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IMPROVEMENT OF BIOLOGICAL BASES FOR CULTIVATION OF WORMWOOD (*ARTEMISIA ABSINTHIUM* L.)

Doctoral (Ph.D.) Dissertation

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DECLARATION

I declare that the thesis titled “Improvement of biological bases for cultivation of wormwood (*Artemisia absinthium* L.)” is my work, that it has not been submitted before for any degree or examination in any other University.

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Abbreviations

AAE: Ascorbic acid equivalents

AC: Antioxidant capacity

ANOVA: One-way analysis of variance

BBCH: Biologische **B**undesanstalt, **B**undessortenamt und **CH**emische Industrie

CV: Coefficient of variation

DNA: Deoxyribonucleic acid

DW: Dry weight

EDTA: Ethylenediaminetetraacetic acid

EMA-HMPC: European Medicines Agency – Committee on Herbal Medicinal Products

EO: Essential oil

FRAP: Ferric reducing antioxidant power

GAE: Gallic acid equivalent

GC: Gas chromatography

HSD: Honestly significant difference

IBA: Indole-3-butyric acid

ISSR: Inter simple sequence repeat

KMO: Kaiser-Meyer-Olkin's statistic

LRI: Linear retention indices

MANOVA: Multivariate analysis of variance

MAP: Medicinal and aromatic plants

Max: Maximum

Min: Minimum

MS: Mass spectrometry;

PC: Principal components

PCA: Principal component analysis

PCR: Polymerase chain reaction

RAPD: Random amplified polymorphic DNA

RT: Retention time

RWC: Ralative water content

SA: *trans*-Sabinyl acetate chemotype

SD: Standard deviation

SZIU: Szent István University

TPC: Total phenolic content

T: Thujone chemotype

TIC: Total ion current

UPGM: Un-weighted Pair Group Method

UK: United Kingdom

UV: Ultraviolet

CHAPTER 1. INTRODUCTION

1.1 Introduction

The genus *Artemisia* is a member of the Compositae (Asteraceae) family which is a large, diverse genus of plants. *Artemisia absinthium* L. has been known by various names as wormwood (UK), absinthe (France), bitter wormwood and wermut (Germany). It grows from 40 to 150 cm in height (Maw et al., 1985) with a woody, hardy rosette and high, branching stems. In folk medicine, it is used against various diseases like mental exhaustion and nervous depression, otalgia, chronic fever, anaemia, amenorrhoea, ect (Ahmad et al., 2010). The herb is also used in treating anorexia nervosa (an eating disorder), atherosclerosis (a hardening of the arteries, as prevention), to improve one's mental state, against insect and spider bites, nervous breakdown, labor pains, stomach ailments, herpes, parasitic worm infections, sclerosis (Guarrera, 2005). The essential oil of this plant has been used as an anthelmintic, anticold, anti-inflammatory, antimicrobial, antiseptic, antidepressant, digestive, carminative, stimulant, choleratic, tonic and balsamic agent (Goud and Swamy, 2015).

The main secondary metabolites of wormwood are volatiles in the leaves and flowers which lend the plant a characteristic and strong aromatic aroma. The content of the essential oil may depend on the origin of the sample (Nguyen and Németh, 2016). The essential oil of *A. absinthium* is usually known and reported to be rich in bicyclic monoterpene thujone (Juteau et al., 2003; Meschler and Howlett, 1999) but several other main compounds have also been detected, e.g. myrcene, sabinene, linalool, *cis*-epoxyocimene, chrysanthenyl acetate and *trans*-sabinyl acetate (Nguyen and Németh, 2016). The main component of *A. absinthium* seems to vary from country to country and the major components are different in relation to the plant origin (Basta et al., 2007, Mohammadi et al., 2015b). For example, in Lithuania the predominant compounds of wormwood such as thujones (*cis* and *trans*) and *trans*-sabinyl acetate were indicated (Judzentiene and Budiene, 2010; Judzentiene et al., 2009) while bornyl acetate was the major component in Cuba (Pino et al., 1997) and the most dominant constituents in Greece were caryophyllene oxide, p-cymene, cineole and (Z)-lanceol acetate (Basta et al., 2007). Although many references describe the large variability of EOs from wormwood samples, the origin of the samples is frequently not defined adequately. In several cases they are commercial samples with of unknown original habitat; in other studies, different plant parts have been investigated and/or bulk samples were taken from the populations which can not represent individual chemical differences. Furthermore, the main components of wormwood essential oils may be affected aside from the origin by many other factors such as soil characteristics, seasonal variations, extraction

methods as well as storage conditions (Judzetiene and Budiene, 2016).

Consumption of wormwood plants is widespread and increasing. There are many types of products such as tea, powder, spirits, medicinal products etc. made from wormwood with big market potential. It is necessary to optimize yield and achieve standardized, uniform, high quality products and avoid species misidentification, variability and instability of extracts, toxic components and contaminants. Stable quantity and quality can only be achieved by cultivation as wild growing populations are of uncertain quality and supply by collection may not fulfill the requirements. For economic production, detailed knowledge on morphology, plant development, yield and chemical features as well as factors influencing the plant production are absolutely necessary. This requirement formed the basis of our investigations.

Existing studies on wormwood describe the evaluation of samples from diverse geographical conditions. However, in the huge majority of these references the effects of genotype, biotic factors, climate and other abiotic circumstances could not be separated from each other (Nguyen and Németh, 2016). Experiments on wormwood species in controlled conditions are very rare. Ontogenetic and morphogenetic factors are also frequently very difficult to distinguish from each other as well as from outside influencing factors. It can be established that the effect of genotype or climatic conditions on growth, development and bioactive substances of wormwood has not been adequately detected or recorded.

In this study we started a detailed investigation of 12 accessions of *A. absinthium*. Seeds of these materials were collected from different regions but the plants were grown at the same place under uniform circumstances to exclude the influence of the environment or technology. As a rare approach, individual sampling was applied to reveal the chemical potential of the species which would be not possible based on bulk samples as it is the drawback of many investigations.

1.2 Goals of research

The main goal of the research was to provide basic knowledge and information about the biology and chemistry of the species and to get practical information for the introduction and elaboration of cultivation. We wanted to obtain and characterize prosperous genotypes for domestication and further breeding. In the frame of this, we wanted to:

- detect the intraspecific variability of *A. absinthium* concerning some important morphological features, biomass production, accumulation and spectrum of volatile compounds and level of phenolics;
- reveal the connection between the investigated characteristics;
- detect the connection between the origin of the intraspecific accessions and their

characteristic features in order to obtain optimal plant material;

- evaluate the biotic and abiotic factors which might influence the chemical features and drug quality of wormwood;
- develop basic methods for effective propagation and maintenance of plantations for assuring good quality raw material with high yields.

CHAPTER 2. LITERATURE REVIEW

2.1 General distribution and scientific classification

The genus *Artemisia* is a member of the Compositae (Asteraceae) family which is a large, diverse genus of plants located in the temperate and cold regions of Eurasia and North America (Wang, 2004). Belonging to this genus, *Artemisia absinthium* L. (wormwood) is native to Europe and can be found throughout temperate Asia northwards to Lapland, Karelia and Southern Siberia. It has become naturalized in North and South America and New Zealand (Maw et al., 1985).

Scientific classification:

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivison:	Spermatophyta
Division:	Magnollophyta
Class:	Magnollopsida
Subclass:	Asteridae
Order:	Asterales
Family:	Asteraceae
Genus:	*Artemisia* L.
Species:	*Artemisia absinthium* L.

2.2 Brief botanical description of wormwood

This plant is an aromatic, bitter, shrubby, perennial herb. It may grow up to 150 cm in height (Maw et al., 1985). The stem is multibranched and firm, almost woody at the base, is erect, angular, hoary and leafy. The stem branch twigs have prominent ridges and furrows covered by white hair and the leaf twigs are silvery hoary on both surfaces (Ahmad et al., 2010; Goud and Swamy, 2015). Leaves are ovate to obovate, silvery grey, up to 20 cm in length (Nguyen et al., 2018) and 3-7 cm in broad, abundantly pinnate with linear or lanceolate, obtuse segments, hoary on both surfaces, radical and lower cauline narrowed into winged petiole (Maw et al., 1985). Flowers are usually produced from July to October, are small and globular, and arranged as loose clusters of small yellow umbels on erect panicles. The various flower heads are short stemmed and hang in many flower panicles. Each flowering head is surrounded by 8-10 bracts. The size of a single flower is approximately 3-4 mm. Capitula flowers are pale yellow and tubular. The fruits produced is a cylindrical, slightly flattened acheme, with no pappus. The fruit is 1.5 mm long.

