4.69	3.82	5.58	6.67
Second cut, 2009									
Minimum	3.91	0.03	4.67	13.83	25.14	3.28	0	0.59	7.03
Maximum	6.78	0.72	45.21	32.96	49.66	15.47	4.95	8.87	19.1
Median	5.36	0.2	19.54	18.89	33.48	7.60	2.16	2.20	10.99
s^2	0.69	0.18	11.53	4.54	5.86	3.55	1.51	2.42	3.64
Ct. germacrene D, 10 accessions first cuts 2009 and 2010									
Minimum	3.47	0.03	0.00	0.00	0.00	2.44	5.05	3.94	19.98
Maximum	7.16	0.12	0.30	0.28	0.49	25.71	59.11	38.39	58.27
Median	5.13	0.04	0.01	0.02	0.06	12.47	31.40	18.47	36.31
s^2	0.94	0.02	0.06	0.08	0.14	6.92	15.07	9.83	10.2
Second cut, 2009									
Minimum	2.45	0.01	0.00	0.00	0.00	7.90	28.12	2.81	21.55
Maximum	6.57	0.08	0.49	0.39	0.76	29.92	50.48	15.64	55.25
Median	4.66	0.04	0.07	0.04	0.11	19.15	39.93	8.19	28.83
s^2	1.14	0.02	0.15	0.21	0.11	7.32	7.29	4.00	8.77
Unidentified type, 3 accessions, first cuts 2009 and 2010									
Minimum	4.03	0.03	0.00	0.00	0.00	1.45	1.10	16.78	30.4
Maximum	6.67	0.10	1.59	1.93	3.20	31.36	29.22	43.71	66.05
Median	5.08	0.06	0.00	0.51	0.88	20.87	19.20	24.68	45.4
s^2	0.77	0.02	0.51	0.67	1.12	11.30	11.31	9.70	12.94
Second cut, 2009									
Minimum	3.74	0.03	0.00	0.00	0.00	13.90	7.88	5.22	20.85
Maximum	5.84	0.12	4.88	3.70	7.21	35.88	36.48	27.89	44.23
Median	4.94	0.03	1.11	1.29	2.13	24.61	23.32	15.44	29.5
s^2	0.80	0.04	2.30	1.66	3.31	7.28	9.61	9.45	8.50

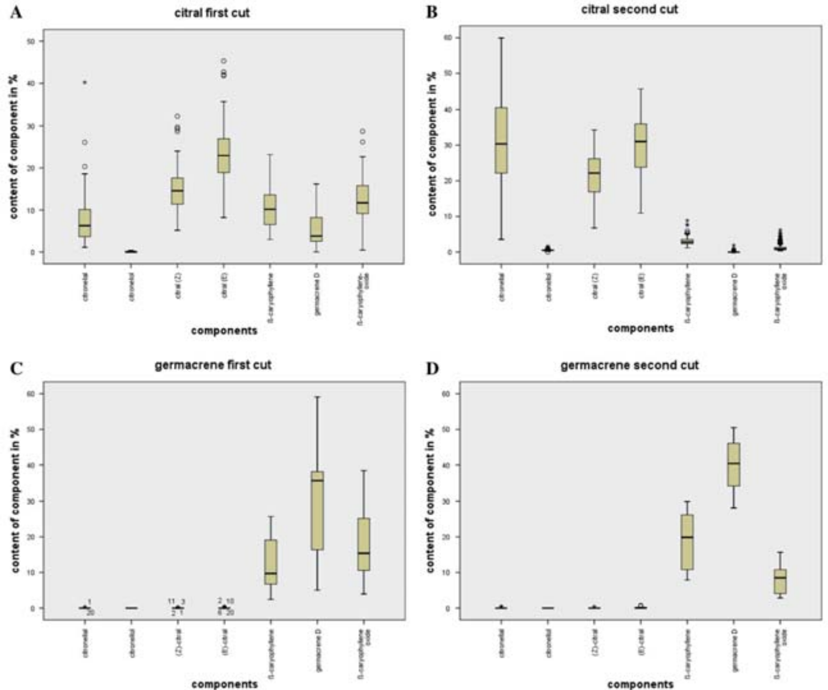

Fig. 4 Box plots for 25 accessions of *M. officinalis* grouped for chemotypes of essential oil: chemotype citral: 15 accessions (**A**, **B**) and chemotype germacrene D: 10 accessions (**C**, **D**) for the first cuts 2009 and 2010 and second cut 2009. Content of essential oil in %. Components in % of essential oil; boxes: interquartile range, including 50% of the values; bar: median; whiskers: maximum and minimum value, excluding outliers; circle: aberration, star: extreme value

(0–1.45%), (*E*)-citral (0–2.58%), β-caryophyllene (1.45–22.87%), germacrene D (1.1–29.18%), β-caryophyllene oxide (21.52–43.71 in the second cut 2009: citronellal (0–4.88%), (*Z*)-citral (0–3.7%), (*E*)-citral (0–7.21%), β-caryophyllene (13.9–35.88%), germacrene D (7.88–36.48%), β-caryophyllene oxide (5.22–27.89%) and in the first cut 2010: citronellal (0–0.52%), (*Z*)-citral (0–1.93%), (*E*)-citral (0–3.2%), β-caryophyllene (19.37–31.36%), germacrene D (15.52–29.22%), β-caryophyllene oxide (16.78–24.84%). These three accessions were named non identified type. The accessions MELI 09 and MELI 12 may found in the left and right boundaries of the dendrogramme (Fig. 1). Accession MELI 20 can be found on the sixth from the left and in the middle of the green group.

Content of rosmarinic acid

The rosmarinic acid content ranged in the first cuts of 2009 and 2010 from 3.28% (diploid *M. officinalis* MELI 8) to 8.78% (diploid *M. officinalis* MELI 1) and in the second cut 2009 from 2.45% (tetraploid *M. officinalis* subsp. *altissima* MELI 17) to 6.78% [diploid *Melissa* sp. D 9597 (MELI 29), Table 3, Electronical Supplement Table 1]. The MEDIAN

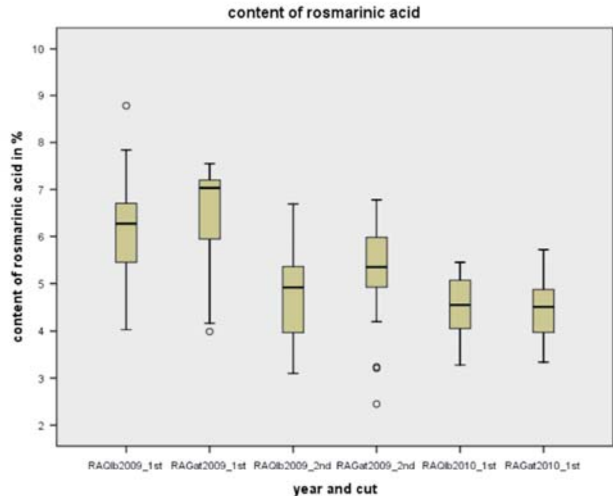

Fig. 5 Box plots for relative rosmarinic acid content (RA) of the collection for the years 2009 (first and second cut) and 2010 (first cut) for both trial sites Quedlinburg (Qlb) and Gatersleben (Gat). Boxes: interquartile range, including 50% of the values; bar: median; whiskers: maximum and minimum value, excluding outliers; circle: outliers; no extreme values are in the data sets

values of first cuts 2009 at trial sites Quedlinburg and Gatersleben were 6.28% and 7.03% respectively. In second cut of 2009 and first cut of 2010 at both trial sites the MEDIAN values were lower than in first cut 2009: Quedlinburg second cut 2009 4.52% and first cut 2010 4.93% and Gatersleben second cut 2009 5.35% and first cut 2010 4.55% (Fig. 5).

Discussion

Content of essential oil

The variation of essential oil content between accessions, cuts and years can be remarkable. Askari and Sefidkon (2004) and Bomme et al. (2002) reported values between 0.14 and 0.25%. Bahtiyarca Bagdat and Cosge (2006) reported values between 0.01 and 0.25%. Under Spanish conditions values up to 0.8% are possible (Bomme et al. 2002). Adzet et al. (1992a) describes the improvement by selection of balm varieties with individual spices up to 0.68% and more. Basker and Putievsky (1978) depict in their evaluation of two cultivars values between 0.6 and 0.7%. Mrlianova et al. (2002) reported contents from 0.06 to 0.16% in the analysis of 16 accessions.

The content of essential oil in the evaluated collection ranged from 0.01 to 0.78%. Authors of previous evaluations reported very low contents of essential oil in the first cut, which were harvested shortly before flowering (Bomme et al. 2002). We also found very low contents in samples of the first cut (Table 3). The data confirms the data of the literature. Essential oil content shows a strong dependence from biotic and abiotic conditions in different growing periods and genetic constitution of the accessions. But for the tested accessions of *M. officinalis* subsp. *altissima* a strong correlation exists to tetraploidy and very low contents of essential oil. All this accessions belong to chemotype germacrene D. The data could not be related to literature, because those chemotypes are not explicitly mentioned. Accession MELI 23 belongs unquestionable to ct. germacrene D and is tetraploid. Accession MELI 9 is diploid and belongs to *M. officinalis* subsp. *officinalis*. The cluster analysis positioned it with bigger phylogenetic distance to the group of tetraploid ct. germacrene D. But the composition of the essential oil allowed no assignment to a chemotype. This is also true for tetraploid MELI 12 and MELI 20, both *M. officinalis* subsp. *altissima*.

Composition of essential oil

Tavares et al. (1996) state the composition of (*E*)-citral plus (*Z*)-citral 48%, citronellal 39.47% and β-caryohyllene with 2.37% and Bahtiyarca Bagdat and Cosge (2006) 39% citronellal, 33% citral [citronellol, linalool, (*E*)-citral and geraniol (*Z*)-citral]. We determined the components citronellal, (*Z*)-citral, (*E*)-citral, citronellol, β-caryophyllene, germacrene D, and β-caryophyllene-oxide and found different chemotypes in the collection. The variation of essential oil content depending on biotic and abiotic conditions is higher than variation in composition of essential oil. This is true if we accept ranges for the main components like Sharafzadeh et al. (2011) and Azizi et al. (2009). There are prominent differences between compositions of first and second cut. But no shift between citronellal, (*Z*)-citral and (*E*)-citral to germacrene D could be found. There is no transition from chemotype citral to chemotype germacrene D. Literature mentions changes between citronellal, (*Z*)-citral and (*E*)-citral. Hefendehl (1970) observed ranges from 8.7 to 96.6% citral and citronellal 0.9–39%. He suggested that young leaves show a high content of citral, while older leaves show a higher content of citronellal. He assumed the age of leaves could be a reason for that phenomenon or that the content and composition of essential oil could be a matter of leaf position. Because of this it is not justified to subdivide chemotype citral for amount of citronellal, (*Z*)-citral and (*E*)-citral (Fig. 2).

The handbook of essential oil (Baser and Buchbauer 2010) reports that phytochemical polymorphism can often occur between different plant organs. In *Origanum vulgare* subsp. *hirtum* different oil glands of the same plant showed a polymorphism (Johnson et al. 2004). Hose et al. (1997) also showed that the composition in essential oil glands of *M. officinalis* changes during ontogenese. This is mentioned for other species (*Salvia sclarea* L., *Satureja hortensis* L., *Salvia officinalis* L.) as well (Grassi et al. 2004; Johnson et al. 2004; Novak et al. 2006; Schmiderer et al. 2008).

Content of rosmarinic acid

The proven pharmaceutical effects of rosmarinic acid make it an important component of balm. The content of rosmarinic acid is an important quality requirement and a raised content is a desired aim of breeding programs.

The aim of the evaluation was first time characterization of a set of lemon balm and balm for their content of rosmarinic acid according to the HPLC method, which is obligatory in Pharmacopoeia Europaea from 2009 (Ph. Eur. 6 2009). All tested 28 accessions fulfil the requirements of Ph. Eur. for rosmarinic acid. But tetraploid types are excluded from pharmaceutical use because of the different composition of the essential oil, which has no lemon like scent. The differences between years (first cut 2009 and first cut 2010) are high with 2.48% of MEDIAN values for GAT and 1.35% QLB. Also differences between first and second cuts are high with 1.68% for GAT and 1.7% for QLB between MEDIAN values. Lower differences were found between experimental places JKI Quedlinburg and IPK Gatersleben: first cut 2009: 0.3%, second cut 2009: 0.5%, first cut 2010: 0.1%.

M. officinalis produces a high amount of rosmarinic acid in comparison with other species of family Lamiaceae. This can be used for special breeding programme to create lines with stable very high yield of rosmarinic acid. The better winter hardiness and higher fresh mass production of tetraploid accessions can be used for production of rosmarinic acid even they are not conforming to Ph. Eur.

Conclusions

The screening of 28 accessions of *M. officinalis* showed an individual pattern of essential oil for every accession, which shifted in quantity as well in quality. The presented results suggest the existence of two different chemotypes in the species *M. officinalis*. We declare the two chemotypes ct. citral and ct. germacrene D. The chemotype citral showed a shifting between citronellal, (*Z*)-citral and (*E*)-citral, in the first and second cut. But the main component always was a citral component. In this study the chemotypes coincide with the ploidy level. The chemotype citral is diploid whereas the ct. germacrene D is always tetraploid. To verify this appearance more non-citral accessions need to be characterised. Three accessions did not belong to ct. citral but could also not assigned to ct. germacrene D. Content of rosmarinic acid is a quality requirement of the Ph. Eur. There is also a

rising demand for lemon balm with high content of rosmarinic acid. This evaluation depicts accessions, which can be useful for breeding new varieties with high content of rosmarinic acid.

The presented data are valuable contribution for characterization of the rosmarinic acid content in the species *M. officinalis* and offer in connection with status of ploidy (Kittler et al. 2015) and amount and composition of essential oil a prerequisite for taxonomical studies within the species. For this a core collection should be selected with maximum of variability for characterized traits. Candidate accessions should be tested more intensive again.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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4 CONTENT AND COMPOSITION OF ESSENTIAL OIL AND CONTENT OF ROSMARINIC ACID IN LEMON BALM AND BALM GENOTYPES (*MELISSA OFFICINALIS*):

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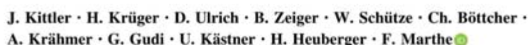
RESEARCH ARTICLE

Content and composition of essential oil and content of rosmarinic acid in lemon balm and balm genotypes (*Melissa officinalis*)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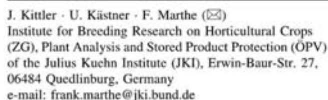
Abstract Lemon balm (*Melissa officinalis* L.) is used since ancient times because of its sedative, spasmolytic and antiviral effects. Its therapeutic impact is due to the content of essential oil and rosmarinic acid. A set of 68 *M. officinalis* genotypes was evaluated for content and composition of essential oil and the content of rosmarinic acid. For all genotypes the level of ploidy was determined. The 68 genotypes were clone plants grown and evaluated for two years at Quedlinburg. For analysis of secondary metabolites distillation, gas chromatography and high performance liquid chromatography was used. The content of essential oil varied in this study in ranges from 0.03 to 0.33% for the second cut 2010 and 0.01–0.35% for the second cut 2011. The rosmarinic acid content ranged in the year 2010 from 3.67 to 7.55% and in the year 2011 from 4.92 to 8.07%. Via statistical analyses two chemotypes of essential oil were found: chemotype citral and chemotype β-caryophyllene oxide. Ploidy was determined for all genotypes and two cytotypes were found: diploid 2n = 2x = 32 (62 of 68 genotypes) and triploid 2n = 3x = 48 (6 of 68 genotypes).

Keywords Content and composition of essential oil · Genetic resources · Chemotypes

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10722-018-0635-4) contains supplementary material, which is available to authorized users.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Introduction

The perennial crop plant lemon balm (*Melissa officinalis* L.), family Lamiaceae (syn. Labiatae) originating from Mediterranean Region or Western Asia (Hanelt and IPK 2001), is grown worldwide in temperate and subtropical regions. Beside essential oil (Aziz and El-Ashry 2009) a wide spectrum of other active constituents was found in lemon balm like phenolic acid derivatives, flavonoids, and triterpenes (Bomme et al. 2013).

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Lemon balm is used for phytopharmaceuticals (*Melissa herba* and *Melissa folium*), as an aromatic plant and in traditional folk medicine. The essential oil, which contains mainly citronellal, (*E*)-citral, (*Z*)-citral and rosmarinic acid, a caffeic acid ester with 3,4-dihydroxyphenyllactic acid are the active pharmaceutical ingredients in lemon balm (Schilcher 2016), which has proven sedative, antibacterial and antivirus activity (Toth et al. 2003). There is also strong evidence that lemon balm has a positive effect on patients, who suffer from Alzheimer's disease (Moradkhani et al. 2010).

Lemon balm collections have been compiled for practical aspects therefore most accessions have lemon like scent (Adzet et al. 1992a, b; Bahtiyarca Bagdat and Cosge 2006; Basker and Putievsky 1978; Bomme et al. 2008; Mrlianova et al. 2002; Seidler-Łożykowska et al. 2013). In a set of 28 accessions Kittler et al. (2018) describe beside diploid populations with lemon like scent also 12 tetraploid accessions. Of these tetraploid accessions ten had a soap-like scent and belong to a chemotype which had germacrene D as the main component of the essential oil.

This study presents evaluation data for content and composition of essential oil, content of rosmarinic acid and level of ploidy. The collection includes beside accessions with typical lemon like scent also genotypes with off odours. These genotypes are named balm whereas genotypes with lemon like scent are called lemon balm in this study. This evaluation depicts genotypes, which can be useful for breeding new varieties with high content of rosmarinic acid and essential oil.

Materials and methods

Material and cultivation

The collection of the Bavarian State Institute for Agriculture at Freising, Germany (LfL) consisted of 68 genotypes of *M. officinalis*, preserved by vegetative maintenance (electronical supplement Table 1). These genotypes originated from botanical gardens, private collections, breeding material or varieties. Wild collected material was not included. The field trials were conducted in Quedlinburg in 68 plots of 20 clone plants and two repeats planted in a scheme of 50 cm × 45 cm. The trial was planted on 10/04/2009. The inner 6 plants of the plot were harvested as a representative probe. The second cut of the plants was harvested on 19/09/2010 and 21/09/2011, respectively, dried at Quedlinburg and subsequently analysed. Between the plots no ontogenetic differences could be found which were more extensive than within the plots, so all accessions of one trial were harvested on the same day, due to the mostly homogeneous developmental stage of the plants. The plants were cut 10 cm above the ground and were dried on a bench drying system (Lü-Ku GmbH, Germany, 30 °C, 72 h). Leaves and stems were separated by hand. To ensure a representative sample at least 100 g air-dried leaf material per sample were used. The experimental

Table 1 Summary of 6 essential oil components and rosmarinic acid of lemon balm (*Melissa officinalis*)

	Substance	Kovats retention index RI	CAS	Substance group	Odour	Co-el
1	Citronellal	1159	106-23-0	Monoterpene aldehyde	Sweet, floral, rose	1
2	(*Z*)-citral (neral)	1255	106-26-3	Monoterpene aldehyde	Sweet, citrus, lemon	1
3	(*E*)-citral (geranial)	1287	141-27-5	Monoterpene aldehyde	Citrus, lemon	1
4	β-caryophyllene	1442	87-44-5	Sesquiterpene, bicyclic	Spicy, woody, citrus	1
5	germacrene-D	1504	37839-63-7	Sesquiterpene, monocyclic	–	0
6	β-caryophyllene oxide	1615	1139-30-6	Sesquiterpene oxide	Woody, spicy	0
7	Rosmarinic acid		20283-92-5	Caffeic acid ester	–	1

CAS chemical abstracts service registry Number. co-el—coelution of authentic reference substances. All compounds were purchased by Sigma-Aldrich, Taufkirchen, Germany or Carl Roth, Karlsruhe, Germany. Number 1–6 were semi-quantified by GC-FID. Number 7 was analyzed by HPLC

station Quedlinburg, Germany was located at the northern foreland of Harz Mountains. Altitude: 140 m, black earth, valuation index of field: 91–97, annual depth of rainfall and average annual temperature 2009: 683 mm, + 9.59 °C 2010: 751.2 mm, + 7.88 °C and 2011: 324 mm, + 10.01 °C.

Methods

Determination of ploidy level

Measuring of relative DNA amount of nuclei occurred by flow cytometry. The measurement followed the procedure described in Kittler et al. (2015).

Determination of essential oil content

For the collection distillation was performed at PytoLab Gmbh Co. KG, Vestenbergsgreuth, Germany in 2010 and 2011 following Ph. Eur. 6.0, 2.8.12 (Ph. Eur. 6 2008). All genotypes were analysed separately in a double assay and both repeats averaged. A sample consisted of the leaves from six plants. The content of essential oil is displayed in % (v/m), which conforms mL/100 g air-dried leaf material.

Determination of essential oil composition

The composition of the essential oil was analysed by gas chromatography (GC, 6890 N, Agilent Technologies, U.S.) equipped with flame ionisation detector and HP-5 capillary column 50 m × 0.32 mm ID × 0.52 µm film thickness. Injector temperature was 250 °C and detector temperature was 280 °C. The oven temperature started at 60 °C and then programmed to 120 °C at a rate of 3 °C/min, then to 250 °C at a rate of 8 °C /min and held at final temperature for 10 min. The carrier gas was hydrogen at 2 mL/min constant flow. The sample volume injected was 1 µL and the split rate was 1:20. The essential oil obtained by distillation was diluted in isooctane 1:1000 before injection. The relative amounts (norm-%) of individual components are based on the peak areas. The components of essential oil were determinated by MS identification, retention time and co-elution of authentic references (Table 1). The used spectra databases were NIST and WILEY. All essential oil probes were analysed with two analytical replications. The targeted semi-quantification of essential oil components was focussed on the six compounds mentioned in Table 1.

Determination of rosmarinic acid

Air-dried and crushed balm leaves (approx. 2 g) were powdered for 7 min at 80 s^{-1} using a mixer mill (MM2, Retsch) and a steel ball of 8 mm diameter. Ground leaf material (50 ± 1 mg) was weighed into a 2–mL polypropylene tube and 1.5 mL 50% (v/v) aqueous ethanol was added. The mixture was thoroughly vortexed for 20 s and sonicated for 10 min at 50 °C. After centrifugation (10 min, 12.000×*g*, 22 °C), the supernatant was transferred to a 10—mL volumetric flask. The remaining residue was extracted twice again with 1.5 mL 50% (v/v) aqueous ethanol as described above. The resulting extracts were combined and their volume adjusted to 10 mL using 50% (v/v) aqueous ethanol. An aliquot of the resulting solution was filtered into a vial using a syringe filter of 0.45 µm pore size and stored at 6 °C until analysis. Rosmarinic acid analyses were performed on an AGILENT 1100 Series HPLC system comprising a degasser (G1322A), binary pump (G1312A), autosampler (G1329A), autosampler thermostat (G1330A), column compartment (G1316A) and diode array detector (G1315A). Extracts (injection volume 5 µL) were separated on an Accucore C18 column (3 × 150 mm, 2.6 µm particle size, Thermo Scientific) using 0.1% (v/v) aqueous formic acid and methanol as eluent A and B, respectively. The following binary gradient programme at a flow rate of 500 µL/min was used: 0 – 20 min, linear from 20 to 100% B; 20 – 24, isocratic, 20% B. The column temperature was maintained at 40 °C. Rosmarinic acid was detected at 320 nm with a spectral bandwidth of 4 nm. ChemStation software (version B.03.02) was applied for controlling the instrument, data acquisition and quantitative analysis. Rosmarinic acid was quantified using an external standard calibration (calibration range 10–5000 ng on column). A linear calibration model was used resulting in R^2>0.999.

Statistical analysis

The statistical software package Statistica 7.1 from StatSoft (Tulsa, USA) was used for the calculation of the principal component analysis (PCA) and the

cluster analysis (agglomerative method, single linkage, euclidic distances). The heat map was created with the Multi Experiment Viewer TM4 from the MEV Development Team (www.tm4.org). Descriptive statistic was carried out with SPSS 16.0 (IBM, USA).

Results

Evaluation of ploidy level by flow cytometry

All 68 accessions of the collection were measured for their relative amount of DNA and ploidy level was deduced (electronical supplement Table 1). Of the genotypes 62 were diploid (2n = 2x = 32) and six were triploid (2n = 3x = 48): BLBP75, BLBP78, BLBP88, BLBP111 (73B), BLBP112 (75B), BLBP113 (78B). No tetraploid accessions were found.

Content of essential oil

The genotypes of the collection generated in the second cut of 2010 essential oil contents between MIN = 0.03, MEDIAN = 0.21 and MAX = 0.33% and in the second cut of 2011 MIN = 0.01, MEDIAN = 0.20 and MAX = 0.35% (electronical supplement Table 1). The mean essential oil content of all genotypes for the second cut was 0.2% in 2010 and 0.19% in 2011 (Fig. 1). A lemon-like scent was determined in 62 out of 68 genotypes. In both years, genotypes BLBP75, BLBP78, BLBP85, BLBP88, BLBP94, BLBP111, BLBP112 and BLBP113 showed very low contents of essential oil. These accessions except BLBP85 and BLBP94 had a soap-like off-scent.

Composition of essential oil

For the essential oil of all 68 genotypes the following main components were characterised: citronellal, (*E*)-citral, (*Z*)-citral, citronellol, β-caryophyllene, germacrene D, and β-caryophyllene oxide. Substances with a concentration less than 1% were defined as minor components. The number of these mostly non-identified substances and their concentration differed but always ranged as minor components. There were up to 30 such substances and they were grouped as "sum of unknown substances". The concentrations ranged for citronellal from 1.32 to 59.95%, (*E*)-citral from 0.78 to

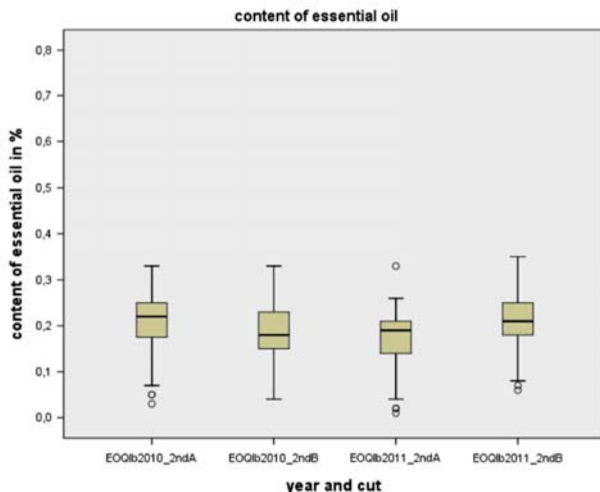

Fig. 1 Content of essential oil (EO) at trial location Quedlinburg (Qlb) of 68 genotypes of *Melissa officinalis* for the second cut 2010 and 2011, two repeats (**a**, **b**), Boxes: interquartile range, including 50% of the values; bar: median; whiskers: maximum and minimum value, excluding outliers; circle: aberration

Table 2 Minimum, maximum, median and s^2 values for content of rosmarinic acid and content and composition of essential oil (second cut 2010 and 2011) of 68 balm and lemon balm genotypes (*Melissa officinalis*) specified for chemotypes (ct.) citral and ct. ß-caryophyllene-oxide are shown for content of rosmarinic acid, content of essential oil and composition of essential oil

Chemotype	Content of rosmarinic acid	Content of essential oil	Citronellal	(*Z*)-citral	(*E*)-citral	ß-caryophyllene	Germacrene D	ß-caryophyllene-oxide	Sum of unknown substances
ct. citral, 62 accessions, second cut 2010									
Minimum	3.67	0.01	3.58	15.67	21.65	1.24	0.00	0.39	7.51
Maximum	7.55	0.35	45.17	34.27	45.65	5.09	0.49	4.28	27.42
Median	4.91	0.20	25.61	25.10	33.96	2.59	0.00	0.90	10.38
s^2	0.67	0.06	12.81	5.41	7.61	1.18	0.25	1.15	5.71
ct. citral, 62 accessions, second cut 2011									
Minimum	4.92	0.04	3.80	6.79	10.95	1.17	0.00	0.45	5.66
Maximum	8.07	0.33	59.95	32.80	44.94	8.87	1.93	6.21	38.00
Median	6.65	0.21	38.43	17.22	24.03	3.04	0.00	0.88	12.94
s^2	0.61	0.06	9.02	3.71	4.87	0.91	0.09	0.79	2.27
ct. β—caryphyllene oxide, 6 accessions, second cut 2010									
Minimum	4.39	0.03	1.32	0.50	0.78	4.70	2.01	29.61	34.39
Maximum	6.23	0.10	6.90	3.02	4.54	10.16	8.52	54.07	43.35
Median	5.09	0.07	2.99	1.43	2.23	9.51	5.50	38.90	37.52
s^2	0.49	0.02	1.57	0.82	1.24	2.01	2.39	9.17	2.91
ct. β—caryphyllene oxide, 6 accessions, second cut 2011									
Minimum	6.48	0.02	1.98	0.98	1.54	7.70	1.79	18.83	31.56
Maximum	7.95	0.11	7.26	5.47	8.45	18.64	13.61	46.98	44.39
Median	7.49	0.07	4.24	4.05	5.36	14.07	7.07	28.10	36.82
s^2	0.57	0.03	1.56	1.70	2.54	3.47	3.80	8.40	3.37

45.65%, (*Z*)-citral from 0.50 to 34.27%, β-caryophyllene from 1.17 to 18.64%, germacrene D from 0 to 13.61% and β-caryophyllene oxide from 0.39 to 54.07% (electronical supplement Table 2).