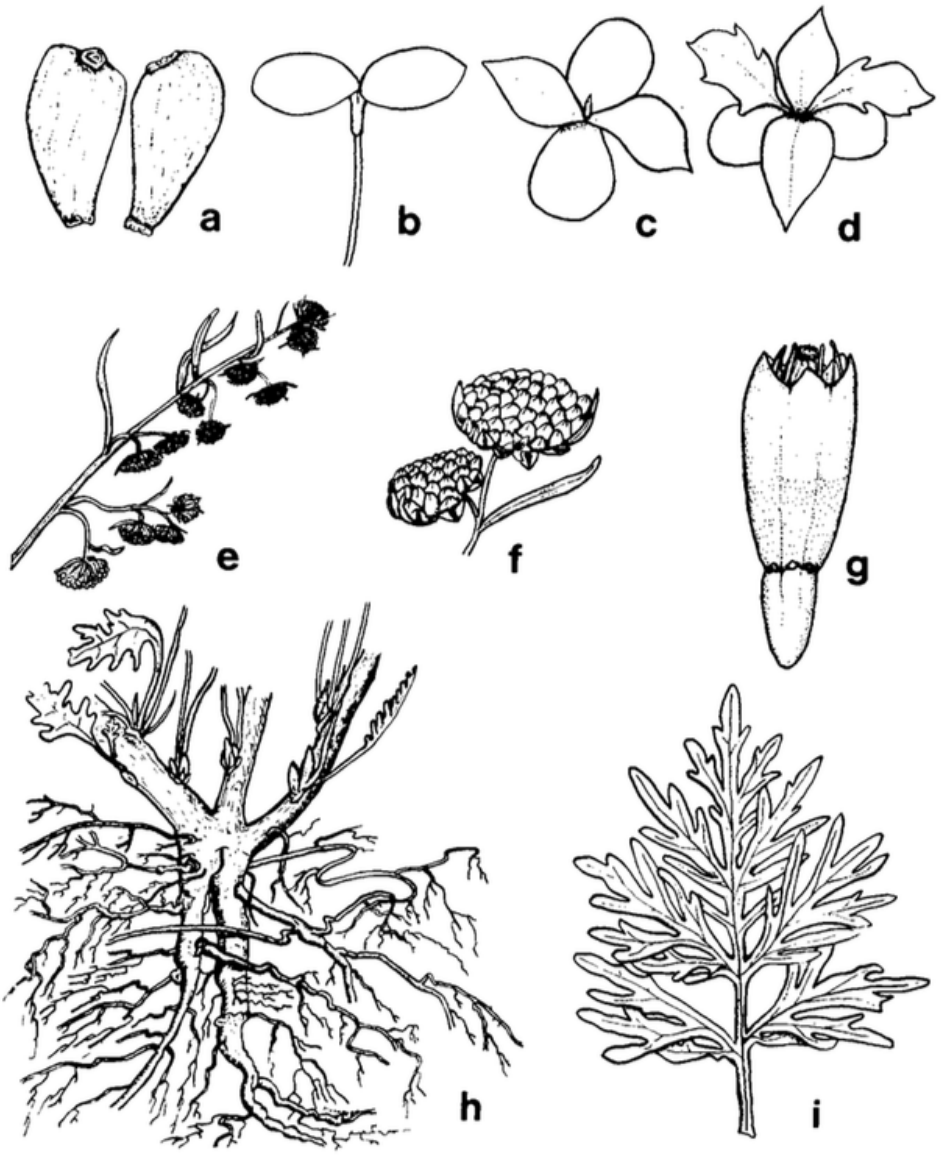

Fig. 1. *Artemisia absinthium*: (a) seeds (× 36); (b) seedling, cotyledon (× 18); (c) seedling, two leaf stage (× 7); (d) seedling, four leaf stage (× 5); (e) flowering branch (× 3); (f) flower head (× 10); (g) flower (× 40); (h) caudex and root (× 0.25); (i) leaflet (× 1).

Fig 1. Morphology of *Artemisia absinthium* L. Source: (Maw et al., 1985)

2.3 Main active ingredients of wormwood

Various secondary metabolites and other products have been isolated from *A. absinthium*, the most important being the essential oil obtained from glands on the aerial parts. Because of high concentrations of volatile terpenes, especially in leaves and flowers, the essential oil of this species has a strong aromatic smell.

References to the content of essential oil indicate different levels depending on the origin of the sample. Orav and co-workers (2006) obtained 0.1% to 1.1% essential oil in plant material coming from different European regions. Relatively high concentrations (1.10% - 1.46%) were

determined from wormwood collected in Tunisia (Msaada et al., 2015). Plant material from Cuba contained 1.25% essential oil (Pino et al., 1997) while the one from Greece gave a yield of 0.31% (Basta et al., 2007).

The color of the oils has been also reported in different literature. Hydrodistillation of dried wormwood resulted in a dark blue essential oil (Msaada et al., 2015) obviously with considerable chamazulen content. According to Pino et al. (1997) the oil extracted from the dried leaves and flowering tops of *A. absinthium* was found to be a dark brownish green liquid. The essential oil of *A. absinthium* is usually known and reported to be rich in bicyclic monoterpene thujone which therefore may be considered as the most characteristic constituent of wormwood oil (Juteau et al., 2003; Meschler and Howlett, 1999). Both isomeric forms, α- and β-thujones, were found in wormwood oil, but the concentration of β-thujone is usually higher than that of α-thujone. However, the actual proportion may change on a large scale. α- and β-thujones were detected in 18.6% and 23.8% respectively in a study of wild collected Iranian plants (Rezaeinodehi and Khangholi, 2008). In Serbian natural populations, β-thujone was the absolute major component representing up to 63.4% of the total oil isolated from the aerial parts of wormwood, while α- thujone occupied only 0.4% (Blagojević et al., 2006). Lachenmeier and Nathan-Maister (2007) reviewed 24 references regarding the wide variations of thujone content in wormwood oil. Based on these, they reported that mean proportions of α- and β-thujones in the essential oil of *A. absinthium* were 5.8% and 12.5%, respectively.

Other major compounds of *A. absinthium* volatile oil observed in several studies are myrcene, sabinene, linalool, *cis*-epoxyocimene, chrysanthenyl acetate (Martín et al., 2011) and *trans*- sabinyl acetate. A study by Ariño et al. (1999) indicated that the most abundant composition in all analyzed samples from Spain was *cis*-epoxyocimene. Llorens-Molina and Vacas (2015) also found *cis*-epoxyocimene (49.3% - 71.5%), *cis*-chrysanthenyl acetate (7.6% - 18%) and linalool (0.7% - 10.4%) as the main compounds in samples collected in Teruel, Spain. *Cis*-epoxyocimene (24.6%) was also detected in an Italian material by Mucciarelli et al. (1995). However, according to Judzentiene et al. (2009), *trans*-sabinyl acetate (8.8% - 55.2%) as the dominant compound was found in 15 investigated oil samples of wormwood from wild growing sites in Vilnius city (Lithuania). Besides thujones, β-pinene was the second most important constituent of Iranian wormwood essential oil (Rezaeinodehi and Khangholi, 2008). In addition, Martín et al. (2011) reported *cis*-epoxyocimene, chrysanthenol, and chrysanthenyl acetate as major compounds. Table 1 provides an outlook on the most frequently detected major compounds of the essential oil of wormwood.

Table 1. Major essential oil (EO) components (>10% of EO) in *A. absinthium* reported in different references

Compound	Plant part	Origin	Ratio (% EO)	References
Bornyl acetate	R	Spain (W)	21.1	(Llorens-Molina and Vacas, 2015)
	AP	Cuba (W)	23.0	(Pino et al., 1997)
Caryophyllene-oxide	AP	Greece (W)	25.5	(Basta et al., 2007)
Camphor	AP	Italy (W)	17.1	(Mucciarelli et al., 1995)
	AP	Iran (W)	14.8	(Nezhadali and Parsa, 2010)
Chamazulene	AP	Turkey (W)	17.8	(Kordali et al., 2005)
	AP	Tunisia (W)	39.9	(Msaada et al., 2015)
cis-Chrysanthenol	FT	France (W)	69.01	(Carnat et al., 1992)
	AP	Spain (C)	15.7	(Martín et al., 2011)
cis-Chrysanthenyl acetate	L	Spain (W)	32.6	(Ariño et al., 1999a)
	FT	Spain (W)	43.0	
	AP	France (W)	33.6	(Juteau et al., 2003)
	AP	Tajikistan (W)	17.9	(Sharopov et al., 2012)
trans-Chrysanthenyl acetate	AP	Italy (W)	21.6	(Mucciarelli et al., 1995)
p-Cymene	AP	Iran (W)	10.3	(Nezhadali and Parsa, 2010)
	AP	Greece (W)	16.8	(Basta et al., 2007)
cis-Epoxyocimene	L	Spain (W)	44.7	(Ariño et al., 1999a),
	FT	Spain (W)	37.3	
	AP	Italy (W)	24.6	(Mucciarelli et al., 1995)
	AP	Croatia (W)	30.8	(Juteau et al., 2003)
	L	France (W)	49.7	
	AP	Russia (P)	21.1	(Orav et al., 2006)
	L&F	France (W)	54.4	(Chialva et al., 1983)
	AP	Tajikistan (W)	17.9	(Sharopov et al., 2012)
α-Fenchene	R	Spain (W)	23.7	(Llorens-Molina and Vacas, 2015)
Myrcene	AP	Tajikistan (W)	22.7	(Sharopov et al., 2012)
	FT	France (W)	10.4	(Carnat et al., 1992)
	AP	Canada (W)	10.8	(Lopes-Lutz et al., 2008)
	AP	Estonia (P)	29.9	(Orav et al., 2006)