Hierarchical cluster analysis and heat map

A hierarchical cluster analysis was carried out with the content of essential oil and the relative amounts of the essential oil components: citronellal, (*E*)-citral, (*Z*)-citral, β-caryophyllene, germacrene D, β-caryophyllene oxide and non identified substances as sum of remaining, mostly unknown minor substances ("sum of unknown substances"). Citronellol was excluded because of the very low contents in all accessions. For calculation analytic data from hydro-distillations of the second cut 2010 and 2011 (electronical supplement Table 2) were used. In the resulting dendrogram accessions are clearly divided into two groups (Fig. 2). The smaller group consist of six genotypes which were all triploid [BLBP75, BLBP78, BLBP88, BLBP111 (73B), BLBP112 (75B), BLBP113 (78B)] and the bigger group includes 62 genotypes, all diploid.

After standardisation of essential oil content and concentrations of essential oil components hierarchical cluster analysis was diagrammed as a heat map (Fig. 3). In the heat map two main groups were found. The first includes content of essential oil and essential oil components citronellal, (*E*)-citral and (*Z*)-citral. The second group is characterised by β-caryophyllene, germacrene D, β-caryophyllene oxide and non identified substances.

In the second cut the citral-component as the sum of the citronellal and diastereomeres (*E*)-citral, (*Z*)-citral can represent up to 85% of the essential oil. In connection with higher essential oil content the citral-

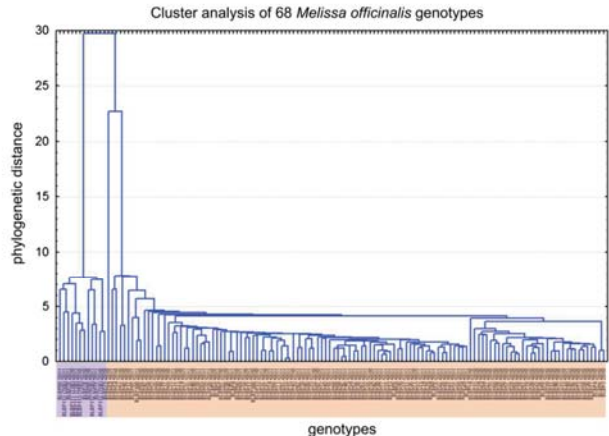

Fig. 2 Cluster analysis for content and relative amount of essential oil components [citronellal, (*Z*)-citral, (*E*)-citral, β-caryophyllene, germacrene D, β-caryophyllene oxide and non identified substances] of 68 *Melissa officinalis* genotypes. Citronellol was excluded. Data for second cut 2010 and 2011 are mean values of two replications. Orange: bigger group, 62 genotypes; Violet: smaller group, 6 genotypes

components characterises the first group. This group is established as chemotype (ct.) citral. The concentrations of essential oil components in this group reached for citronellal 3.58–59.95%, (*E*)-citral 10.95–45.65%, (*Z*)-citral 6.79–34.27%, β-caryophyllene 1.17–8.87%, germacrene D 0–1.93%, β-caryophyllene oxide 0.39–6.21% (Table 2, Fig. 4A). As chemotype citral 62 of 68 accessions were defined.

In the collection genotypes exist with the main component β-caryophyllene oxide. They belong to the second group in connection with relative content of β-caryophyllene, germacrene D and non identified substances. This group is established as chemotype β-caryophyllene oxide. The concentration of essential oil components for chemotype β-caryophyllene oxide where β-caryophyllene 4.7–18.64%, germacrene D 1.79–13.61% and β-caryophyllene oxide 18.38–54.07% (Table 2, Fig. 4B). For the genotypes of this chemotype traces of citral-components could be detected. As chemotype β-caryophyllene oxide, six of 68 genotypes were defined which had no lemon scent [BLBP75, BLBP78, BLBP88, BLBP111 (73B), BLBP112 (75B), BLBP113 (78B)].

The six genotypes of chemotype β-caryophyllene oxide are identical with the smaller group of hierarchical cluster analysis and the triploid group.

Content of rosmarinic acid

In the collection the rosmarinic acid content ranged for the second cut in the year 2010, from MIN = 3.67%, MEDIAN = 4.94% to MAX = 7.55%. Replication A of diploid genotype of *M. officinalis* BLBP8 reached 3.67% and, replication A of diploid genotype of *M. officinalis* BLBP22 7.55%. In the year 2011 the values for the second cut ranged from MIN = 4.92%, MEDIAN = 6.68% to MAX = 8.07%. Replication B of diploid genotype of *M. officinalis* BLBP33 reached 4.92%, replication A of diploid genotype of *M. officinalis* BLBP52 8.07% (electronical supplement Table 1). The mean values of 2010 for replication A were 5.26% and for replication B 4.86%. For both replications in 2011 the mean values were higher: A 6.82% and B 6.54%. The mean and median values do not differ (Fig. 5).

Discussion

Content of essential oil

The content of essential oil varies in a great range between accessions, cuts and years. Askari and Sefidkon (2004) and Bomme et al. (2002) reported values between 0.14 and 0.25%. Bahtiyarca Bagdat

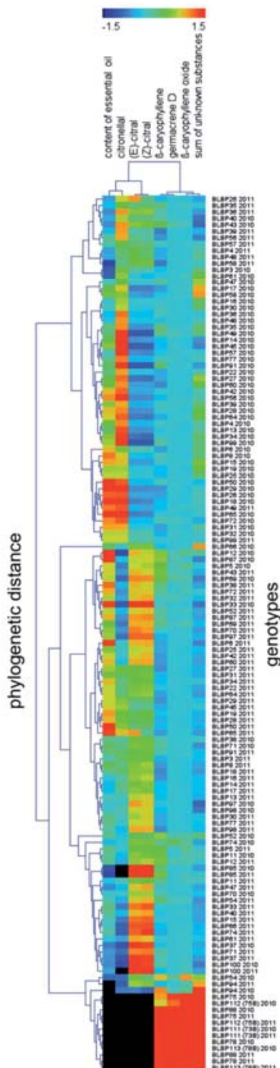

Fig. 3 Heat map for amount and relative concentration of essential oil components [citronellal, (*Z*)-citral, (*E*)-citral, β-caryophyllene, germacrene D, β-caryophyllene oxide and non identified substances, without citronellol] of 68 *Melissa officinalis* accessions. The relative concentrations were standardised before hierarchical cluster analysis (HCL). Colour code: deep blue/black (low/absent) − 1.5 to red (high) + 1.5

and Cosge (2006) reported values between 0.01 and 0.25%. Bomme et al. (2002) also stated that values up to 0.8% could only be realised under Spanish conditions. He cited the data of Adzet et al. (1992a), which describes the improvement by selection of balm varieties with an average content 0.3% essential oil to cultivars with an average of more than 0.5% and extreme values up to 0.68% and more. But also Basker and Putievsky, (1978) depict values between 0.6 and 0.7% in their evaluation of two cultivars. Mrlianova et al. (2002) analysed 16 accessions and reported contents from 0.06 – 0.16%.

The content of essential oil in the evaluated collection ranged from 0.01 – 0.35%. The data confirms the values of the literature. The range shows a strong dependence from biotic and abiotic conditions, different harvesting years and genetic make-up of the genotypes. Especially the chemotype ct. β-caryophyllene oxide had very low contents of essential oil (Table 2). The data could not be related to other literature, because those chemotypes are not explicitly mentioned. The data suggest that the β-caryophyllene oxide chemotype had very low content of essential oil. Authors of previous evaluations reported very low contents of essential oil in the first cut, which were harvested shortly before flowering (Bomme et al. 2002).

Composition of essential oil

The composition of essential oil for the genotypes showed an individual pattern of ingredients. Tavares et al. (1996) state the composition of (*E*)-citral + (*Z*)-citral 48%, citronellal 39.47% and β-caryohyllene with 2.37% and Bahtiyarca Bagdat and Cosge (2006) 39% citronellal, 33% citral (citronellol, Linalool, (*E*)-citral and geraniol (*Z*)-citral). We determined the components (*E*)-citral, (*Z*)-citral, citronellal, citronellol, germacrene D, β-caryophyllene and β-caryophyllene-oxide and found different chemotypes in the collections. Every genotype had its own pattern of metabolites (Fig. 3). That is the reason why we suggest ranges for the main components like Sharafzadeh et al. (2011) and Azizi et al. (2009). A major shift between (*E*)-citral, (*Z*)-citral, citronellal to germacrene or β-caryophyllene could not be investigated. There is no transition from ct. citral to ct. β-caryophyllene. If an accession showed a special chemotype, it could not be transferred to another

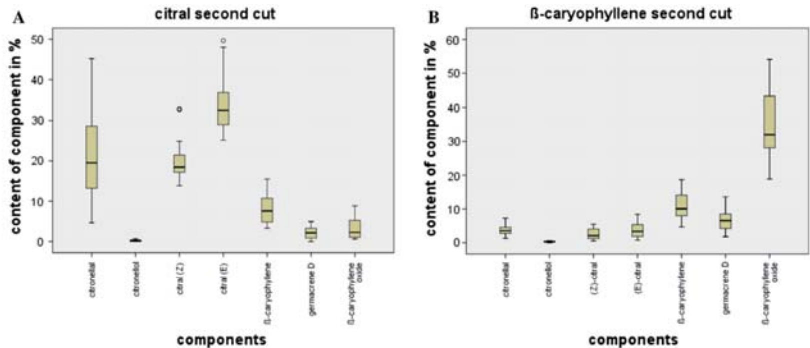

Fig. 4 Composition of essential oil of 68 genotypes for separate chemotypes: ct. citral: 62 genotypes (**A**) and ct. β-caryophyllene oxide: 6 genotypes (**B**). Values for two repeats of second cut 2010 and 2011; Boxes: interquartile range, including 50% of the values; bar: median; whiskers: maximum and minimum value, excluding outliers; circle: aberration

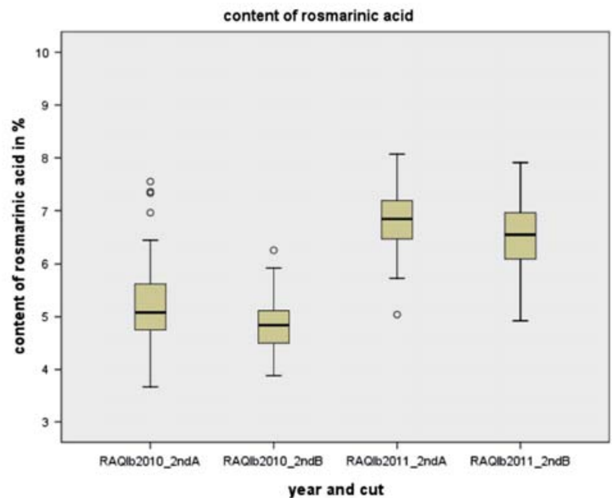

Fig. 5 content of rosmarinic acid (RA) for the years 2010 and 2011, second cut (2nd) for two repeats (**A**, **B**) at trial location Quedlinburg (Qlb)Boxes: interquartile range including 50% of the values; Bar: median; Whiskers: maximum and minimum value, excluding outliers; Circle: outliers; No extreme values are in the data sets

chemotype. Literature mentions changes between (*E*)-citral, (*Z*)-citral and citronellal. Hefendehl (1970) observed ranges from 8.7–96.6% citral and citronellal 0.9–39%. He suggested that young leaves show a high content of citral, while older leaves show a higher content of citronellal. He assumed the age of leaves could be a reason for that phenomenon or that the content and composition of essential oil could be a matter of leaf position. Because of this it is not justified

to subdivide ct. citral for amount of (*E*)-citral, (*Z*)-citral and citronellal (Fig. 4).

The handbook of essential oil (Baser and Buchbauer 2010) state that phytochemical polymorphism is often the case between different plant organs. In *Origanum vulgare* subsp. *hirtum* even different oil glands of the same plant showed a polymorphism (Johnson et al. 2004). Hose et al. (1997) also showed that the composition in essential oil glands of *M. officinalis* changes during ontogenesis. This is mentioned for other species (*Salvia sclarea* L., *Satureja hortensis* L., *Salvia officinalis* L.) as well (Grassi et al. 2004; Johnson et al. 2004; Novak et al. 2006; Schmiderer et al. 2008).

Of the 68 evaluated genotypes 62 belong to ct. citral and conform to Pharmacopoeia Europaea (Ph. Eur. 8 2014). The described variation offers the required basis for breeding programs to increasing essential oil content. The breeding process can be expedited by using haploid induction as reported for balm (Kästner et al. 2016).

Content of rosmarinic acid

For the pharmaceutical use next to essential oil, rosmarinic acid is the substance of interest, because of its proven pharmaceutical effects. Until 2008 rosmarinic acid had to be calculated by a photometric method as the sum of all hydroxycinnamic acid derivatives (Ph. Eur. 6 2008). Since 2009 the Pharmacopoeia Europaea changed to calculate specific rosmarinic acid content by using high performance liquid chromatography (HPLC) (Ph. Eur. 6 2009). Krüger et al. (2010) compared HPLC and photometry methods to evaluate the content of rosmarinic acid in 2009. The determined values ranged between 2.8 and 9%, HPLC method and 7.4 and 15.5% sum of hydroxycinnamic acid derivatives. The content of rosmarinic acid is an important quality requirement and a raised content is a desired aim of breeding programs. The aim of the evaluation was first time characterization of large sets of lemon balm and balm for their content of rosmarinic acid according to the HPLC method. All tested 68 accessions fulfil the requirements of Ph. Eur. for rosmarinic acid. For most accessions from this collection the sum of hydroxycinnamic acid derivatives were measured by photometric method (Bomme et al. 2008). There is no correlation between this data and the presented data because of the insufficient correlation between both methods (Krüger et al. 2010) and the high impact of the year and the ontogenetic status of measured plants. The presented data are valuable contribution for characterization of rosmarinic acid in the species *M. officinalis* and offer in connection with status of ploidy (Kittler et al. 2015) and amount and composition of essential oil a prerequisite for taxonomical studies inside the species.

Lemon balm produces a high amount of rosmarinic acid in comparison with other species of family Lamiaceae. This can be used for special breeding programme to create lines with stable very high yield of rosmarinic acid. The better winter hardiness and higher fresh mass production of triploid balm accessions can be used for production of rosmarinic acid even they are not conforming to Ph. Eur.