	AP	Moldova (P)	38.9	
	AP	Scotland (P)	18.0	
	R	Spain (W)	29.2	(Llorens-Molina and Vacas, 2015)
	R&AP	Turkey (W)	44.3	(Altunkaya et al., 2014)
Neryl butanoate	AP	France (P)	13.9	(Orav et al., 2006)
β-Pinene	AP	Iran (W)	23.8	(Rezaeinodehi and Khangholi, 2008)
Sabinyl acetate	AP	Croatia (W)	13.5	(Juteau et al., 2003)
	AP	Canada (W)	26.4	(Lopes-Lutz et al., 2008)
	AP	Lithuania (W)	51.3	(Judzentiene et al., 2010)
	AP	Siberia (P)	31.5	
	AP	France (P)	84.5	
	AP	Belgium (P)	18.6	(Orav et al., 2006)
	AP	Estonia (P)	70.5	
	AP	Armenia (P)	34.2	
Sabinene	AP	Hungary (P)	18.1	(Orav et al., 2006)
	AP	Estonia (P)	25.3	
Terpinen-4-ol	L&F	United State (C)	11.6	(Nin et al., 1995)
	AP	Italy (C)	28.8	
α-Thujone	AP	Lithuania (W)	36.8	(Judzentiene et al., 2010)
	L	Morocco (W)	39.6	(Derwich et al., 2009)
β-Thujone	AP	Greece (P)	38.7	(Orav et al., 2006)
	AP	Estonia (P)	64.6	
	AP	Iran (W)	18.6	(Rezaeinodehi and Khangholi, 2008)
	L	Croatia (W)	48.6	(Juteau et al., 2003)
	AP	Canada (W)	10.1	(Lopes-Lutz et al., 2008)
	AP	Lithuania (W)	48.9	(Judzentiene et al., 2010)
	L&F	United State (C)	69.9	(Nin et al., 1995)
	AP	United State (P)	33.1	(Tucker et al., 1993)
	AP	Iran (W)	35.1	(Morteza-Semnani et al., 2005)

- *AP: aerial parts; F: flowers; FT: flowering tops; L: leaves; R: roots*

- *W: wild collected material; C: cultivated variety; P: purchased on the retail market*

The bitter taste and the activity of the herb are ascribed to several sesquiterpene lactones. Wright (2003) has reported that the appetite stimulant property of *A. absinthium* is caused by the bitter substances anabsinthin (sesquiterpene lactone) and absinthin (dimer of sesquiterpene lactone) present in plant extracts.

The phytochemical study of Ashok and Upadhyaya (2013) showed the presence of various chemical constituents, i.e. carbohydrate, glycoside, oils and fats, saponins, phytosterols, proteins and amino acids, tannins, phenolic compounds and flavonoids in hexane and alcoholic extracts of wild Indian wormwood. In detail, the ratio of starch, sugar, tannin and total phenolic contents of the leaves extracts were 11.66%, 6.38%, 0.20% and 2.78%, respectively.

Wormwood contains also fatty acids. Palmitic (33.39%), arachidic (26.2%), linoleic (27.5%), lauric, myristic, steric and oleic acids have been detected in the lipid fraction of volatile oils of leaf (0.22%), flower (0.35%), and herb (0.3%) by TLC (Wasim Ahmad, 2010). Nikhat et al. (2013) reported that the seeds contain a mixture of 9-hydroxy-trans, trans, 10, 12-octadecadienoic acid and 13-hydroxy-trans, trans, 9, 11-octadecadienoic acid in the ratio of 2:1.

2.4 Human physiological and therapeutic effects

In traditional medicine, *A. absinthium* is believed to treat mental exhaustion and nervous depression, otalgia, chronic fever, anaemia, amenorrhoea, etc. (Wasim Ahmad, 2010). According to Guarrera (2005) and Wake et al. (2000), wormwood has been used in folk medicine as an antispasmodic, febrifuge, stomachic, cardiac stimulant, anthelmintic agent and for the restoration of declining mental function and inflammations of the liver.

It is also folk remedy in Asia, and a long list of medicinal uses have been attributed to wormwood, including antimalarial, antiviral, antitumor, spasmolytic and others (Tan et al., 1998). In China, *A. absinthium* has commonly been used as an aromatic substance and additive to rice wine and to grape wine, while it was an additive to beer in ancient Egypt (Tan et al., 1998).

It is still being used in Yemen to alleviate the pain associated with parturition (Rätsch, 2005). According to the same author, wormwood is one of the most important gynecological agents in European folk medicine for abortion and to induce menstruation or delivery. In tea form, it is consumed primarily for stomach pains, against lack of appetite, feelings of fullness, gallbladder problems, vomiting, and diarrhea. Moreover, in homeopathy, wormwood is used in accordance with medical descriptions to treat epilepsy and nervous or hysterical spasms (Rätsch, 2005).

Although obviously not all of the traditional uses could be justified by modern tools, pharmacological and clinical investigations, there are a lot of data on possible sophisticated

utilizations. In a study at Yale University, patients from five locations in Germany with Crohn's disease were administered a herbal blend containing wormwood herb (3 x 500 mg/day), or placebo, for a ten week period. It was observed that patients consuming the herb blend were able to come off steroids - the conventional treatment for this disease - and the treatment improved their mood and quality of life (Omer et al., 2007). Similar findings were published by Krebs et al. (2010) administering 3 x 750 mg dried powdered wormwood for 6 weeks in addition to their basic Crohn's disease therapy. In animal experiments, Bora and Sharma (2010) detected neuroprotective effects of wormwood on focal ischemia and reperfusion-induced cerebral injury and this finding was ascertained by Lachenmeier (2010), too.

As with many other plants, wormwood represents a rich source of antioxidants which may support healing of skin wounds (Bora and Sharma, 2011; Craciunescu et al., 2012). A study on oxidative stress conducted by Kharoubi and co-workers (2008) has indicated that oral administration of wormwood extract (200 $mg.kg^{-1}$ body weight) for 11 weeks to rats stimulated the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase. The results of this study also suggested that *A. absinthium* extract had a protective role against lipid peroxidation. Similarly, Altunkaya et al. (2014) reported that the essential oil of *A. absinthium* showed significant antimicrobial activity against 6 bacteria and 2 yeasts in disk diffusion trial.

The benefits of this plant are not limited to human health; its efficacy is also known in veterinary therapies. As a result of a study by Tariq and co-workers (2009), wormwood extract may be a natural alternative to commercial drugs for addressing intestinal invaders in sheep. Wormwood essential oil has been shown to have insecticidal activity (Kordali et al., 2005) and repellent effects against fleas, flies and mosquitoes (Erichsen-Brown, 1979). It is suggested that the essential oil of this plant might play an important role in the development of natural or biological insecticides, thus contributing to the reduction of environmental pollution by chemicals.

2.5 Contrary and adverse effects of using *A.absinthium* and its legislation

The use of this herb as a source of natural products has attracted the interest of many researchers and producers. The special utilization of wormwood in the spirit absinthe has gained the highest reputation, but it plays an important role as flavouring agent of some other alcoholic beverages, as well. *Absinthe* was produced in French-speaking Switzerland in the late 18th century, and in the late 19th century Absinthe became the most popular spirit drink in Europe, called the "green fairy" (Lachenmeier et al., 2006). Vogt (1981) described that the consumption of absinthe was

between five and six o'clock when Parisians gathered to sit outside of the cafés and drink their customary glasses of this green, anise-flavored spirit. Nearly all of the 33,000 bars and cafés were filled with patrons sipping absinthe in that period, and it was noted that "the sickly odor of absinthe lies heavy on the air" in the old village of Montmartre, France (Holstege et al., 2002). Because of the rising interest in anise-based spirits as well as increased promotion and advertising, the production of Pernod's absinthe climbed up to 125,000 liter in 1896. The annual per capita French consumption of absinthe grew dramatically in the period from 1875 to 1913 and the production reached 239.492 hectoliters in 1913, representing 60 liters per inhabitant of France (Padosch et al., 2006). Indeed, the 1890s were called the *Absinthe decade* (Baker, 2001). However, at the beginning of the 20th century, the production of this bitter spirit was prohibited in several countries as it was blamed for a range of severe symptoms, called absinthism.

The syndrome called absinthism originates from the name of the plant. Symptoms of so-called absinthism included convulsions, blindness, hallucinations and mental deterioration (Lachenmeier et al., 2006). As a consequence of its supposed negative effects on human health, absinthe, the bitter spirit containing wormwood, was banned for a period at the beginning of the 20th century (Padosch et al., 2006).

There are some detailed cases reported in literature from the very early times untill recent times. Smith (1863) described a case of a salesman who was insensible, convulsed, foaming at the mouth and showed a tendency to vomit due to using wormwood oil. Another recent case reported by Weisbord and co-workers (1997) from the U.S. was a 31-year-old man who had purchased from a website and consumed 10 ml of wormwood essential oil used in aromatherapy, which he assumed was the spirit absinthe. A few hours after ingesting this oil, the patient became listless, suffered seizures and finally developed rhabdomyolysis and the acute renal failure. It was supposed that thujone was to blame for the symptoms (Weisbord et al., 1997). The study of Dettling et al. (2004) on 25 healthy subjects who consumed alcoholic beverages of different amounts of thujone content shown that the simultaneous administration of alcohol and high concentrations of thujone (100 mg/l) may severely affect attention performance.