Conclusions

The screening of 68 balm and lemon balm genotypes showed that every genotype had its own pattern of essential oil, which shifted in ranges as well in quantity as in quality. The presented results suggest the existence of two different chemotypes in the tested collection of *M. officinalis*. We declare the ct. citral and ct. β-caryophyllene oxide. In this study the chemotypes coincide with the ploidy level. The genotypes of ct. citral were always diploid and the genotypes of ct. β-caryophyllene oxide were triploid. To verify this appearance more non-citral accessions need to be characterised. Content of rosmarinic acid is a quality requirement of the Ph. Eur. But there is also a rising demand for lemon balm with high content of rosmarinic acid. The data of rosmarinic acid evaluation in connection with ploidy level and amount and composition of essential oil can contribute to taxonomical studies inside the species *M. officinalis*.

The presented results could be used for generation of a core collection for *M. officinalis*. The evaluation results of 28 M. *officinalis* accessions which include 10 accessions of ct. germacrene D and 15 of ct. citral (Kittler et al. 2018) should also be include in the selection of the core collection to reach the maximum of variability for characterized traits. Candidate accessions should be tested more intensive again.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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5 GENERAL DISCUSSION:

5 GENERAL DISCUSSION:

This general discussion consists of the results of the generation of doubled haploid plants, the variability of the content and composition of the essential oil, the subspecies of *M. officinalis* and their cytologic properties.

5.1 BREEDING-STRATEGIES IN BALM:

The aim of the project ”Development of generative propagatable high yielding lines of lemon balm (*Melissa officinalis* L.) via conventional generating of homozygote lines as a prereqisite for synthetic and hybrid variaties”, was to find a breeding-strategy for developing high yielding varities of lemon balm, which express a very good winter hardiness and show a high content of both essential oil and rosmarinic acid. The attempt to develop a hybrid systems for balm failed through the lacking of the natural occurance of genotypes exhibiting male sterility. To develop homogene breeding lines the convential way of inbreeding and the development of doubled haploid plants were tested.

The development of double haploid plants was an aim of the project “Development of a procedure to generate double haploid plants of lemon balm (*Melissa officinalis* L.) and search for elements of creating a system for the regulation of fertilisation based on male sterility“. In context of this project, different methods were used to create double haploid plants to reduce the time needed to produce stable inbreed lines. Such a creation of stable inbreed lines is time consuming for many cultures, especially in *M. officinalis*. Blossoms are formed during the second vegetation year of a balm plant. Through a vernalisation of 12 weeks, young plants could be enabled to blossom in the first year of development to create an inbreed generation after just one year. In the family *Lamiaceae,* successful creation of a double haploid plant is unknown. Swelling and development of a callus is described for 14 examined *Lamiaceae* (Ferry et al. 2007). The regeneration of haploid plants in *Lamiaceae* and especially *Melissa* sp. is complicated (Kästner et al. 2016). In *Salvia sclarea* Bugara et al. (1986) created embryos but could not develop a plant. Hadian et al. (2012) reported about embryos in a microspore culture from *Satureja khuzistanica* and *S. rechingeri*, but also failed to regenerate a plant. Kästner et al. (2016) report all methods used with *M. officinalis*. They achieved first cell divisions to microcullus.

The development of homozygous lines via inbreeding held the possibility of severe depression of the inbreed-lines in all agricultural traits. Lemon balm is described as a strong cross breeding plant, but the self-pollination did not have a negative impact on the performance of the genotypes and caused no reduction in yield or any quality requirement. Through the developed vernalisation programme, it was possible to create one inbreed generation per year. Four generations of inbreed-lines could be developed and were used in the following project to further homogenise the breeding-lines. For the

development of a new variety of lemon balm, two systems are possible: An inbreed-line variety or a synthetic variety.

5.2 VARIABILITY OF THE CONTENT AND THE COMPOSITION OF THE ESSENTIAL OIL OF BALM AND LEMON BALM:

The described chemotypes citral, germacrene D and β-caryophyllene-oxide (ct. citral, ct. germacrene D, ct. β-caryophyllene-oxide) and subspecies *Melissa officinalis officinalis* L. and *Melissa officinalis altissima* (Sibth. & Sm.) Arcangeli differ in content and composition of the essential oil. The highest determined values in the evaluation trails were 0.11 % essential oil per 100 g drymatter (ct. β-caryophyllene-oxide), 0.12 % essential oil per 100 g drymatter (ct. germacrene D) und 0.72 % essential oil per 100 g drymatter (ct. citral).

For the pharmacological use of lemon balm leaves (*Melissae folium*) the drug has to provide a lemon-like scent (Ph. Eur. 2014). There are no detailed contents or compositions required. However, saleable material for tea production and other commodities needs to exhibit a content of 0.3 to 0.4 % essential oil per 100 g dry matter.

The publications „Evaluation of 28 balm and lemon balm (*Melissa officinalis*) accessions for content and composition of essential oil and content of rosmarinic acid" and "Content and composition of essential oil and content of rosmarinic acid in lemon balm and balm genotypes (*Melissa officinalis*)",

describe germacrene D- and β-caryophyllene-oxide-types and the variability of the citral consisting accessions and genotypes. These citral-consisting genotypes conform to the pharmacopeia and therefore could be used as a phytopharmaceutical. The content of citral (*E*) and (*Z*) and citronellal in the accessions of the IPK collection varied strongly. It ranged from 8.22 to 45.33 %. The content of (*Z*)-citral ranged from 5.17 and 32.36 % and the values for Citronellal from 1.13 to 26.1 %. In the trial of the LfL-collection the values for (*E*)-Citral varied between 10.95 and 44.94%, for (*Z*)-Citral between 6.79 and 32.80 % and for Citronellal from 3.8 to 59.95 %.

The content of essential oil varies during ontogenesis and in the different levels of the leaves. According to Mrlianova et al. (2003) the composition of the essential oil changes, with the young leaves containing the highest values of essential oil. Mrlianova et al. (2003) determined the variability of the content and the composition of leaves and herb of *M. officinalis* in relation to the height of cutting. The upper third of the herb contained 0.13 % essential oil, which is 27 % of the total dry matter. The upper and middle part altogether reached a content of essential oil of 0.08 % and counted for 46 % of the dry matter. The last third of the plant contained 0.06 % essential oil and was 54 % of the dry matter. The dried leaves contained 0.39 % essential oil in the upper third and 0.14 % in the basal third of the plant.

The basal part of the plant contained lesser citral, linalool and β-caryophyllene, but had higher contents of β-caryophyllene-oxide and citronellal. (*E*)-citral and (*Z*)-citral made 55.79 % of essential oil in the upper part and 48.46 % of the complete harvest. The leaves had a content of 59.74 % and 56.87 %.

Basker and Putievsky (1978) showed that the content of essential oil in dried leaves is highest during summer. However, depending on the aim of harvest, different harvest dates need to be chosen.

Therefore, requirements such as highest dried leaf mass, highest essential oil content in dried leaves or highest essential oil content per hectare determine specific time points for harvesting the plant. Two *M. officinalis* cultivars were examined including drying rate (18 and 11.3 %), mass of dried leaves (10190 and 6880 kg/ha), content of essential oil (0.7 and 0.6 % w/v), as well as essential oil yield per hectare (75 and 41 l/ha). All these results were evaluated on different dates of harvesting. It shows that specific yield maxima cannot be reached on the same harvesting dates and thus need to be considered separately. The development stage of a plant and the conditions while the harvest seem to have an influence on the chemical and biological activity of secondary ingredients, as shown in various culinary and medicinal plants (Papageorgiou et al. 2008).

An experiment to investigate ontogenetic differences in the proportion of essential oil and the composition of these was set up. The aim was to evaluate different levels of leaves of a single plant. By doing so, Hose et al. (1997) determined drastic differences in the quantitative composition of essential oil between the basal and upper leaves. Especially the proportions of monoterpene-aldehydes citronellal and (*E*)- and (*Z*)-citral varied to a high extend. In the literature, a proportion of 8.7 and 96.6 % of (*E*)- and (*Z*)-citral in essential oil and 0.9 % up to 39 % of citronellal are stated. Various attempts to explain these differences can be found in the literature. Hefendehl et al. (1970) observed that young leaf pairs had a higher proportion of (*E*)- and (*Z*)-citral in the essential oil. The remainder of 70 % of the leaves showed a higher percentage of citronellal in the essential oil. That fact was explained by the age of the leaves. However, it might also be the position of the leaves, which could influence the composition of the essential oil and thus, might be independent of the age of the plant. Another hypothesis assumes different biotypes or chemotypes. Enjalbert et al. (1983) were the pioneers in assuming chemotypes when they compared plants from France with plants from Germany. In contrast the French plants were characterised by a higher content of (*E*)- and (*Z*)-citral, the German ones showed a higher percentage of citronellal. Tittel et al. (1982) did investigations on Spanish and German plants and detected a high proportion of (*E*)- and (*Z*)-citral for both. However, Mulkens and Kapetanidis (1988) disproved this hypothesis by comparing French and Swiss *Melissa* cultures, which did not feature significant differences in (*E*)- and (*Z*)-citral or citronellal. But they observe a diminishment of monoterpene-aldehyde and a rise in sesquiterpene throughout 4 years of culturing. Adzet et al. (1992b) wrote that neither the point of harvest nor the position of the leaves has a significant influence on the composition of the essential oil. Hose et al. (1997) and Adzet et al. (1992b) postulated that especially the ontogenetic state of the plant determines the composition of the essential oil. Their investigation on whole plants showed that the essential oil of *M. officinalis* can be divided into 52 components with terpenes exhibiting the greatest proportion. The total proportion in mono- and sequiterpenes made up 67 % up to 30.2 %. Monoterpenes were mostly oxidised (92.8 %). The monoterpenes could be broken down into citronellal (22.8 %) and citral (26.4 %) ((*E*)- citral (16.3 %) and (*Z*)-citral (10.1 %)). β-caryophyllene dominated the sequiterpenes with 10.4 %. All these values represented average values of the whole plant.

Hose et al. (1997) analysed the correlation between the position of the leaves and content of essential oil, as well as the composition of the essential oil. Especially the proportion in citral and citronellal varied a lot between different positions of the leaves. The proportion of citral decreased from the upper young

leaves down to the basal and older leaves from 37.2 to 0.5 %. In contrast to this, citronellal increased from young to old leaves from 1.1 to 52.4 %. The content of monoterpene esters exhibited distinctive proportional fluctuations. Whereas the proportion in geranylacetat decreased in the upper three levels of leaves from 10.2 to 0%, methylcitronelllale showed a rise from 0 up to 17.9 %.

To determine whether the composition of the essential oil correlates with the position of the leaves or with the age of the leaves, levels of leaves in the middle region of the plant were marked and analysed every 6 weeks. The result of this analysis showed a strong correlation of the position and the age of the leaves. Thus, a lower leaf corresponds with an old leaf and an upper leaf with a young one. The content of citronellal rose from 11 to 32.2 % while the content of citral sank from 37.2 to 12.9 %. The content of geranylacetat sank from 10.2 to 0 % and the content of methylcitronellate rose from 0 to 3.8 %. These values confirm that the difference in monoterpenes is more dependent on the age of the leaves than on the position. However, the age does not seem to influence the content of sequiterpenes to a large extend.

Moreover, an investigation was undertaken to analyse whether a change in components is due to biosynthesis or remodelling processes. The leaf tips of pairwise opposite leaves are almost identical. Therefore, these leave pairs were used to analyse the temporal effect on the composition of the essential oil. In an experimental setting, location and quantity of essential oil stores on the tips of the leaves was determined. It turned out that location and quantity were about the same. Five essential oil glands of one side of the pair of leaves were analysed, the glands of the other side after two months. The investigated changes where compared with the leaves experiments.

Therefore, the assumption that the content of essential oil is changing in the essential oil stores was verified. Seen from a quantitative perspective, content of essential oil is rising per leave up to the middle part of the plant and is decreasing above. The content of essential oil per leave is highest in the youngest leave and decreases much in the first three levels of leaves. Due to the high variability in essential oil composition within the whole plant, the total amount of essential oil is always a mixture of various components in the individual levels of leaves. Furthermore, intense variation can occur in a time span of only two weeks and might include the transformation of citral to citronellal.

5.3 SUBSPECIES:

M. officinalis belongs to the family Labiatae (syn. Lamiaceae). This medicinal and aromatic plant is cultivated all over the world, but its origin is difficult to determine due to its long and intense use and distribution. The regions around the Mediterranean Sea and Western Asia are assumed to be the regions of origin. The Mediterranean region, Anatolia, Caucasus, Iran, Iraq and Pakistan as well as the Asian mountains Kopet-Dag, Alai and West Tian Shan belong to the possible regions of origin of *M. officinalis* (Hanelt and IPK 2011). Northern Iran and Iraq as well as the Mediterranean region are mentioned to be the regions with great diversity (Davis 1982). Worldwide cultivation of Melissa in the moderate and subtropical climate zone caused imbrutement in Europe, including England, Sweden and central Russia, as well as North America, leading to a worldwide distribution (Hoppe 2013). Two to three

subspecies are described: *M. officinalis* L. ssp. *officinalis* (Tutin 1972, Davis 1982, Hanelt 2001), *M. officinalis* L. ssp. *altissima* (Sibth. & Sm.) Arcangeli (Tutin 1972, Davis 1982) and *M. officinalis* L. ssp. *inodora* Bornm. (Davis 1982). Morphological differences such as hair growth, blossom structure and scent are used to distinguish between these subspecies. An important morphological characteristic of the blossom is the tooth of the upper lip of the calyx (Hoppe 2013). *M. officinalis ssp. officinalis* distinguishes itself from other species by almost no hair growth on the upper side of the leaves, three distinct teeth on the upper lip of the calyx, and a citric smell. In contrast, ssp *altissima* develops extreme hair growth but almost no teeth on the upper lip of the calyx, or the middle tooth can be reduced a lot compared to the two other ones. Ssp. *inodora* defined by its hairy stem and a big middle tooth is known as another subspecies described by Davis (1982). All tetraploid species 2n = 4x = 64 that are typified by intense hair growth, reduced teeth in the upper lip of the calyx and citrus-like aroma belong to this group. Additionally, there are transition forms between all subspecies (David 1982). Although the wild type is tetraploid, it is supposed to belong either to ssp. *altissima* (Hanelt 2011) or to *M. romana* (Pignatti 2002).

In cross-breeding trials conducted in 2013 at JKI Quedlingburg between di- and tetraploid plants, no offspring could be generated. The trial was repeated with tetraploid genotypes, which were generated due a cholchizine treatment of diploid genotypes in the project "Development of a procedure to generate double haploid plants of lemon balm (*Melissa officinalis* L.) and search for elements of creating a system for the regulation of fertilisation based on male sterility". The tetraploid plants had mainly tetraploid offspring but also di- and tri-ploid genotypes could be found. This phenomon could be a cause for further investigation.

The compositions of essential oils show distinct differences between species of various origins (Moradkhani 2010). The essential oils of ssp. *officinalis* contain amongst others monoterpene-aldehyde such as citronellal and citral, which cause the lemon-like smell as a specific characteristic of this species. Ssp. *altissima* does not have such a high proportion in monoterpene-aldehydes but contains more of sesquiterpenes including β-caryophyllene, germacrene D, β-caryophyllene oxide and cubebene. Sesquiterpenes can also be found in ssp. *inodora* as well as small amounts of geranial citral (Moradkhani 2010). With a content of essential oils < 0.04 % ssp. *altissima* and ssp. *inodora* are below ssp. *officinalis*. The content of essential oil is dependent on the stage of development, as well as the biotic and abiotic growth factors.

The basic number of chromosomes in the family Lamiaceae goes from x = 5 up to x = 11. However, also chromosome numbers of x = 13, 15, 17 and 19 have been measured (Harley et al. 2004). Such high numbers might have been developed due to a structural rearrangement of the chromosome set. For M. *officinalis* ssp. *officinalis* and ssp. *altissima* chromosome numbers of 32 and 64 respectively are stated (Tutin et al. 1972). It is hypothesised that the tetraploid ssp. *altissima* is the origin of the diploid ssp. *officinalis* (Hanelt and IPK 2001). Darlington and Wylie (1955) specified a chromosome number of x = 8 for the haploid state of M*. officinalis*. Investigation using FISH succeeded to determine the chromosome number of the haploid state of *Melissa* as x = n = 16 (Kittler et al. 2015).

5.4 DIFFERENCES IN SIGNAL STRENGTH OF FLUORESCENCE IN SITU HYBRIDISATION:

The strength in signal in different haplotypes is varying. In the examined diploid accessions the signals of 18/25S and 5S rDNA seemed to exhibit fluorescence in both genomes with about the same intensity. By contrast, triploid genotypes showed one strong and two weaker signals, two strong and two weak signals could be measured in the tetraploid accessions.

These results might give insight into the development of the genome forms as the strength of a signal could give rise to different chromosomes. This might lead to a conclusion of whether a plant is allo- or auto-tetraploid or what chromosome pairing of allo-tetraploid plants might look like. In addition, knowledge about the development of the described triploid forms could be gained.

The genome-regions which were detected by the probes for the 18/25S and 5S rDNA are highly conserved in all plants. Another way of finding hints for question of the origin of di-, tetra- and triploid genotypes and the controversial question - if there are species or subspecies - could be the approach of genotyping by sequencing. With the success and availability of next generation sequencing, it might be possible to sequence enough genomic sequences of enough genotypes to calculate the genetic distance of the genotypes and produce a very detailed dendrogram of the involved genotypes. The different chemo- and haplotypes and diverse genotypes in the collection would be an interesting set. Crossings between the different haplotypes, which may give an insight in development of the different haplotypes, could be performed by the developed cross-breeding method.

The results of the evaluation-papers show, that all tested accessions and genotypes of diploid *Melissa officinalis* L. conform to European Pharmacopoeia and the German Pharmacopoeia. There is a high demand for genotypes with a high rosmarinic acid content, so future breeding aims should be to stabilise a high content of essential oil and rosmarinic acid, a high yield and winter-hardiness. Further investigations should be directed to the ontogenesis of lemon balm and the different contents of essential oil and rosmarinic acid in different stages of development and between the harvesting cuts, to gain better information, when to harvest for the best results in yield and/or essential oil and/or rosmarinic acid.

6 CONCLUSION:

This thesis contains results of the project ”Development of generative propagatable high yielding lines of lemon balm (*Melissa officinalis* L.) via conventional generating of homozygote lines as a prereqisite for synthetic and hybrid variaties”(original title in German: „Entwicklung generativ vermehrbarer Hochleistungslinien von Zitronenmelisse (*Melissa officinalis* L.) durch konventionelle Erzeugung homozygoter Linien als Voraussetzung für Synthetiks oder Hybridsorten“) and builds a basis for the establishment of modern breeding methods in *Melissa.* The results could be used to generate a basis for the selection of breeding material. The data could be used for choosing partners for crossovers, the development of inbreed lines and the establishment of performance tests. Also the adapted cross-pollination method formed the foundation for the application of modern breeding methods in lemon balm. Moreover, the results can be used for a marker-assisted selection or the generation of double haploid plants as shown in the publication „Comparison of *in vitro* haploid induction in balm (*Melissa officinalis*)“ (Kästner et al. 2016). This intensive evaluation of numerous accessions and genotypes revealed the agronomical and economical value of lemon balm and its sub-species. Characteristic traits such as content and composition of essential oil and content of rosmarinic acid were examined and add to the essential information needed for further breeding activities in lemon balm. The practical use of the described work in further breeding projects, up to the possible production of new varieties are signs of the success, importance and usability of the results. The publishing of further results, such as the identification of ingredients and the variability of the valued ingredients during ontogenesis facilitates a more detailed image of *Melissa*. Performance tests of selected lines retrieved from inbreeds and crossovers were undertaken in another project. The findings can be seen as an important catalyst for breeding practice.

To conclude, not only the acquired data but also the used methods contribute to the knowledge about medicinal and aromatic plants as well as possibilities of evaluating and investigating them. On the one hand, detailed information starting from a genetic level of the species allows follow-up experiments on *M. officinalis*. On the other hand, the fine-tuned methods might be adapted and become established in experiments with other species. This demonstrates that the impact of this thesis is much broader and goes across various medicinal and aromatic plants.

7 SUMMARY:

7.1 SUMMARY:

This thesis contains the published results of the ”Development of generative propagatable high yielding lines of lemon balm (*Melissa officinalis* L.) via conventional generating of homozygote lines as a preregisite for synthetic and hybrid variaties” (original title in German: „Entwicklung generativ vermehrbarer Hochleistungslinien von Zitronenmelisse (*Melissa officinalis* L.) durch konventionelle Erzeugung homozygoter Linien als Voraussetzung für Synthetiks oder Hybridsorten“) which was part of the project “Improving the international position of the German production of medical herbs and spices, by taking the example of the optimisation of breeding and cultivation of valerian, camomile and lemon balm.“ and was funded by the Fachagentur für Nachwachsende Rohstoffe . The papers “Chromosome number and ploidy level of balm (*Melissa officinalis*)”, “Evaluation of 28 balm and lemon balm (*Melissa officinalis*) accessions for content and composition of essential oil and content of rosmarinic acid” and “Content and composition of essential oil and content of rosmarinic acid in lemon balm and balm genotypes (*Melissa officinalis*)” contain important results for modern breeding attempts in lemon balm. The publications show the characteristics of the accessions and genotypes such as ploidy status, content and composition of essential oil and content of rosmarinic acid. Of particular importance are the first descriptions of triploid *Melissa* genotypes, as well as three *Melissa* chemotypes, which have never been outlined before.

7.2 ZUSAMMENFASSUNG:

Diese Arbeit stellt die grundlegenden Ergebnisse des Projektes „Entwicklung generativ vermehrbarer Hochleistungslinien von Zitronenmelisse (*Melissa officinalis*) durch konventionelle Erzeugung homozygoter Linien als Voraussetzung für Synthetiks oder Hybridsorten“ dar, welches im Rahmen des Demonstrationsprojektes „Verbesserung der internationalen Wettbewerbsposition des deutschen Arznei- und Gewürzpflanzenanbaus am Beispiel der züchterischen und anbautechnologischen Optimierung von Kamille, Baldrian und Zitronenmelisse“ durch die FNR gefördert wurde vor. Ziel dieser Arbeit ist es im Konkreten das Grundwissen über die Gattung *Melissa* bereitzustellen und im Allgemeinen Grundlagen für neue Züchtungsmethoden in der Familie der *Lamiaceae* und anderen Arznei- und Gewürzpflanzen zu schaffen und durch den Züchtungsfortschritt den Anbau von Arznei- und Gewürzpflanzen zu unterstützen. In Form einer kumulativen Disseration werden die veröffentlichten Ergebnisse der Untersuchungen der Genpools der uns zur Verfügung gestellten Akzessionen zusammenfassend dargestellt. Die Veröffentlichungen „Chromosome number and ploidy level of balm (*Melissa officinalis*)”, „Evaluation of 28 balm and lemon balm (*Melissa officinalis*) accessions for content and composition of essential oil and content of rosmarinic acid” und „Content and composition of essential oil and content of rosmarinic acid in lemon balm and balm genotypes (*Melissa officinalis*)” befassen sich mit der Untersuchung der züchterischen Grundlagen an Melisse. In den Arbeiten werden die grundlegenden Eigenschaften der Akzessionen und Genotypen wie Ploidiestatus, Gehalt und Zusammensetzung des ätherischen Öles und der Gehalt an Rosmarinsäure beschrieben. Besonders hervorzuheben ist dabei die Erstbeschreibung triploider Melissegenotypen sowie der drei beschriebenen Chemotypen der Melisse. Diese Arbeit enthält somit wichtige und wertvolle Informationen zur Kulturpflanze Melisse und darüber hinaus Ansatzpunkte für moderne Züchtungsverfahren in Arznei- und Gewürzpflanzen der Familie *Lamiaceae*.

8 ELECTRONICAL SUPPLEMENTS:

8 ELECTRONICAL SUPPLEMENTS:

Table 9: Electronical supplement Table 1: Collection of lemon balm and balm (*Melissa officinalis*) for evaluation of essential oil; main component of essential oil: citral type - orange, germacrene D type - green, undetermined type - grey; content of essential oil (EO) in % and rosmarinic acid content (RA) in % of air-dried leaf material in the years 2009 and 2010 for two trial sites (Qlb: Quedlinburg; Gat: Gatersleben)

Acc. No.*	Scientific name**	Ploidy	Origin	EO Qlb 2009 first cut	EO Gat 2009 first cut	EO Qlb 2009 second cut	EO Gat 2009 second cut	EO Qlb 2010 first cut	EO Gat 2010 first cut	RA Qlb 2009 first cut	RA Gat 2009 first cut	RA Qlb 2009 second cut	RA Gat 2009 second cut	RA Qlb 2010 first cut	RA Gat 2010 first cut
MELI 1	*M. officinalis* subsp. *officinalis*	*diploid*	unknown	0.16	0.1	0.18	0.56	0.12	0.12	8.78	7.52	5.76	5.71	5.06	4.59
MELI 2	*M. officinalis* subsp. *officinalis*	*diploid*	unknown	0.04	0.03	0.06	0.2	0.03	0.06	6.8	7.32	5.03	4.2	4.94	4.44
MELI 4	*M. officinalis* ssp. *officinalis*	*diploid*	GDR	0.03	0.04	0.14	0.52	0.08	0.06	7.81	7.1	5.81	5.65	4.34	3.91
MELI 5	*M. officinalis* ssp. *officinalis*	*diploid*	GDR	0.03	0.03	0.1	0.28	0.08	0.08	6.83	7.2	6.09	4.47	4.98	4.73
MELI 6	*M. officinalis* ssp. *officinalis*	*diploid*	Germany	0.04	0.04	0.16	0.38	0.06	0.08	7.37	7.02	5.5	5.53	3.89	3.7
MELI 7	*M. officinalis* ssp. *officinalis*	*diploid*	unknown	0.03	0.08	0.03	0.14	0.08	0.08	7.84	7.25	6.69	6.25	4.59	4.49
MELI 8	*M. officinalis* ssp. *officinalis*	*diploid*	Georgia	0.04	0.08	0.03	0.12	0.06	0.08	6.57	7.14	3.91	5.22	3.28	3.73
MELI 9	*M. officinalis* ssp. *officinalis*	*diploid*	France	0.03	0.03	0.03	0.03	0.06	0.06	5.4	6.67	3.8	4.93	5.03	5.13
MELI 10	*M. officinalis* ssp. *officinalis*	*diploid*	France	0.08	0.16	0.3	0.72	0.48	0.4	6.09	7.38	5.28	6.35	3.81	4.13
MELI 11	*M. officinalis* subsp. *officinalis*	*diploid*	Italy	0.04	0.04	0.18	0.36	0.1	0.12	6.61	7.55	5.15	5.42	4.52	4.14
MELI 12	*M. officinalis* subsp. *altissima*	*tetraploid*	Italy	0.03	0.04	0.03	0.12	0.06	0.06	6.26	5.59	4.96	5.84	4.49	4.17
MELI 13	*M. officinalis* subsp. *officinalis*	*diploid*	Georgia	0.03	0.08	0.04	0.1	0.03	0.04	7.79	7.54	5.36	5.25	4.09	4.59
MELI 14	*M. officinalis* L. subsp. *altissima*	*tetraploid*	Italy	0.03	0.04	0.04	0.03	0.04	0.06	5.6	6.47	4.9	6.15	5.3	5.32
MELI 15	*M. officinalis* subsp. *altissima*	*tetraploid*	Italy	0.03	0.03	0.03	0.04	0.06	0.08	6.51	7.04	5.09	5.28	5.12	4.87
MELI 16	*M. officinalis* subsp. *officinalis*	*diploid*	unknown	0.02	0.04	0.03	0.24	0.08	0.1	5.81	7.16	5.36	4.94	4.06	4.73
MELI 17	*M. officinalis* subsp. *altissima*	*tetraploid*	Greece	0.04	0.12	0.08	0.08	0.04	0.06	4.7	4.17	3.1	2.45	3.95	3.82

MELI 18	*M. officinalis* subsp. *altissima*	*tetraploid*	unknown	0.03	0.03	0.03	0.03	0.03	0.03	5.49	6.95	4.87	4.67	5.11	5.72
MELI 19	*M. officinalis* subsp. *altissima*	*tetraploid*	Italy	0.03	0.08	0.01	0.08	0.06	0.04	5.76	5.81	4.03	5.72	5.45	4.9
MELI 20	*M. officinalis* subsp. *altissima*	*tetraploid*	Italy	0.08	0.08	0.03	0.1	0.08	0.1	4.03	4.88	3.74	4.95	5.16	4.9
MELI 21	*M. officinalis* subsp. *altissima*	*tetraploid*	Albania	0.03	0.03	0.06	0.08	0.03	0.06	5.16	4.68	3.35	3.21	4.16	3.57
MELI 22	*M. officinalis* subsp. *altissima*	*tetraploid*	Turkey	0.03	0.04	0.04	0.06	0.03	0.08	4.6	4.44	3.54	3.24	3.47	3.58
MELI 23	*M. officinalis* subsp. *altissima*	*tetraploid*	Italy	0.03	0.04	0.08	0.08	0.03	0.04	5.41	3.99	4.59	6.57	4.82	5.05
MELI 24	*M. officinalis* subsp. *altissima*	*tetraploid*	Italy	0.03	0.03	0.03	0.03	0.06	0.08	6.24	7.16	4.65	5.21	5.43	5.15
MELI 25	*M. officinalis* subsp. *officinalis*	*diploid*	unknown	0.03	0.08	0.28	0.46	0.12	0.18	6.37	6.54	4.7	6.4	4.61	4.54
MELI 26	*M. officinalis* subsp. *officinalis*	*diploid*	Armenia	0.04	0.04	0.08	0.26	0.04	0.12	6.29	7.05	4.57	5.19	3.63	3.34
MELI 27	*M. officinalis* subsp. *officinalis*	*diploid*	Italy	0.04	0.08	0.2	0.28	0.12	0.16	6.45	6.81	5.36	6.03	4.18	4.27
MELI 28	*M. officinalis* subsp. *altissima*	*tetraploid*	Italy	0.03	0.03	0.01	0.03	0.03	0.06	5.14	6.08	3.72	5.93	5.08	4.84
D 9597 (MELI 29)	*M. officinalis* subsp. *officinalis*	*diploid*	unknown	0.03	0.08	0.2	0.54	0.12	0.24	6.47	7.2	4.97	6.78	4.05	4.04

* Acc. No.: accession number;

** Taxonomical classification according to information of collection holder;

Table 10: Electronical supplement Table 2: Complete dataset for content and composition of essential oil of 28 accessions of balm (green and grey) and lemon balm (orange).