On the contrary, numerous investigations proved that thujone plays none or only a minor role in the clinical picture of absinthism (Lachenmeier et al., 2006). According to these authors, thujone should not be the main reason for the symptoms, but absinthism is predominantly caused by high alcoholic concentration (>50 vol%) that may lead and might have led both today and in the 18th century to major health and social problems. Strang and co-workers (1999) also suggested that acute alcohol intoxication was the main reason leading to the syndrome of absinthism. Similarly, in a pilot study of Kroner et al. (2005) blood concentrations of both thujone and ethanol were

examined after the consumption of absinthe. They detected high blood alcohol concentrations (>1 g/l) but - as expected - no thujone in the blood samples.

For bitter spirit drinks, such as absinthe, a maximum limit of 35 mg/kg thujone was introduced in the EU Council Directive 88/388/EC. According to Max (1990), in a typical recipe for absinthe, 2.5 kg of wormwood were used in preparing 100 litres of absinthe. This is equivalent to 4.4 mg wormwood oil or 2 mg to 4 mg thujone per drink, which would be far below the level at which acute toxicity effects were observed. Pharmaco-dynamical and toxicological studies revealed that even by consuming a relatively high amount of absinthe and reaching a blood alcohol concentration of 2.5 g/l it might mean only a thujone level of approximately 3.5 mg (0.005 mg/kg body weight). In a recent study, Lachenmeier and Uebelacker (2010) provided evidence that the current EU limits assure a sufficient protection for consumers. Based on the previously mentioned studies, it seems to be acceptable that thujone content in the spirit absinthe is hardly able to cause the characteristic syndromes of absinthism and these symptoms may easily be confused with those of chronic alcoholism.

Therefore, after a nearly century-long prohibition, absinthe has seen a resurgence after recent deregulation in many European countries. However, it should be added, that the huge majority of the mentioned studies have focused especially on thujone as the potentially toxic agent of the spirit. In addition, for the time being, no studies on the differences between the effects of α- and β- thujones with regard to the symptoms called absinthism are available. Besides, other compounds have seldom been investigated, thus the question on the source of potential health problems does not seem to be fully resolved even today.

2.6 Sources of chemical variability of wormwood

Studies have shown that *A. absinthium* displays a significant intraspecific chemical variation. Especially studies on the constituents of the essential oil have been published while manuscripts on other compounds are rather restricted. Besides the genetic potential and inherited properties of the accession, many other factors, such as growing conditions, stage of development, organic differentiation and harvesting time may affect the constituents of the essential oil to a great extent (Müller-Riebau et al., 1997).

2.6.1 Origin of raw material

The chemical constituents of essential oils from wormwood have been intensively studied in

different countries around the world and different chemotypes have been reported in literature (Table 1). Frequently, both "pure" chemotypes and "mixed" chemotypes (when the plants contain two or more components in higher proportions) have been defined. As an example, Chialva et al. (1983) determined "pure" chemotypes including *cis*-epoxyocimene, sabinyl acetate and β-thujone types and "mixed" ones such as β-thujone + *cis*-epoxyocimene, β-thujone + sabinyl acetate, *cis*- epoxyocimene + chrysanthenyl acetate + sabinyl acetate ones, etc,. Nin et al. (1995) defined the samples from the United States (69.7%), from Germany (49.8%) and from Italy (49.2%) as characteristic β-thujone chemotypes. According to Juteau et al. (2003) pure thujone chemotype plants (48.6% oil) were found in Croatia, as well.

Chemotypes with the presence of thujone together with other major compounds were observed in several studies. Besides the above mentioned accessions in the study of Chialva et al. (1983), Rezaeinodehi and Khangholi (2008) reported β-thujone + β-pinene chemotypes in Iran, Tucker et al. (1993) determined β-thujone (33.11%) + α-sabinyl acetate (32.75%) types in the United States. In Lithuania, "mixed" thujone chemotypes were indicated as the oil contained predominantly 18.0%–71.7% thujones (both α and β isomers) together with β-sabinyl acetate (in 5.6%-23%) (Judzentiene and Budiene, 2010).

Surprisingly, in several other cases thujone could not be demonstrated among the main components of the oil or was totally missing in the samples. Orav et al. (2006) described sabinene + myrcene type samples from items purchased at retail pharmacies in Estonia, while Carnat et al. (1992) reported a *cis*-chrysanthenol type population from Auvergne (France). In Cuba, bornyl acetate type plants could be identified with 23% bornyl acetate and only 0.29% thujone (Pino et al., 1997). Mixed types with the main components of caryophyllene oxide + p-cymene + 1,8- cineole were described by Basta et al. (2007) from wild populations in Greece, while *cis*- epoxyocimene and *cis*-epoxyocimene + chrysanthenyl acetate chemotypes were determined by Ariño et al. (1999a) in Spain. These two latter ones were free of thujone. Similarly, wormwood from Tajikistan has been reported as a new chemotype with a myrcene and *cis*-chrysanthenyl acetate and very low concentration of thujone (0.4%–7.3%) (Sharopov et al., 2012).

It can be established that the essential oil profile of wormwood is variable and thus raw material originating from different countries and different regions may show great differences in quality. Nevertheless, the majority of publications do not reveal the background of this variability and frequently not even the origin of the sample is correctly described. In this situation, it is almost impossible to determine the real significance of the genotype and discern it from any other biotic and abiotic factors.

Principally, thujone-free chemotypes offer an alternative for the production of food industrial items, beverages and spice products. However, there are no appropriate studies published on

whether the use of these plant materials has any connection with flavour and aroma of the extract. The absence or the presence of even a minimal amount of thujones might have an important role in food processing sector in avoiding potential toxicity and keeping concentration limits.

According to Ghazghazi and co-workers (2017), the total phenolic content of fresh wormwood leaves harvested from four regions in Tunisia varied significantly from one site of harvest to another with a wide range. The highest levels of phenolic compounds were recorded for Gafsa site (TPC=127.5±5.22 mg GAE/g DW) while the lowest contents were observed for Ghar Dimaou locality (TPC=94.23±4.81 mg GAE/g DW). Other research from Tunisia dealing with aerial parts of wormwood materials harvested at flowering stage from four different regions has been shown variability of total phenolic content. The total polyphenol contents reached the highest amount in the region of Kairouan (99.89 ± 3.30 mg GAE/g DW), followed by the region of Bou Salem, Boukornine, and Je´rissa where their levels were 83.70 ± 1.31, 72.05 ± 1.83, and 49.39 ± 2.20 mg GAE/g DM, respectively (Msaada et al., 2015).

2.6.2 Ontogenetic factors

The stage of development can be a determinant for the accumulation of secondary compounds during plant life (Németh, 2005). Concomitantly, the spectrum of volatile compounds and the quality of the essential oil may undergo major changes.

In a Lithuanian study the content of *trans*-sabinyl acetate dramatically increased from 0.8% at leaf stage to make up 52.6% of total oil during fruiting, while sabinene content dropped from 16.8% (first leaves) to 2.9% (fruiting stage) (Judzentiene and Budiene, 2010). Variation of essential oil composition of *Artemisia absintium* has been investigated in a Spanish native population, as well. According to the study of Llorens-Molina and Vacas (2015) bornyl acetate and neryl-isovalerate were detected in highest concentrations at the beginning of summer and showed a significant quantitative shift from this early vegetation period (June) untill the winter sampling (January). In parallel, other compounds, such as camphor, borneol, *cis*-geraniol and geranyl acetate exhibited significant increases from the vegetative phenological stage and reached the highest percentages at the period from August to October.

Fluctuations of the most important component, thujone, were investigated by Carnat and co-workers (Carnat et al., 1992) in several wormwood samples from a wild growing site in Auvergne (France). The proportions of both α- and β-thujones and *cis*-chrysanthenol changed dramatically during the harvesting period from June to November. Data in Table 2 show that the content of thujones decreased while *cis*-chrysanthenol increased significantly in the mentioned period. Unfortunately, the July samples do not follow this general tendency, therefore it is questionable

if any other factors like organic differentiation, presence of flowers or weather conditions could play a role in these changes.

Table 2. Variations of thujone and *cis*-chrysanthenol proportions (% in EO of *A. absinthium*) at the different harvesting times based on a 4 year trial (Carnat et al., 1992)

Constituents	**Harvesting period**					
	June	**July**	**August**	**September**	**October**	**November**
α-thujones	21.60	2.55	21.54	18.04	8.07	3.75
β-thujones	25.90	3.75	28.33	22.70	10.80	4.22
cis-chrysanthenol	15.70	54.90	19.67	24.46	51.03	69.01

An Iranian study conducted by Mohammadi et al. (2015) has reported that the highest concentration of total phenols found in essential oils from the aerial parts of *A. absinthium* was 48.49 mg GAE/g DW (preflowering stage), followed by 48.89 mg GAE/g DW (flowering stage) and the essential oil showed the lowest concentration of total phenols in the after-flowering stage (31.73 mg GAE/g DW).

2.6.3 Morphogenetic factors

The accumulation of secondary compounds can be largely dependent on the plant part used. Either they are aerial parts (leaves, stems, flowers) or roots. As mentioned above, ontogenetic and morphogenetic factors are frequently very difficult to divide from each other as well as from outside influencing factors. To make firm conclusions, well designed experiments are necessary and these are practically missing in the case of wormwood untill now. Comparison of underground and overground organs was carried out first by Blagojević et al. (2006) who indicated that the major constituents in the essential oil isolated from aerial parts of wormwood were β-thujone, *cis*-β-epoxyocimene, *trans*-sabinyl acetate, sabinene, and linalyl 3-methylbutanoate, while α-fenchene is the main component found in wormwood root oil. In the aerial parts the oil was dominated by monoterpenes which took 84,6% of the oil, while major compounds of the root oil were aliphatic esters in 64.5%. Interestingly, while the investigated genotype was obviously a thujone type with β-thujone contents up to 63.4%, this constituent could not be detected in root oil (Blagojević et al., 2006).

Similarly, a relatively high ratio of other compounds was detected in the roots of a wild growing

population from Spain. According to research results of Llorens-Molina and Vacas (2015), a predominance of oxygenated monoterpenes (81.4-89.1%) was observed in aerial parts while the root essential oil showed high ratios of hydrocarbon monoterpenes (43.8–55.1%) and monoterpenic esters (36.6–41.5%). The major constituents found in aerial parts were *cis*- epoxyocimene (49.3–71.5%), (Z)-chrysanthenyl acetate (7.6–18%) and linalool (0.7–10.4%), while β-myrcene and α-fenchene were the main components of the root oils. Mostly quantitative, but significant changes were observed also inside the shoots, between leaves and flowering tips. The study of Riahi et al. (2013) indicated that two dominant compounds, such as chamazulene and β-thujone were found both in leaves and flowers. However, camphor was found only in flower oil in a proportion of 16.2% and it could not be detected in the leaves. To the contrary, leaf oil was characterized by bornan-2-one (17.33%), whereas flower essential oils were lacking of this compound.

2.6.4 Environmental factors

Environmental and weather conditions significantly affect the content and composition of secondary metabolites. In the case of wormwood, results of investigations carried out under controlled conditions are not available. Gholami et al. (2005) showed that there were significant differences among the compositions of oils obtained from *A. absinthium* grown *in vitro*, in greenhouse and under field conditions. While thujone was the predominant compound of the greenhouse and field grown plants (41% and 60%, respectively), this constituent was absent in the regenerated *in vitro* plantlets. Meanwhile, the regenerated plantlet oil was found to be rich in citronelyl-isovalerate (22%) and terpinyl-isobutyrate (11%), but these compounds were not present in the in vivo samples. Interestingly, α-copaene (in 27.5%) was detected only in the oil of greenhouse-grown plants.

At the first glance, the effect of habitat and environmental factors may be anticipated from the data of Orav and co-workers (2006), as well, who published the occurrence of several different chemotypes of wormwood. Large variability was observed among samples from Estonia, Scotland, Moldova, Hungary and other European countries. Unfortunately, based on the published data, the real effect of ecological factors cannot be concluded, because these research samples were collected from retail pharmacies. Thus, the origin of the variability might be any factor (original growing habitat, genotype, sample homogeneity, phenophase, plant organs, etc).

2.6.5 Extraction methods and storage conditions

It has been demonstrated with several species that the essential oil content and composition may be largely influenced by the methods used to extract and analyze the volatiles (Figueiredo et al., 2008). In order to get the most reliable picture of the composition of the intact plant, considerable efforts have been invested in research optimizing the extraction and analysis (Jean et al., 1992; Reineccius, 1993).

According to a recent study, organic solvent extraction (OSE) provided the highest yield (23.81%) over supercritical fluid extraction (SFE) and hydrodistillation (HD) (Martín et al., 2011).

Different extraction methods may result in not only different yields but in changing composition of the extracts. Neither sabinene nor α-thujone were found in wormwood products extracted by direct steam distillation (DSD) method, while these compounds could be detected in extracts produced by microwave assisted process (MP) and distillation in water (DW). Other compounds like an unidentified sesquiterpene ($C_{15}H_{24}$) was present in DSD at 4.2%, however, it was absent in MP and DW (Chiasson et al., 2001).

In another study, *cis*-epoxyocimene, chrysanthenol and chrysanthenyl acetate were the major constituents found both in distillation in water (DW) essential oils and supercritical fluid extracts (SFE). However, quantitatively, the extracts were different. *Cis*-epoxyocimene was the major compound of DW oils and SFE extracts (22.2% and 39.5%, respectively), but it was a minor one in both OSE hexane oils and OSE ethanol oils (6% and 0.3%, respectively) (Martín et al., 2011).

According to the study of Arino et al. (1999b), the composition of wormwood volatile oil obtained by HSE (headspace extraction) showed an increase in the concentrations of most volatile compounds when compared to the SDE (simultaneous distillation-extraction) extract. Chamazulene – which is also a characteristic compound of only a few species, among others wormwood – only appeared in the oil produced by the SDE method (0.18% oil in a fresh sample). However, this constituent was absent in the remaining oils produced by HSE, MP (microwave extraction) and USE (ultrasonic extraction) (Arino et al., 1999b). It can be established that choosing the proper techniques for the isolation of wormwood oil plays an essential role in the appropriate utilization and obtaining the required quality.

In regard to the effects of primary processing methods on wormwood samples, there are hardly any relevant data. Arino et al. (1999b) investigated the essential oil of samples produced by different drying methods. Treatments included fresh wormwood materials, freezing (fresh wormwood branches were stored for 4 weeks at -18°C) and air-drying (fresh wormwood branches were allowed to dry at room temperature for about 1 week). The results indicated no significant

differences in the composition of the oil extracted from fresh plants compared to plants being processed and between samples produced by two different processing methods.

Storage conditions, such as light, temperature and moisture status play an important role in maintaining the quality of essential oil containing drugs and of the oils themselves. Although there are some data on the effect of storage conditions on wormwood products, the available information is still scarce. According to a study by Blagojević et al. (2006), after one year of storage of the dried herb at ambient temperature (25°C ± 2°C) in a sealed container, not only the concentration of the oil decreased from 0.29% to 0.08% but the number of detected components dropped, as well. The ratios of some compounds, such as *cis*- and *trans*-linalool oxides, 1,8-cineole and neryl 2-methylpropanoate proved to be higher, while the percentages of sabinene and β-myrcene decreased after one year of storage.

Lachenmeier et al. (2006) reported that the thermal exposure of absinthe at 50°C from 5 to 25 hours had no significant effect on the contents of either α- and β-thujone isomers.. In the same study, however, after using a treatment of ultraviolet (UV) light irradiation for 25 hours, there was a decrease of β-thujone content in commercial absinthe from 9.7 mg/l to 1.8 mg/l, while the α- thujone content of both products and the β-thujone concentration of the self-produced absinthe did not change significantly. The authors have explained the difference by changing amounts of natural antioxidants extracted from the plant material. In addition, Lee et al. (2013) have indicated that the total phenolic content of the different type of extracts from leaves of *A. absinthium* reached the highest (134.47 mg/g DW) amount of phenolic compounds (water extract) followed by methanol (131.18 mg/g DW), and ethyl acetate (51.49 mg/g DW). Different dried conditions of raw materials have influenced the total polyphenol content as well. Monica et al. (2008) showed that total polyphenol content of wormwood aerial parts collected in Romania using Folin Ciocalteau reagent was 98.20 mg/g DW (normal dried condition) and 115 mg/g DW (dried at 105°C for 4 hours)

2.7 Aspect of wormwood cultivation

2.7.1 Germination

Studying on the storage duration and condition of seeds plays an important role in the long-term conservation of plant genetic resources. Since very early days, Bradbeer (1988) has reported that the storage temperature and condition strongly affect the seed germination capacity. Seeds storage in inappropriate temperature in room condition may reduce rate of seed germination, seed deterioration and loss of viability (Hezewijk et al., 1993). In case of wormwood, Aghilian et al.

(2014) have conducted germination tests of forty species including *A. absinthium* at the beginning of storage, 9 months and 12 months after storage in both cooled conditions (4℃) and at room temperature. According to this study, the germination rate of wormwood seeds was very low (2% - 3%). In addition, Heeger (1956) measured a 69% - 100% reduction of the germination capacity after five years of storage; however, the storage conditions were not mentioned in this study.