Acc. No.*	repeat	year	cut	content of essential oil	citronellal	citronellol	(*Z*) - citral	(*E*) - citral	ß-caryophyllene	germacrene D	ß-caryophyllene-oxide	sum of unknown substances
					[in %]	[in %]	[in %]	[in %]	[in %]	[in %]	[in %]	
MELI 1	QLB	2009	1 st	0.16	11.60	0.00	19.40	29.22	8.47	4.20	9.28	17.84
MELI 1	GAT	2009	1 st	0.10	26.10	0.19	12.98	20.58	6.42	2.96	9.91	20.85
MELI 1	QLB	2010	1 st	0.12	7.18	0.00	14.22	21.98	16.31	11.19	6.95	22.17
MELI 1	GAT	2010	1 st	0.12	14.29	0.00	15.34	24.63	11.12	8.24	6.98	19.40
MELI 1	QLB	2009	2 nd	0.18	40.21	0.33	19.54	29.66	2.99	0.00	0.47	6.80
MELI 1	GAT	2009	2 nd	0.56	40.20	0.46	15.64	28.96	3.54	0.54	0.64	10.02
MELI 2	QLB	2009	1 st	0.04	9.32	0.19	12.20	19.51	7.94	3.26	18.72	28.86
MELI 2	GAT	2009	1 st	0.03	18.58	0.23	11.47	18.26	7.51	3.20	13.96	26.78
MELI 2	QLB	2010	1 st	0.03	6.93	0.00	12.92	20.38	14.74	7.59	12.37	25.05
MELI 2	GAT	2010	1 st	0.06	8.18	0.00	15.52	24.81	11.82	8.24	9.01	22.42
MELI 2	QLB	2009	2 nd	0.06	12.28	0.00	16.11	26.03	12.65	4.95	8.87	19.10
MELI 2	GAT	2009	2 nd	0.20	28.60	0.43	19.11	34.25	6.61	2.14	1.70	7.16
MELI 4	QLB	2009	1 st	0.03	7.86	0.16	8.66	14.51	6.96	3.68	21.53	36.64
MELI 4	GAT	2009	1 st	0.04	14.60	0.25	6.89	11.55	6.11	2.55	21.51	36.53
MELI 4	QLB	2010	1 st	0.08	5.51	0.00	16.03	26.35	12.42	7.04	11.26	21.40
MELI 4	GAT	2010	1 st	0.06	7.44	0.00	13.79	22.72	11.16	8.91	11.70	24.29
MELI 4	QLB	2009	2 nd	0.14	42.41	0.45	15.62	25.14	4.94	0.94	1.65	8.84
MELI 4	GAT	2009	2 nd	0.52	45.21	0.67	13.83	27.73	3.68	0.95	0.91	7.03
MELI 5	QLB	2009	1 st	0.03	6.76	0.00	12.39	20.63	8.30	4.06	20.14	27.72
MELI 5	GAT	2009	1 st	0.03	12.20	0.18	10.63	17.70	7.76	3.05	19.21	29.26
MELI 5	QLB	2010	1 st	0.08	2.04	0.00	10.69	16.60	20.86	14.99	8.95	25.87
MELI 5	GAT	2010	1 st	0.08	4.70	0.00	14.55	23.61	14.10	9.30	10.78	22.97
MELI 5	QLB	2009	2 nd	0.10	20.62	0.00	18.05	29.13	10.72	2.59	5.27	13.61
MELI 5	GAT	2009	2 nd	0.28	27.21	0.49	19.00	37.17	5.79	1.82	1.49	7.05
MELI 6	QLB	2009	1 st	0.04	12.27	0.12	11.24	19.03	8.95	5.94	13.32	29.13
MELI 6	GAT	2009	1 st	0.04	18.45	0.30	9.02	14.64	6.08	2.94	15.43	33.14
MELI 6	QLB	2010	1 st	0.06	6.23	0.00	11.37	18.85	13.55	11.08	10.94	27.99
MELI 6	GAT	2010	1 st	0.08	6.11	0.00	15.19	23.64	11.43	9.53	9.93	24.17
MELI 6	QLB	2009	2 nd	0.16	26.09	0.23	17.16	29.15	7.41	3.30	3.24	13.42
MELI 6	GAT	2009	2 nd	0.38	32.87	0.31	17.46	32.01	4.83	2.31	0.73	9.48
MELI 7	QLB	2009	1 st	0.03	3.56	0.00	7.36	13.19	8.64	3.68	26.29	37.28
MELI 7	GAT	2009	1 st	0.08	5.77	0.00	5.17	8.22	6.64	2.73	28.75	42.72
MELI 7	QLB	2010	1 st	0.08	3.82	0.00	12.21	19.73	17.29	9.79	11.82	25.34
MELI 7	GAT	2010	1 st	0.08	3.58	0.00	12.36	19.47	16.36	9.67	13.24	25.33
MELI 7	QLB	2009	2 nd	0.03	17.13	0.32	14.42	28.20	13.42	2.17	6.61	17.74
MELI 7	GAT	2009	2 nd	0.14	19.60	0.34	17.45	33.06	9.59	4.15	2.31	13.50

MELI 8	QLB	2009	1 st	0.04	2.42	0.00	5.21	8.69	21.71	16.27	11.69	34.01
MELI 8	GAT	2009	1 st	0.08	3.66	0.00	5.34	8.27	17.77	14.15	12.59	38.23
MELI 8	QLB	2010	1 st	0.06	2.20	0.00	10.63	17.22	23.18	12.18	9.77	24.82
MELI 8	GAT	2010	1 st	0.08	1.77	0.00	13.77	21.76	21.12	11.11	8.69	21.78
MELI 8	QLB	2009	2 nd	0.03	6.86	0.00	20.99	34.26	15.47	3.81	3.95	14.65
MELI 8	GAT	2009	2 nd	0.12	13.54	0.27	21.84	37.65	10.36	3.66	1.48	11.18
MELI 9	QLB	2009	1 st	0.03	0.96	0.00	1.45	2.58	3.13	1.35	42.75	47.77
MELI 9	GAT	2009	1 st	0.03	1.59	0.00	1.19	1.95	2.24	1.45	41.21	50.37
MELI 9	QLB	2010	1 st	0.06	0.00	0.00	0.91	1.58	22.37	21.14	18.10	35.90
MELI 9	GAT	2010	1 st	0.06	0.52	0.00	1.93	3.20	19.37	15.52	23.80	35.67
MELI 9	QLB	2009	2 nd	0.03	1.63	0.00	1.70	2.78	13.90	7.88	27.89	44.23
MELI 9	GAT	2009	2 nd	0.03	4.85	0.00	3.70	7.10	21.89	20.61	9.74	32.12
MELI 10	QLB	2009	1 st	0.08	3.16	0.00	28.78	42.03	4.54	1.18	5.42	14.90
MELI 10	GAT	2009	1 st	0.16	6.64	0.00	29.38	41.83	3.78	0.90	3.87	13.59
MELI 10	QLB	2010	1 st	0.48	1.58	0.19	29.74	42.78	8.91	2.91	3.19	10.70
MELI 10	GAT	2010	1 st	0.40	1.13	0.23	32.36	45.33	6.37	2.06	2.37	10.16
MELI 10	QLB	2009	2 nd	0.30	6.45	0.00	32.70	48.04	3.28	0.00	0.78	8.75
MELI 10	GAT	2009	2 nd	0.72	4.67	0.00	32.96	49.66	4.26	0.34	0.59	7.52
MELI 11	QLB	2009	1 st	0.04	6.29	0.00	14.90	23.68	8.54	3.46	16.88	26.25
MELI 11	GAT	2009	1 st	0.04	16.51	0.18	14.13	22.12	6.18	2.29	13.82	24.77
MELI 11	QLB	2010	1 st	0.10	7.89	0.00	17.49	28.24	12.65	6.15	9.14	18.44
MELI 11	GAT	2010	1 st	0.12	4.48	0.00	17.81	27.31	12.17	6.63	11.00	20.61
MELI 11	QLB	2009	2 nd	0.18	16.11	0.00	21.45	33.87	8.13	2.52	4.41	13.50
MELI 11	GAT	2009	2 nd	0.36	28.34	0.31	18.10	35.80	5.96	2.51	1.05	7.93
MELI 12	QLB	2009	1 st	0.03	0.42	0.00	0.62	1.01	4.51	4.66	32.70	56.08
MELI 12	GAT	2009	1 st	0.04	0.00	0.00	0.00	0.27	1.45	1.10	43.71	53.48
MELI 12	QLB	2010	1 st	0.06	0.00	0.00	0.41	0.74	23.78	23.84	24.51	26.71
MELI 12	GAT	2010	1 st	0.06	0.00	0.00	1.23	2.17	25.60	23.96	24.84	22.20
MELI 12	QLB	2009	2 nd	0.03	0.60	0.00	0.88	1.48	23.52	18.57	21.14	33.82
MELI 12	GAT	2009	2 nd	0.12	4.88	0.00	3.54	7.21	28.32	27.13	5.22	23.69
MELI 13	QLB	2009	1 st	0.03	7.53	0.00	18.24	30.11	8.93	0.81	11.75	22.62
MELI 13	GAT	2009	1 st	0.08	6.25	0.00	24.07	35.82	5.77	0.66	9.32	18.10
MELI 13	QLB	2010	1 st	0.03	4.71	0.30	21.18	30.90	9.34	1.61	9.16	22.79
MELI 13	GAT	2010	1 st	0.04	3.58	0.00	23.08	34.88	11.94	2.58	9.59	14.35
MELI 13	QLB	2009	2 nd	0.04	9.78	0.00	24.85	39.04	9.17	0.64	4.00	12.52
MELI 13	GAT	2009	2 nd	0.10	15.31	0.41	23.42	39.03	7.79	1.00	2.23	10.81
MELI 14	QLB	2009	1 st	0.03	0.27	0.00	0.28	0.47	7.73	16.18	26.10	48.96
MELI 14	GAT	2009	1 st	0.04	0.00	0.00	0.24	0.38	2.44	5.05	38.12	53.78
MELI 14	QLB	2010	1 st	0.04	0.00	0.00	0.00	0.00	19.38	40.94	12.86	26.82
MELI 14	GAT	2010	1 st	0.06	0.00	0.00	0.00	0.00	20.91	35.58	16.21	27.31
MELI 14	QLB	2009	2 nd	0.04	0.00	0.00	0.00	0.00	23.09	32.49	11.87	32.54
MELI 14	GAT	2009	2 nd	0.03	0.00	0.00	0.00	0.22	24.97	51.64	2.80	20.37
MELI 15	QLB	2009	1 st	0.03	0.00	0.00	0.21	0.34	9.51	17.46	19.27	53.22
MELI 15	GAT	2009	1 st	0.03	0.00	0.00	0.00	0.00	5.81	13.53	22.38	58.27

MELI 15	QLB	2010	1 st	0.06	0.00	0.00	0.00	0.00	18.06	37.73	7.17	37.05
MELI 15	GAT	2010	1 st	0.08	0.00	0.00	0.00	0.00	18.86	36.63	8.63	35.88
MELI 15	QLB	2009	2 nd	0.03	0.00	0.00	0.00	0.00	18.63	36.57	7.62	37.18
MELI 15	GAT	2009	2 nd	0.04	0.00	0.00	0.00	0.22	18.76	44.96	3.25	32.82
MELI 16	QLB	2009	1 st	0.02	4.57	0.00	16.39	26.55	5.48	1.95	19.65	25.40
MELI 16	GAT	2009	1 st	0.04	9.12	0.00	12.22	19.47	4.77	1.08	22.74	30.59
MELI 16	QLB	2010	1 st	0.08	3.69	0.00	16.80	27.11	12.26	5.37	14.99	19.79
MELI 16	GAT	2010	1 st	0.10	3.58	0.00	17.28	25.55	12.39	6.23	14.82	20.15
MELI 16	QLB	2009	2 nd	0.03	13.21	0.00	17.19	29.40	12.68	3.79	7.25	16.47
MELI 16	GAT	2009	2 nd	0.24	13.60	0.21	18.79	34.34	11.07	4.92	3.08	13.99
MELI 17	QLB	2009	1 st	0.04	0.00	0.00	0.25	0.49	6.76	57.12	9.15	26.23
MELI 17	GAT	2009	1 st	0.12	0.00	0.00	0.00	0.00	6.41	48.86	13.12	31.62
MELI 17	QLB	2010	1 st	0.04	0.00	0.00	0.00	0.00	9.29	55.76	9.68	25.27
MELI 17	GAT	2010	1 st	0.06	0.00	0.00	0.00	0.00	9.95	36.40	12.37	41.28
MELI 17	QLB	2009	2 nd	0.08	0.00	0.00	0.00	0.00	8.46	46.10	8.13	37.31
MELI 17	GAT	2009	2 nd	0.08	0.00	0.00	0.00	0.28	10.80	38.61	8.93	41.39
MELI 18	QLB	2009	1 st	0.03	0.00	0.00	0.00	0.00	13.61	27.64	22.02	36.73
MELI 18	GAT	2009	1 st	0.03	0.00	0.00	0.00	0.00	8.04	13.14	35.57	43.26
MELI 18	QLB	2010	1 st	0.03	0.00	0.00	0.00	0.00	25.71	37.90	11.11	25.28
MELI 18	GAT	2010	1 st	0.03	0.00	0.00	0.00	0.00	21.81	31.05	18.15	29.00
MELI 18	QLB	2009	2 nd	0.03	0.33	0.00	0.00	0.00	27.44	30.77	13.50	27.96
MELI 18	GAT	2009	2 nd	0.03	0.00	0.00	0.00	0.00	29.92	42.23	3.78	24.07
MELI 19	QLB	2009	1 st	0.03	0.00	0.00	0.00	0.00	8.23	16.57	30.79	44.42
MELI 19	GAT	2009	1 st	0.08	0.00	0.00	0.00	0.00	5.74	10.80	38.39	45.07
MELI 19	QLB	2010	1 st	0.06	0.00	0.00	0.00	0.00	23.43	36.98	12.62	26.98
MELI 19	GAT	2010	1 st	0.04	0.00	0.00	0.00	0.00	23.19	34.93	15.46	26.42
MELI 19	QLB	2009	2 nd	0.01	0.00	0.00	0.00	0.00	22.59	34.27	13.25	29.89
MELI 19	GAT	2009	2 nd	0.08	0.00	0.00	0.00	0.00	23.88	44.08	2.81	29.22
MELI 20	QLB	2009	1 st	0.08	0.00	0.00	0.00	0.00	22.87	29.18	21.52	26.43
MELI 20	GAT	2009	1 st	0.08	0.00	0.00	0.19	0.31	13.53	17.25	33.44	35.28
MELI 20	QLB	2010	1 st	0.08	0.00	0.00	0.00	0.00	31.36	29.22	16.78	22.63
MELI 20	GAT	2010	1 st	0.10	0.00	0.00	0.00	0.00	30.78	27.39	21.33	20.50
MELI 20	QLB	2009	2 nd	0.03	0.00	0.00	0.00	0.00	25.70	26.03	22.30	25.97
MELI 20	GAT	2009	2 nd	0.10	0.00	0.00	0.00	0.00	35.88	36.48	6.79	20.85
MELI 21	QLB	2009	1 st	0.03	0.00	0.00	0.00	0.23	8.31	55.50	7.84	28.13
MELI 21	GAT	2009	1 st	0.03	0.00	0.00	0.00	0.00	6.64	43.81	10.24	39.30
MELI 21	QLB	2010	1 st	0.03	0.00	0.00	0.00	0.00	12.11	37.72	9.29	40.88
MELI 21	GAT	2010	1 st	0.06	0.00	0.00	0.00	0.00	12.13	36.32	10.80	40.75
MELI 21	QLB	2009	2 nd	0.06	0.00	0.00	0.00	0.00	11.98	50.48	9.99	27.55
MELI 21	GAT	2009	2 nd	0.08	0.00	0.00	0.00	0.00	10.42	28.12	6.21	55.25
MELI 22	QLB	2009	1 st	0.03	0.00	0.00	0.00	0.00	4.54	52.44	9.28	33.74
MELI 22	GAT	2009	1 st	0.04	0.00	0.00	0.00	0.00	4.98	59.11	3.94	31.97
MELI 22	QLB	2010	1 st	0.03	0.00	0.00	0.00	0.00	12.78	38.30	15.36	33.57
MELI 22	GAT	2010	1 st	0.08	0.00	0.00	0.00	0.00	12.80	37.43	22.35	27.43