2.7.2 Cultivation

Detailed information about wormwood cultivation is missing. According to the brief description for a small scale garden mentioned by Simon et al. (1984) the plant can be propagated from cuttings, by root division in autumn or by seeds sown in autumn. It is stated that the species grows well in even relatively poor, dry soils. Plantations of wormwood may last from seven to ten years, peaking in production during the second or third year and the plant can be harvested twice per year, during the late spring in vegetative stage and during full bloom (Simon et al., 1984). Another study, by Galambosi (1979), was conducted in Hungary regarding the examination of propagation, spacing and herbicides influencing wormwood cultivation. He indicated that the best time for transplantation of seedlings into open field was from the end of July untill the end of August. In addition, the spacing of plantation had no influence on the height of plants and the width of stem the next year; however, it influenced the number of shoots with the highest value at spacing of 50x30 cm and the production of wormwood reaching maximum yields at the plant spacing of 70x30 cm. According to Heeger (1956), wormwood seedlings should be raised and planted out in May-June with a distance of 50x30 cm and the optimal harvesting time is at the beginning of flowering which based on the attention of the bitter substance (Absinthin) content with no hints on the EO or phenolic contents. As an alternative solution suggested by this author, a planting out in August -September is also advisable after a longer seedling growing period at controlled place; however, no vegetative propagation methods were mentioned here (Heeger, 1956).

2.7.3 Genetic resources and varieties

As mentioned above, *A. absinthium* is a widespread and diverse species. Nevertheless, the basics of the variability have not been adequately revealed until now. Inheritance and/or population genetic as well as breeding studies are not known. Molecular genetic markers have only exceptionally been used for studying the relationship of intraspecific accessions of Artemisia species. RAPD investigations in *Artemisia annua* L. have already been performed in India 17

years before (Sangwan et al., 1999). Nazar and Mahmood (2011) established the genetic diversity measured through the analysis of RAPD data of 15 sample from some collected *Artemisia* species including *A.absinthium* in Pakistan. UPGMA analysis produced two main groups, all of *A. absinthium* individuals and one individual of *A. roxburghiana* were in the same group which showed a resemblance to individuals of *A. absinthium* at 0.73 similarity coefficient. Application of ISSR analysis has been used for *A. herba-alba* in Tunisia to detect genetic polymorphism within this species.

2.7.4 Phytotoxic activities

The phytotoxic properties of plants and their compounds against other plants or seeds are being increasingly investigated. Many secondary plant products as allelopathic compounds are released from donor plants into the environment in several ways including volatilization, leaching, root exudation, and decomposition of plant residues (Roger et al., 2006). This liberation of substances could kill other plants or inhibit their development such as seed germination, root growth, seedling growth etc. In case of *A. absinthium*, an early study conducted by Funke (1943) reported that eighteen species sown beside the hedge of *A. absinthium* within a distance of 100 cm were injured or even killed by the chemical excretions of this plant. The percentage of germination and development of seedlings were also reduced remarkably when sowing seeds into soil mixed with fresh wormwood leaves (Funke, 1943). However, no hints on the responsible compounds could be made that time. Even today there is a lack of sophisticated results and a need for further studies on the inter- and intra-population allelopathic effects in wormwood plantations as this kind of information might contribute to a sustainable cultivation.

CHAPTER 3: MATERIALS AND RESEARCH METHODS

3.1. Detecting intraspecific variability of *A. absinthium*

3.1.1 Plant materials and cultivation

The basic plant material for the studies included gene bank accessions, market items and seeds collected from wild habitats are shown in the Table 3.

Table 3. List of the investigated *A. absinthium* accessions

Accession			Origin of population
No.	Sign	Denomination	
1	Bel	Belgian wild	Accession of Gatersleben genebank (collected in Belgium)
2	Eng	English market	Market item from England ('Premier seeds direct' company)
3	Ger0	German market	Market item from Germany (Gabriele Köhn, Kranichblick 7, 18442 Duvendiek, Deutschland)
4	Ger1	Leipzig (German wild 1)	Accession of Gatersleben genebank (collected in Leipzig - Germany)
5	Ger2	German wild 2	Accession of Gatersleben genebank (collected in Germany)
6	Hum	Hungarian market	Market item from Hungary ('Herbaria' company)
7	HuW1	Hungarian wild 1	Wild collected population: Csór, Hungary
8	HuW2	Hungarian wild 2	Wild collected population: Pákozd, Hungary
9	HuW3	Hungarian wild 3	Wild collected population: Soroksár, Hungary
10	HuW4	Hungarian wild 4	Wild collected population: Öskü, Hungary
11	Nor	Norwegian wild	Accession of Gatersleben genebank (collected in Norway)
12	Spa	Spanish wild	Wild collected population: Teruel, Spain

Propagation was carried out by seed sowing in March 2016 and growing the seedlings in a greenhouse for 2 months. The seedlings with 5-6 leaves were planted into open field plots in June, installing three replicates at the Experimental Station of Szent István University in Budapest. The soil is sandy-loam, pH 7.8, humus content is low. The plants were grown without additional fertilization; mechanical weed control and irrigation were applied in dry periods.
Measurements and samplings were carried out according to the goals of the experiments as follows.

3.1.2 Studying the relationship of wormwood genotypes by morphological traits

3.1.2.1 Qualitative morphological characteristics

Morphological assessments were carried out in the year of plantation, in a vegetative stage, at the beginning of August, 2016. 18 individuals of each accession were evaluated. The following morphological characters were observed for each plant individually and for each character a numerical code (0, 1, 2, 3) was defined. The following characteristics were chosen (modified from the study of Nazar and Mahmood, 2011).

- Leaf color ad axial: silvery (1); green (2)
- Leaf color ab axial: light green (1); grayish (2)
- Stem color: purplish (1); silvery (2).
- Branch form: many branches (1); few branches (2)
- Growth character: erect (1); prostrate (2)
- Leaf form: deltoid (1); flabellate (2); ovate (3)

3.1.2.2 Quantitative morphological characteristics

At the same time as the above, plant height (from ground level to tallest shoot) and width (widest diameter) were measured on each plant (10 replicates/accession).
In addition to characterise the leaves more exactly, five leaves were collected from the central part of the shoots of each individual. In total, 200 leaves were studied in the leaf rosette period. The leaves were pressed and a herbarium was prepared following the techniques of DeWolf (1968). The blade thickness (as the mean of five target points midway between the margins and midrib at the widest part of the leaf) was measured using a Digimatic Thickness Gauge. The lengths of the total leaf and total blade were measured using a vernier caliper on the herbarium specimen and the ratio between them was calculated (Figure 2).

Fig 2. Devices for leaf measurements and herbarium leaves of *A. absinthium*

3.1.2.3 Biomass production

After the morphological measurements, all individuals were cut at about 5 cm height above the soil surface. The mass of the fresh harvested material of each plant was measured separately (10 replicates) by analytical scale. The plant material was dried at room temperature (20-25°C) in shade for two weeks and measured again to determine the dry mass.

3.1.3 Studying the relationship of wormwood genotypes by molecular markers

Young leaves from 10-15 plants of nine accessions (Spa, Nor, Bel, Eng, Ger1, Hum, HuW1, HuW2, HuW3) were collected from the plant with the appearance of two true leaves, then grounded in liquid nitrogen to a fine powder. Three other accessions (HuW4, Ger0, Ger2) did not produced enough materials for this investigation. Genomic DNA was extracted from fresh leaves by a DNeasy Plant Mini Kit (Qiagen, BioScience, Hungary). The concentration and quality of extracted DNA from each accession were assessed using NanoDrop (BioScience, Hungary) and visually checked on 1.5% agarose gel.

Out of the primarily screened 13 RAPD and 15 ISSR primers only 11 RAPD and all the 15 ISSR primers produced clear, reproducible and scorable bands, thus, the investigations have been carried out by these ones. Their sequences are presented in Table 10.

The PCR reaction was performed in a 12 µl volume containing the reaction buffer (approximately 15-25 ng genomic DNA, 1 µM primer (for RAPD) or 2µM primer (for ISSR), 6 µl of 2x GoTaq Hot Start Green Master Mix (Promega), 3 mM $MgCl_2$ and nuclease free water). PCR amplification was conducted in a SuperCycler SC-200 thermocycler (Kyratec). The thermal cycle used for RAPD primers was 2min at 95°C followed by 35 cycles at 94°C for 30s; 1 min at specific annealing temperature (the optimal annealing temperature was determined for each individual

primer); 1 min at 72°C and final extension at 72°C for 5 min. The program used for ISSR primers was 3 min at 95°C followed by 35 cycles at 94°C for 30s; 45s at specific annealing temperature; 45s at 72°C and final extension at 72°C for 5 min.
Amplified DNA fragments were separated in a 1.5% agarose gel (SeaKem LE Agarose, Lonza) at 100 V for 90-120 min in 1 x Tris-Acetate EDTA (TAE) buffer (pH 8.0) and stained by 1% (w/v) ethidium bromide. The PCR products were visualized under UV light by AlphaImager EP Imaging System (Cell Bioscience). The 100 bp ladder (Promega) was used as a molecular weight size marker. This work was conducted at the Department of MAPs, SZIU.

3.1.4 Studying the relationship of wormwood genotypes by phytochemical characteristics

The laboratory works (EO distilaation, GC - MS analysis, the measurement of TPC and AC) were carried out at the Department of MAPs, SZIU.

3.1.4.1 Essential oil extraction

From the dried plant material all leaves were separated from stem parts and only the leaves were used for essential oil distillation. Plant samples (g) were distilled in a Clevenger-type apparatus according to the method recommended by the VII. Hungarian Pharmacopoeia using distilled water to maintain a near neutral pH. Essential oils were produced from 50 g dried leaves of each accession (Table 3) by hydro-distillation (500 ml water) for 2.5 hours. The oils of 120 samples were collected, and traces of water removed with anhydrous sodium sulphate. Then, the extracts were separated with a syringe filter, and stored in airtight vials in a refrigerator at 4°C until the analysis took place.

3.1.4.2 Gas chromatographic mass spectrometric analysis

The GC–MS analyses were carried out on each EO sample using an Agilent Technologies 6890N instrument equipped with HP–5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm) and an Agilent Technologies MS 5975 inert mass selective detector. The temperature program was the following: initial temperature 60°C, then increased by a rate of 3°C/min up to 240°C; the final temperature was maintained for 5 min. The carrier gas was helium (1 ml/min), injector and detector temperatures were 250°C. Split ratio: 30:1. 10 ul of EO has been diluted by n-hexane to 1 ml and from this the injected quantity was 0.2 ul. The percentage composition of the essential

oil was computed from the GC peak areas. Ionization energy was 70 eV. The MS were recorded in full scan mode that revealed the total ion current (TIC) chromatograms (mass range m/z 50–550 uma). Composition was identified by comparison of their linear retention indices (LRI) - that were calculated using the generalized equation of Van Den Dool and Dec. Kratz (1963) with literature data and by matching their recorded mass spectra with those in mass spectral library references (NIST MS Search 2.0 library, Wiley 275) and mass spectra library (Adams, 2017).
In some cases the GC–MS analysis was controlled under other conditions (at the Polytechnic University of Valencia, Spain by Prof. J.A. Llorens-Molina). In these cases we used a Clarus 500 GC–MS (Perkin Elmer Inc.) equipped with a ZB-5 capillary column (30 m × 0.25 mm i.d. × 0.25 μm). The GC oven temperature program was set from 50°C to 250°C at a rate of 3°C min^{-1} holding the final temperature for 5 min. Helium was used as carrier gas (1.2 mL min^{-1}), and the injector and detector temperatures were set at 250°C. Injected quantity: 0.2 ul. The ionization source temperature was 200°C, and a 70 eV electron impact mode was used. MS spectra were obtained using the total ion scan (TIC) mode (mass range m/z 45–500 uma). The total ion chromatograms and mass spectra were processed using Turbomass 5.4 (Perkin Elmer Inc.) software. The essential oil components were identified by comparison of their mass spectra with those of the component libraries mentioned above.

3.1.4.3 Total phenolic content (TPC)

For determination of the TPC, 1 g of the dried, powdered plant material was extracted by boiling 100 ml of distilled water, which was allowed to stand for 24 hours. Then the extracts were filtered and stored in a freezer until the measurements were taken. This was carried out by the modified method of Singleton and Rossi (1965). The absorbance was measured at 760 nm in a Thermo Evolution 201 spectrophotometer after 5 min incubation period in hot water (50°C). Gallic acid (0.3 M) was the chemical standard for calibration. The TPC of the sample was expressed as mg of gallic acid equivalents (GAE) per g of dry weight of extract. A blank was prepared containing distilled water instead of extract. Triplicate measurements were carried out for each of the three biological replicates.

3.1.4.4 Antioxidant capacity (AC)

AC of the extracts was measured using the FRAP (ferric reducing antioxidant power) method according to the modified method of Benzie and Strain (1996). The absorbance was measured at 596 nm in a Thermo Evolution 201 spectrophotometer. Ascorbic acid was the chemical standard

for calibration. The antioxidant capacity (cc mg AAE/g DW) of the sample was expressed as mg of ascorbic acid equivalents (AAE) per g of dry weight of extract. A blank was prepared containing distilled water instead of extract. Triplicate measurements were carried out for each of the three field replicates.

3.2 Revealing factors influencing the chemosyndroms of wormwood

3.2.1 Ontogenetic and morphogenetic (organic) factors

3.2.1.1 Plant material and growth conditions

The plant material used in this study consisted of both thujone (T) chemotype (chosen from 6 individuals of "Bel" and 4 individuals of "HuW4" accessions) and *trans*-sabinyl acetate (SA) chemotype (chosen from 6 individuals of "Ger2" and 4 individuals of "Nor" accessions). For revealing the role of plant development in the intraspecific variability of wormwood, the second year old plants were used and sampled in 2017 (Table 4). Each time bulk samples from ten chosen individual plants were harvested.

Table 4. Harvesting times and developmental phases of *A. absinthium*

Harvesting time	**BBCH Code (Hess et al., 1997)**	**Description**	**Denomination of phase**
05.05.2017	40	Harvestable vegetative plant parts or vegetative propagated organs begin to develop	Vegetative
02.07.2017	55	First individual flowers visible (still closed)	Floral budding
15.08.2017	65	Full flowering: 50% of flowers open, first petals may have fallen	Flowering
10.09.2017	67	Flowering finishing: majority of petals fallen or dry	After flowering

3.2.1.2 Essential oil extraction

Leaves and flowers were divided from stem parts and they were distilled separately in three replicates. 50 g dried materials from each sample was hydro-distilled applying the same method as described in chapter 3.1.4.1.

3.2.1.3 Gas chromatography-Mass spectrometry analysis

Using the same method mentioned at chapter 3.1.4.2

3.2.2 Environmental factors

3.2.2.1 Treatments

Two phytotron chambers (Fitotron SGC120 growth chambers, Weiss Gallenkamp Ltd., Loughborough, Leicestershire, United Kingdom) were installed with two growing programs simulating "warm" weather (higher temperature and light intensity) and "cold" weather (lower temperature and light intensity) circumstances (Table 5). The experiment was conducted from September 2016 to February 2017. The treatments started after an acclimatization period of 14 weeks.

3.2.2.2 Plant material and growth conditions

Plant material for the study included seeds collected from wild habitats in Teruel, Spain (accession "Spar") and Csór, Hungary (accession "HuW1"). Propagation consisted of sowing the seeds in August 2016, followed by 2 months of seedling growth in the greenhouse. The seedlings bearing 5-6 leaves were planted into pots (16 cm in diameter and 15.5 cm in height) filled with a standard soil mixture (Florasca B). In order to assure genetic identity, twenty individuals from each of the two accessions were divided into two equal parts and placed in the two climatic chambers (10 individuals of each of the two accessions in each chamber) (Table 5).

Table 5. Main parameters of the controlled environments in two climatic chambers

Date	Warm			Cold		
	Temperature (°C) 14h day/10h night	Relative air humidity (%)	Light intensity during day (klx)	Temperature (°C) 14h day/ 10h night	Relative air humidity (%)	Light intensity during day (klx)
04.11.2016	18/10	55		13/8	55	
14.11.2016	18/10	55		13/7	55	
28.11.2016	19/11	55		13/8	55	
05.12.2016	19/12	55		14/8	55	
12.12.2016	19/12	55		15/9	55	
20.12.2016	20/14	55	16	15/10	55	8
04.01.2017	21/15	55		16/10	55	
09.01.2017	22/15	55		16/11	55	
16.01.2017	23/15	60		17/11	60	
23.01.2017	24/16	60		17/12	60	
30.01.2017	25/17	60		18/12	60	
06.02.2017	27/19	65		18/10	65	

The plants were irrigated three times per week with an equal amount of water for each pot, maintaining 65%-70% RWC in the soil. Measurements and sampling were carried out at the vegetative stage after 14 weeks of cultivation.

3.2.2.3 Essential oil extraction

Leaves were separated from stem parts and only the former were used for EO distillation. The individual samples of the same genotypes from each of the climatic chambers were mixed and divided into three replicates to get representative plant material for both treatments and both accessions. 50 g of dried leaves of each sample were hydro-distilled in a Clevenger-type apparatus applying the same method as written in Chapter 3.1.4.1

3.2.2.4 Gas chromatography-Mass spectrometry analysis

Using the same method mentioned at chapter 3.1.4.2

3.2.2.5 Total phenolic content (TPC)

Using the same method mentioned at chapter 3.1.4.3

3.2.2.6 Antioxidant capacity (AC)

Using the same method mentioned at chapter 3.1.4.4

3.3. Optimization of wormwood cultivation

3.3.1. Study on generative propagation: effect of storage on seed germination

Seeds were collected from 3-year-old mother plants from two accessions ("Hum" and "Spa") in October, 2015. For the 2 chosen accessions, after drying, seeds were tested for their initial germination capacity. The initial test was conducted after collecting the seeds on 18th October 2015. The experimental seed lots were placed in two different experimental conditions. The first was at room temperature (20-24°C) and the second in a refrigerator at standard +4°C. The seeds have been periodically tested for their germination capacity at 3 month intervals through altogether 29 months.