MELI 22	QLB	2009	2 nd	0.04	0.00	0.00	0.00	0.00	7.90	48.29	15.64	28.17
MELI 22	GAT	2009	2 nd	0.06	0.00	0.00	0.00	0.00	9.01	32.04	10.79	48.16
MELI 23	QLB	2009	1 st	0.03	0.00	0.00	0.00	0.00	7.48	14.55	28.07	49.89
MELI 23	GAT	2009	1 st	0.04	0.00	0.00	0.00	0.00	4.07	7.46	36.72	51.75
MELI 23	QLB	2010	1 st	0.03	0.00	0.00	0.00	0.00	18.09	22.31	20.71	38.89
MELI 23	GAT	2010	1 st	0.04	0.00	0.00	0.00	0.00	22.17	33.15	15.08	29.60
MELI 23	QLB	2009	2 nd	0.08	0.38	0.00	0.00	0.00	26.81	35.37	10.34	27.11
MELI 23	GAT	2009	2 nd	0.08	0.00	0.00	0.00	0.00	28.10	44.23	3.89	23.77
MELI 24	QLB	2009	1 st	0.03	0.00	0.00	0.00	0.00	7.34	14.79	24.24	53.63
MELI 24	GAT	2009	1 st	0.03	0.00	0.00	0.00	0.00	4.07	9.13	29.57	57.22
MELI 24	QLB	2010	1 st	0.06	0.00	0.00	0.00	0.00	19.34	36.00	10.39	34.27
MELI 24	GAT	2010	1 st	0.08	0.00	0.00	0.00	0.00	18.78	38.36	11.77	31.09
MELI 24	QLB	2009	2 nd	0.03	0.49	0.00	0.34	0.55	20.23	32.29	8.90	37.19
MELI 24	GAT	2009	2 nd	0.03	0.00	0.00	0.00	0.00	19.50	47.97	4.09	28.44
MELI 25	QLB	2009	1 st	0.03	12.74	0.10	16.91	26.45	5.54	2.91	11.74	23.62
MELI 25	GAT	2009	1 st	0.08	14.77	0.28	14.67	22.39	5.77	2.49	13.68	25.96
MELI 25	QLB	2010	1 st	0.12	6.92	0.00	18.94	30.06	11.13	6.34	8.78	17.83
MELI 25	GAT	2010	1 st	0.18	4.89	0.00	20.80	30.79	10.99	6.61	7.19	18.73
MELI 25	QLB	2009	2 nd	0.28	25.66	0.23	23.09	36.64	4.71	0.52	1.19	7.96
MELI 25	GAT	2009	2 nd	0.46	25.38	0.30	22.04	36.74	4.96	1.64	0.86	8.08
MELI 26	QLB	2009	1 st	0.04	4.56	0.00	10.80	18.58	13.64	2.00	21.98	28.44
MELI 26	GAT	2009	1 st	0.04	7.64	0.13	12.68	20.40	9.00	0.87	21.68	27.60
MELI 26	QLB	2010	1 st	0.04	3.11	0.00	15.08	24.36	15.51	4.29	18.45	19.20
MELI 26	GAT	2010	1 st	0.12	1.64	0.00	18.69	26.89	16.49	3.54	14.45	18.30
MELI 26	QLB	2009	2 nd	0.08	7.46	0.00	23.15	36.82	12.42	0.84	7.23	12.08
MELI 26	GAT	2009	2 nd	0.26	17.39	0.29	24.17	40.09	6.72	0.55	2.67	8.12
MELI 27	QLB	2009	1 st	0.04	12.67	0.24	8.72	15.16	8.09	3.92	19.62	31.57
MELI 27	GAT	2009	1 st	0.08	17.83	0.39	11.44	18.35	7.27	3.20	15.42	26.10
MELI 27	QLB	2010	1 st	0.12	8.14	0.00	13.54	23.26	13.96	8.39	11.71	21.01
MELI 27	GAT	2010	1 st	0.16	4.97	0.00	16.87	24.53	14.86	8.18	9.68	20.91
MELI 27	QLB	2009	2 nd	0.20	19.48	0.27	17.36	26.38	9.83	2.40	6.86	17.43
MELI 27	GAT	2009	2 nd	0.28	27.31	0.40	16.05	29.69	8.76	4.30	2.17	11.33
MELI 28	QLB	2009	1 st	0.03	0.00	0.00	0.00	0.20	8.63	19.55	29.91	41.71
MELI 28	GAT	2009	1 st	0.03	0.30	0.00	0.00	0.29	5.71	11.30	36.59	45.81
MELI 28	QLB	2010	1 st	0.03	0.00	0.00	0.00	0.00	19.58	34.78	15.52	30.12
MELI 28	GAT	2010	1 st	0.06	0.00	0.00	0.00	0.00	24.53	43.68	11.81	19.98
MELI 28	QLB	2009	2 nd	0.01	0.00	0.00	0.00	0.21	24.15	35.87	11.63	28.14
MELI 28	GAT	2009	2 nd	0.03	0.00	0.00	0.39	0.76	26.16	46.50	4.64	21.55
D 9597 (MELI 29)	QLB	2009	1 st	0.03	10.90	0.00	14.92	22.59	5.35	2.56	16.29	27.39
D 9597 (MELI 29)	GAT	2009	1 st	0.08	20.34	0.21	18.44	26.73	4.47	1.75	7.63	20.44
D 9597 (MELI 29)	QLB	2010	1 st	0.12	6.13	0.00	15.74	23.83	12.23	7.13	10.59	24.35
D 9597 (MELI 29)	GAT	2010	1 st	0.24	3.45	0.00	21.66	30.69	11.32	6.45	7.20	19.23

D 9597 (MELI 29)	QLB	2009	2 nd	0.20	35.68	0.31	20.20	30.89	3.48	0.68	1.14	7.62
D 9597 (MELI 29)	GAT	2009	2 nd	0.54	35.12	0.33	19.20	33.09	3.69	0.93	0.62	7.04

* Acc. No.: accession number;

Table 11: Electronical supplement Table 1: Collection of lemon balm and balm (*M. officinalis*) for evaluation of essential oil, field trial Quedlinburg (Qlb); main component of essential oil - citral type: orange, - β-caryophyllene oxide type: violet; Content of essential oil (EO in %) and content of rosmarinic acid (RA in % of air-dried material) average for replicates A and B for the years 2010 and 2011, field trial Quedlinburg (Qlb). Taxonomical classification according to information of collection holder; BG: Botanical garden.

no. of genotype	scientific name	ploidy	origin	EO Qlb 2010	EO Qlb 2011	RA Qlb 2010	RA Qlb 2011
BLBP3	*M. officinalis*	diploid	Hungary	0,13	0,19	4,70	6,32
BLBP4	*M. officinalis*	diploid	Germany	0,22	0,16	5,04	6,21
BLBP5	*M. officinalis*	diploid	France	0,24	0,20	5,15	6,61
BLBP6	*M. officinalis*	diploid	Germany	0,25	0,28	4,62	6,89
BLBP8	*M. officinalis*	diploid	Spain	0,26	0,21	3,78	6,49
BLBP11	*M. officinalis*	diploid	Spain	0,19	0,18	5,26	6,84
BLBP12	*M. officinalis*	diploid	B.S.V.	0,28	0,20	5,39	6,61
BLBP13	*M. officinalis*	diploid	Jelitto	0,21	0,19	5,29	6,83
BLBP14	*M. officinalis*	diploid	Bornträger	0,21	0,17	5,78	6,56
BLBP15	*M. officinalis*	diploid	Fetzer	0,25	0,19	5,39	6,99
BLBP16	*M. officinalis*	diploid	Pfann	0,18	0,18	6,32	7,09
BLBP17	*M. officinalis*	diploid	Hild	0,21	0,17	6,50	7,03
BLBP18	*M. officinalis*	diploid	Pötschke	0,28	0,20	6,16	6,81
BLBP19	*M. officinalis*	diploid	Sperling	0,23	0,26	5,49	6,37
BLBP22	*M. officinalis*	diploid	Jugoslavia	0,20	0,24	6,33	7,05
BLBP25	*M. officinalis*	diploid	Austria	0,24	0,23	5,22	6,69
BLBP26	*M. officinalis*	diploid	Austria	0,27	0,16	5,23	6,17
BLBP27	*M. officinalis*	diploid	Germany	0,21	0,23	4,34	6,60
BLBP28	*M. officinalis*	diploid	BG Oldenburg	0,25	0,25	4,39	6,40

BLBP29	*M. officinalis*	diploid	BG Marburg	0,30	0,24	4,93	5,62
BLBP30	*M. officinalis*	diploid	BG Leipzig	0,20	0,20	4,67	6,41
BLBP31	*M. officinalis*	diploid	Switzerland	0,25	0,22	4,67	6,37
BLBP32	*M. officinalis*	diploid	Italy	0,24	0,24	4,97	6,77
BLBP33	*M. officinalis*	diploid	BG Halle	0,28	0,20	5,32	5,72
BLBP34	*M. officinalis*	diploid	BG Krefeld	0,23	0,25	5,19	7,26
BLBP35	*M. officinalis*	diploid	BG Köln	0,20	0,16	4,75	6,80
BLBP36	*M. officinalis*	diploid	BG Aachen	0,17	0,16	4,82	6,95
BLBP37	*M. officinalis*	diploid	BG Stuttgart	0,15	0,16	4,60	6,68
BLBP38	*M. officinalis*	diploid	Germany	0,20	0,27	4,88	6,04
BLBP39	*M. officinalis*	diploid	Bulgaria	0,23	0,17	4,69	6,00
BLBP40	*M. officinalis*	diploid	Germany	0,16	0,20	5,35	6,51
BLBP42	*M. officinalis*	diploid	Germany	0,25	0,24	4,66	6,25
BLBP43	*M. officinalis*	diploid	Germany	0,15	0,22	4,99	6,94
BLBP45	*M. officinalis*	diploid	Germany	0,23	0,24	5,33	6,70
BLBP47	*M. officinalis*	diploid	BG Braunschweig	0,18	0,15	4,28	7,02
BLBP48	*M. officinalis*	diploid	BG Braunschweig	0,17	0,16	4,89	5,45
BLBP49	*M. officinalis*	diploid	Germany	0,20	0,26	5,63	5,87
BLBP50	*M. officinalis*	diploid	Germany	0,29	0,30	4,66	5,66
BLBP52	*M. officinalis*	diploid	Finland	0,20	0,24	4,53	7,69
BLBP54	*M. officinalis*	diploid	B:S:V:	0,14	0,19	4,60	7,16
BLBP56	*M. officinalis*	diploid	BG Basel	0,26	0,20	4,82	5,51
BLBP57	*M. officinalis*	diploid	BG Rostock	0,18	0,19	4,99	7,44
BLBP58	*M. officinalis*	diploid	BG Gent	0,22	0,12	4,84	7,54
BLBP60	*M. officinalis*	diploid	BG Poznan	0,22	0,25	4,56	6,64
BLBP61	*M. officinalis*	diploid	BG Braunschweig	0,14	0,18	5,54	6,74

BLBP64	*M. officinalis*	diploid	USA	0,24	0,25	4,94	6,64
BLBP65	*M. officinalis*	diploid	India	0,31	0,28	5,65	6,39
BLBP66	*M. officinalis*	diploid	Austria	0,21	0,18	4,78	6,28
BLBP69	*M. officinalis*	diploid	Austria	0,24	0,22	4,83	6,63
BLBP70	*M. officinalis*	diploid	France	0,18	0,23	5,07	6,98
BLBP71	*M. officinalis*	diploid	Belgium	0,21	0,16	5,11	7,07
BLBP72	*M. officinalis*	diploid	Germany	0,26	0,25	5,01	7,62
BLBP74	*M. officinalis*	diploid	Austria	0,17	0,14	5,14	6,91
BLBP75	*M. officinalis*	triploid	Czech Republic	0,05	0,07	5,17	7,93
BLBP77	*M. officinalis*	diploid	Hungary	0,20	0,20	5,24	7,08
BLBP78	*M. officinalis*	triploid	France	0,07	0,09	5,10	7,15
BLBP85	*M. officinalis*	diploid	Georgia	0,13	0,12	5,70	5,99
BLBP87	*M. officinalis*	diploid	Georgia	0,29	0,25	4,91	5,96
BLBP88	*M. officinalis*	triploid	BG Liege	0,10	0,09	5,56	7,62
BLBP91	*M. officinalis*	diploid	Germany	0,21	0,21	4,55	7,16
BLBP94	*M. officinalis*	diploid	Germany	0,05	0,04	5,04	6,83
BLBP97	*M. officinalis*	diploid	Poland	0,18	0,23	4,90	6,59
BLBP98	*M. officinalis*	diploid	Czech Republic	0,18	0,19	5,17	6,50
BLBP99	*M. officinalis*	diploid	France	0,23	0,24	4,78	6,97
BLBP100	*M. officinalis*	diploid	Switzerland	0,15	0,16	5,05	6,73
BLBP111 (73B)	*M. officinalis*	triploid	Hungary	0,06	0,05	5,27	6,55
BLBP112 (75B)	*M. officinalis*	triploid	Czech Republic	0,05	0,05	5,18	7,72
BLBP113 (78B)	*M. officinalis*	triploid	France	0,08	0,08	4,69	6,65

Table 12: Electronical supplement Table 2: Composition of essential oil for the second cut of the years 2010 and 2011 average of replication A or B of 68 genotypes of balm and lemon balm (*Melissa officinalis*); orange: citral type, 62 lemon balm of 68; violet: β-caryophyllene oxide type, six balm of 68.

							composition of essential oil		
no. of genotype	year	citronellal [in %]	(*E*)-citral [in %]	(*Z*)-citral [in %]	citronellol [in %]	ß-caryophyllene [in %]	germacrene D [in %]	ß-caryophyllene-oxide [in %]	sum of unknown substances [in %]
BLBP3	2010	33,92	27,39	19,17	0,56	2,61	0,00	0,74	15,61
BLBP3	2011	30,93	31,86	22,92		2,39	0,00	0,72	11,19
BLBP4	2010	42,29	22,65	16,40	0,55	3,17	0,00	0,77	14,18
BLBP4	2011	30,72	31,00	21,28		2,38	0,00	0,79	13,85
BLBP5	2010	18,16	35,43	25,15	0,33	4,61	0,17	4,30	11,85
BLBP5	2011	24,43	31,41	22,87		4,61	0,12	4,27	12,31
BLBP6	2010	34,41	26,76	19,27	0,53	2,16	0,00	0,86	16,02
BLBP6	2011	24,57	36,82	26,98		2,19	0,00	0,87	8,59
BLBP8	2010	39,22	23,69	16,98	0,62	3,25	0,00	0,71	15,53
BLBP8	2011	29,37	33,05	24,86		2,08	0,00	0,58	10,08
BLBP11	2010	22,21	33,25	23,38	0,53	4,76	0,00	2,54	13,33
BLBP11	2011	25,92	32,37	22,79		3,80	0,00	2,23	12,90
BLBP12	2010	12,53	36,76	25,65	0,29	5,10	0,27	4,82	14,57
BLBP12	2011	20,91	33,77	25,11		4,95	0,40	2,83	12,05
BLBP13	2010	45,28	21,13	15,22	0,55	2,77	0,00	0,62	14,41
BLBP13	2011	25,51	34,93	26,13		2,64	0,00	0,52	10,29
BLBP14	2010	50,60	21,19	15,48	0,52	2,59	0,00	0,50	9,12
BLBP14	2011	29,64	32,26	24,08		2,55	0,00	0,58	10,90
BLBP15	2010	39,76	21,53	15,59	0,56	3,09	0,00	0,98	18,49
BLBP15	2011	17,92	39,50	29,19		2,90	0,00	0,82	9,68

BLBP16	2010	36,86	24,66	17,82	0,60	2,93	0,00	0,73	16,40
BLBP16	2011	27,07	33,93	25,43		2,03	0,00	0,58	10,98
BLBP17	2010	39,53	20,15	14,42	0,49	2,06	0,00	1,19	22,15
BLBP17	2011	28,31	33,46	23,98		2,53	0,00	0,74	11,00
BLBP18	2010	48,56	20,23	14,78	0,53	2,54	0,00	0,54	12,81
BLBP18	2011	30,56	33,23	24,47		1,84	0,00	0,44	9,47
BLBP19	2010	36,90	24,18	17,52	0,65	2,53	0,00	0,59	17,64
BLBP19	2011	33,38	30,24	22,71		2,69	0,00	0,69	10,30
BLBP22	2010	41,39	24,22	17,42	0,55	2,25	0,00	0,69	13,49
BLBP22	2011	30,36	32,47	23,83		2,81	0,00	0,60	9,94
BLBP25	2010	38,06	24,37	17,66	0,56	2,46	0,00	0,85	16,02
BLBP25	2011	24,95	35,11	26,14		2,29	0,00	0,63	10,90
BLBP26	2010	45,05	22,03	15,97	0,75	2,19	0,00	0,61	13,40
BLBP26	2011	38,55	27,85	20,68		2,50	0,00	0,66	9,78
BLBP27	2010	43,85	19,28	13,74	0,73	2,27	0,00	0,67	19,46
BLBP27	2011	33,01	31,20	22,92		1,90	0,00	0,54	10,44
BLBP28	2010	42,44	20,89	15,17	0,65	3,02	0,00	0,67	17,16
BLBP28	2011	33,98	30,44	22,34		2,33	0,00	0,59	10,33
BLBP29	2010	48,45	20,80	15,00	0,63	2,02	0,00	0,56	12,54
BLBP29	2011	35,35	29,08	21,55		1,98	0,00	0,68	11,38
BLBP30	2010	37,84	24,12	17,46	0,61	3,13	0,00	0,83	58,01
BLBP30	2011	25,35	34,32	26,10		2,25	0,00	0,57	11,43
BLBP31	2010	40,93	24,04	17,57	0,51	2,17	0,00	0,52	14,25
BLBP31	2011	31,72	31,54	23,52		2,44	0,00	0,57	10,22
BLBP32	2010	37,76	26,56	19,22	0,46	2,36	0,15	0,97	12,53
BLBP32	2011	20,73	36,45	27,13		3,16	0,32	1,17	11,06
BLBP33	2010	14,01	42,59	31,31	0,35	3,07	0,00	0,71	7,96

BLBP33	2011	17,89	39,35	29,44		2,89	0,00	0,71	9,73
BLBP34	2010	46,39	19,32	13,52	0,85	3,03	0,00	0,84	16,04
BLBP34	2011	30,01	31,46	23,10		2,46	0,00	0,93	12,05
BLBP35	2010	42,31	21,01	15,08	0,58	4,07	0,00	1,27	15,68
BLBP35	2011	32,29	28,91	21,26		4,01	0,00	1,57	55,98
BLBP36	2010	43,56	23,42	16,65	0,50	3,24	0,00	0,84	11,79
BLBP36	2011	28,46	33,12	24,49		3,37	0,00	0,97	9,60
BLBP37	2010	13,27	42,30	30,19	0,28	2,70	0,00	1,33	9,94
BLBP37	2011	13,63	41,69	30,15		3,31	0,00	1,58	9,64
BLBP38	2010	27,51	31,85	23,18	0,46	3,42	0,00	1,01	12,58
BLBP38	2011	21,79	36,70	25,95		4,03	0,00	1,79	9,75
BLBP39	2010	40,84	22,49	16,36	0,57	3,11	0,11	0,91	15,61
BLBP39	2011	41,20	26,22	19,59		1,70	0,00	0,69	10,61
BLBP40	2010	37,15	28,91	20,86	0,49	3,58	0,10	1,15	7,76
BLBP40	2011	22,10	36,74	27,34		2,73	0,00	1,12	9,98
BLBP42	2010	44,25	22,18	16,00	0,61	2,97	0,00	0,64	13,35
BLBP42	2011	24,55	36,02	26,70		2,07	0,00	0,75	9,92
BLBP43	2010	28,53	33,35	23,31	0,35	4,59	0,45	2,01	7,42
BLBP43	2011	19,82	36,65	26,04		4,87	0,31	1,95	55,19
BLBP45	2010	57,75	17,53	12,58	0,57	2,47	0,00	0,53	8,57
BLBP45	2011	36,61	27,61	20,46		2,38	0,00	0,88	12,06
BLBP47	2010	32,34	24,97	18,02	0,47	3,56	0,00	1,16	19,48
BLBP47	2011	22,26	36,44	27,29		3,04	0,00	1,00	9,97
BLBP48	2010	41,71	23,87	17,14	1,12	2,70	0,00	0,57	12,87
BLBP48	2011	30,10	30,66	21,58		2,33	0,00	1,04	14,30
BLBP49	2010	49,41	16,89	12,03	0,92	3,89	0,28	1,00	15,58
BLBP49	2011	45,11	22,72	15,81		2,58	0,00	1,07	12,72

BLBP50	2010	44,60	22,97	16,88	0,47	3,30	0,00	0,68	11,11
BLBP50	2011	35,32	29,29	21,60		2,56	0,00	0,81	10,43
BLBP52	2010	23,28	30,16	21,52	0,47	5,25	0,33	3,08	15,91
BLBP52	2011	22,00	36,06	26,38		3,57	0,06	1,87	10,07
BLBP54	2010	16,57	31,99	20,90	0,54	5,99	0,63	4,24	19,15
BLBP54	2011	21,22	36,17	26,88		4,13	0,27	2,19	9,16
BLBP56	2010	47,06	20,57	14,78	0,57	2,57	0,00	0,95	13,49
BLBP56	2011	40,82	28,38	19,62		2,04	0,00	0,79	8,36
BLBP57	2010	47,83	20,72	14,61	0,55	2,73	0,00	1,41	12,16
BLBP57	2011	32,61	29,17	20,92		3,24	0,00	1,39	12,67
BLBP58	2010	35,47	21,88	15,20	0,81	2,72	0,00	1,30	22,62
BLBP58	2011	29,35	31,94	23,39		1,64	0,00	1,24	12,45
BLBP60	2010	42,07	21,85	15,73	0,50	1,83	0,00	0,84	17,19
BLBP60	2011	23,03	37,00	27,14		2,17	0,00	0,78	9,89
BLBP61	2010	36,05	24,28	16,70	0,58	2,77	0,00	2,12	17,51
BLBP61	2011	20,68	37,36	26,26		2,94	0,00	1,29	11,48
BLBP64	2010	44,58	19,85	14,13	0,51	2,36	0,00	1,25	17,32
BLBP64	2011	29,75	33,18	24,59		2,19	0,00	0,79	9,50
BLBP65	2010	48,87	19,99	14,73	0,64	2,88	0,00	0,55	12,33
BLBP65	2011	39,95	27,90	19,67		2,12	0,00	0,75	9,62
BLBP66	2010	24,94	32,59	23,60	0,51	2,65	0,00	1,13	14,59
BLBP66	2011	15,20	40,69	29,78		2,61	0,00	1,29	10,44
BLBP69	2010	16,60	38,67	27,97	0,29	4,74	0,61	2,88	8,23
BLBP69	2011	17,70	37,24	26,98		3,91	0,20	2,94	11,04
BLBP70	2010	24,95	34,13	24,82	0,52	3,33	0,00	0,90	11,35
BLBP70	2011	19,76	39,00	27,57		3,09	0,00	1,03	9,56
BLBP71	2010	29,57	32,74	23,56	0,38	3,70	0,00	0,99	9,06

BLBP71	2011	16,94	38,68	28,56		3,58	0,00	1,26	10,98
BLBP72	2010	44,88	22,94	16,74	0,45	3,34	0,24	1,16	10,24
BLBP72	2011	19,55	36,15	26,15		3,79	0,05	2,22	12,10
BLBP74	2010	23,99	32,57	23,71	0,47	3,81	1,28	4,28	9,88
BLBP74	2011	8,47	22,67	16,81		7,53	3,25	16,01	25,26
BLBP75	2010	2,33	1,31	0,83	0,15	6,26	3,33	49,20	36,60
BLBP75	2011	3,91	1,87	1,24		14,41	7,20	36,05	35,32
BLBP77	2010	42,92	22,79	16,57	0,76	3,10	0,10	0,72	13,04
BLBP77	2011	24,50	35,37	25,93		2,27	0,00	0,68	11,25
BLBP78	2010	4,10	3,63	2,37	0,50	9,76	8,18	31,98	39,49
BLBP78	2011	5,35	6,17	4,19		13,99	9,10	22,64	38,57
BLBP85	2010	7,89	44,89	32,12	0,13	4,09	0,00	1,35	9,53
BLBP85	2011	5,80	43,48	31,61		4,93	0,00	1,74	12,44
BLBP87	2010	23,05	35,32	26,03	0,39	5,23	0,50	1,07	8,41
BLBP87	2011	19,79	37,92	27,96		3,64	0,00	1,03	9,66
BLBP88	2010	2,69	2,09	1,36	0,36	9,92	5,36	43,36	34,86
BLBP88	2011	5,09	6,83	4,73		16,94	8,72	25,74	31,97
BLBP91	2010	55,53	16,68	12,06	0,63	4,31	0,00	1,30	9,49
BLBP91	2011	30,65	30,87	23,04		3,36	0,00	1,29	10,81
BLBP94	2010	17,79	20,15	12,97	0,85	6,50	0,25	4,67	36,82
BLBP94	2011	14,56	34,22	24,04		2,59	0,00	2,43	22,17
BLBP97	2010	24,76	38,00	26,26	0,39	2,77	0,00	0,83	6,98
BLBP97	2011	17,03	39,99	29,04		2,41	0,00	1,05	10,50
BLBP98	2010	26,81	35,74	25,87	0,37	2,49	0,00	0,67	8,06
BLBP98	2011	22,26	37,35	27,08		1,89	0,00	0,69	10,74
BLBP99	2010	50,73	16,66	12,00	0,68	3,62	0,00	0,71	15,60
BLBP99	2011	38,72	26,34	19,15		2,91	0,00	0,80	12,10

BLBP100	2010	12,72	43,55	30,08	0,25	3,44	0,00	1,91	8,05
BLBP100	2011	7,79	44,61	33,07		2,65	0,00	2,02	9,86
BLBP111 (73B)	2010	2,77	2,81	1,76	0,38	8,69	6,96	34,13	42,50
BLBP111 (73B)	2011	3,71	4,49	3,19		11,12	7,70	28,84	40,96
BLBP112 (75B)	2010	1,94	0,86	0,55	0,00	5,66	2,94	51,72	36,33
BLBP112 (75B)	2011	2,83	1,76	1,21		12,57	6,70	37,98	36,96
BLBP113 (78B)	2010	5,94	4,01	2,61	0,57	9,89	7,99	29,98	39,01
BLBP113 (78B)	2011	5,63	7,56	4,99		12,72	7,07	25,43	36,62

n.s. not specified

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12 EIDESSTATTLICHE ERKLÄRUNG / DECLARATION UNDER OATH:

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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