Germination tests were carried out according to the International Rules for Seed Testing formulated by the International Seed Testing Association (ISTA, 2007). For each testing, three replicates of 50 seeds from both storage conditions were used. Using top of paper method, Petri dishes were stored in refrigerator, with alternating temperatures of 20°C applied for eight hours and 30°C for 16 hours per day. The experiment was performed in a randomized design. Seed germination was recorded and counted until constant readings were obtained. Germination capacity was defined as: germinated seeds/all seeds x 100. Additionally, the mean germination time (MGT) for each treatment was calculated by the formula given by Ellis and Roberts (1981) as follows:

$MGT = n_1 \times d_1 + n_2 \times d_2 + n_3 \times d_3 +$--------/Total number of days. Where n= number of germinated seed; d = number of days. Additionally, root length was measured by using a vernier caliper.

3.3.2. Study on vegetative propagation

A Hungarian cultivated material has been maintained as perennial plantation at the genebank of the department. 90 cuttings were taken randomly from ten mother plants at vegetative period in May 2016. 10-15 cm long, half woody cuttings were prepared. Half of the cuttings was treated by 0.5% IBA while the other half was left as control (no IBA). Cuttings were placed in plastic trays 40x60 cm in size and kept in a greenhouse for 2 months. The number of living cuttings and number of their roots were counted and recorded; additionally, the length of roots of each cutting was also measured by using a vernier caliper.

Layering of 10-10 individuals in both perennial populations defined in chapter 3.3.1. was conducted in March 2016 (before the beginning of the vegetation). The arial parts of the mother plant were cut at about five cm height above the soil surface. After cutting the arial parts, soil was used to cover the plants. After two months, the number of newly rooted shoots on each mother plant was counted. The number of roots and length of shoots for each new plantlet were recorded.

3.3.3. Study on allelopathic activity of wormwood

3.3.3.1 Treatments with different types of plant materials

The shoots of the above mentioned Hungarian wormwood accession (3.3.2.) were cut at about 5 cm height above the soil surface. Leaves were separated from stem parts and only the former ones were used for investigation. 300 g of fresh, finely cut leaves were used for each of the following treatments and mixed with 3kg of standard soil mixture (Florasca B) and put into 40cm x 60cm trays. Treatments: a./ fresh leaves (cut into small pieces), b./ dried leaves (starting from 300 g of fresh material and dried at 45°C for 24 hours until a remaining moisture content of 13%, then the dried materials were also powdered) c./ water extract (300g fresh leaves put into 1.5 liters of distilled water for 24 hours under room conditions). The control tray contained only soil.

For test species we used seeds of mustard (*Sinapis alba* L.) and lettuce (*Lactuca sativa* L.) which were sown directly into the trays. 100 seeds from each species were used per tray. Three replicates of each treatment were installed.

The number of germinated seeds and the mean germination time were defined as mentioned in chapter 3.3.1. Ten seedlings were chosen randomly and seedling growth was measured. The number of roots were counted and recorded, additionally, the length of roots of each seedling was also measured by using a vernier caliper.

3.3.3.2 Treatments with wormwood leaf powder

The shoots of two wormwood accessions mentioned in chapter 3.3.1. were cut at about five cm height above the soil surface. Leaves were separated from stem parts and only the former ones were used for the experiment. The leaves were dried in room conditions for 2 weeks and after drying the leaves were ground into powder. Two different dosages (50g and 100g dried leaves powder) were mixed with 3kg standard soil mixture (Florasca B) and filled into 40x60 cm trays. The control tray contained only soil.

Seeds of basil (*Ocimum basilicum* L.), lettuce and mustard were chosen as the tested species. These species germinate easily and uniformly and grow relatively rapidly in order to obtain data without undue delays. In addition, mustard is a less sensitive species in case of using inhibited test, following by basil and lettuce as the most sensitive ones. Seeds were sown directly into the trays, 100 seeds from each species were used per tray. Three replicates of each treatment were installed.

The number of germinated seeds and the mean germination time were defined as mentioned in chapter 3.3.1. Ten seedlings were chosen randomly and seedling growth was measured. The number of roots were counted and recorded; additionally, the length of roots of each seedling was also measured by using a vernier caliper.

3.3.3.3 Treatments with aqueous extracts

The aqueous extracts of leaves of two *A. absinthium* chemotypes - a high thujone chemotype (Belgian accession, Table 3.) and sabinene + ß-myrcene chemotype (Hungarian accession, Table 3) - were used for the experiment. The following concentrations were produced and applied: 0 (control, only distilled water); 0.1; 0.2; 0.3, 0.4 mg of dry leaf powder per ml distilled water. The experimental species were lettuce and basil, the seeds of which were laid onto filter paper in 9 cm diameter Petri dishes. 50 seeds per dish were treated with 5 ml of each extract: 0; 0.1; 0.2; 0.3; 0.4 mg/ml. Three replications were applied. This in vitro experiment was carried out in 2016 in climatic chamber with a program of 30°C/20°C; 16 klx light intensity with 14h day/10h night rhythm.

Germination capacity and the mean germination time were measured by the same method in chapter 3.3.1. The length of roots of each germinated seed was measured by using a vernier caliper.

3.4. Statistical analysis

SPSS version 23 was used to analyze the data. MANOVA test has been conducted for evaluating the measured morphological characters of the leaves (blade-thickness, leaf-length, petiole-length

and ratio of blade/petiole), plant height, width, biomass, dry mass and dry leaf mass (experiment 3.1.2 and 3.2.2). In the case of a significant MANOVA result (Wilks lambda $p<0.05$), a test of between-subjects analysis was performed with Bonferroni's correction. The two-way ANOVA test was conducted to compare the EO content of twelve accessions, EO content of leaves and flowers from the two investigated chemotypes harvested at different phenological stages (experiment 3.2.1) and EO content of two accessions growing in climatic chambers (experiment 3.2.2) as well. The correlation between two variables (experiment 3.1.2.2; 3.1.4.3 and 3.1.4.4) was analysed by bivariate correlations of the Pearson product-moment correlation coefficient. Normality of the residuals was proven by the absolute values of skewness and kurtosis of the residuals. Homogeneity of variances was checked by Levene's test ($p>0.05$).

One-way analysis of variance (ANOVA) and Tukey's HSD post hoc test were carried out to analyze the significant differences of EO content of the twelve accessions. Homogeneity of variances was checked by Levene's test and the normality of variances was checked by Kolmogorov-Smirnov method. In case of violated homogeneity of variances, Games-Howell was used in post hoc test to determine the significant difference between accessions.

Principal component analysis (PCA) (experiment 3.1.4): according to the communalities (>0.15), out of 69, 26 compounds were involved in principal component analysis (PCA). Based on the screen plot, five principal components (PC) were extracted. PC loadings were then rotated by varimax method. The Kaiser-Meyer-Olkin's statistic (KMO=0.51) and Bartlett's test ($Chi2(325)=1200.78$; $p <0.001$) indicated that the data set was adequate and suitable for PCA. Statistical analysis were carried out by using IBM SPSS 23.0.

In experiment 3.1.3, amplified DNA fragments with reproducible bands of each locus were scored as binary present (1) or absent (0) and data matrices of RADP and ISSR loci were assembled for further analysis. The results were summarized in a Microsoft Excel table. Popgene version 1.32 (Yeh et al., 1997) was used to estimate the number of polymorphic bands, percentage of polymorphic bands, Nei's (1972) gene diversity (h) and Shannon's Information Index (I) (Lewontin, 1972) for dominant marker data or all loci and also for each population separately. Genetic relationship among genotypes was studied by UPGMA (Un-weighted Pair Group Method with Arithmetic averages) cluster analysis and principal component (PCA) analysis using PAST software (Hammer et al., 2001).

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Intraspecific variability of wormwood

4.1.1 Study the relationship of wormwood genotypes by morphological traits

4.1.1.1 Qualitative morphological characteristics

Based on Crosstab analysis, all the morphological characters were analyzed and were used for cluster analysis. According to Chi-Square Tests, the value of absoluted adjusted residual higher than 2 was found as significant difference. Significant differences were detected among the accessions in 5 of the 6 investigated morphological characters: $Chi2(11)_{leafcoloerab} = 156.04$, $P<0.001$; $Chi2(11)_{stemcolor} = 92.71$, $P<0.001$; $Chi2(11)_{branchform} = 122.03$, $P<0.001$; $Chi2(11)_{growth} = 107.02$, $P<0.001$; $Chi2(11)_{leafform} = 98.88$, $P <0.001$ (Table 6).

Table 6. Qualitative morphological traits of the studied *A. absinthium* accessions

Acc.	Leaf color ad			Leaf color ab			Stem color			Branch form			Growth			Leaf form			
	nr. of plants		(*)	nr. of plants		(*)	nr. of plants		(*)	nr. of plants		(*)	nr. of plants		(*)	nr. of plants			(*)
Categ	1	2		1	2		1	2		1	2		1	2		1	2	3	
Bel	2	16	c	0	18	b	18	0	c	0	18	b	18	0	a	6	0	12	c
Eng	18	0	a	18	0	a	16	2	c	2	16	a	11	7	a	17	0	1	a
Ger0	18	0	a	18	0	a	5	13	a	9	9	a	6	12	a	17	0	1	a
Ger1	6	12	c	6	12	b	18	0	c	0	18	b	15	3	c	17	0	1	a
Ger2	18	0	a	18	0	a	6	12	b	12	6	c	0	18	a	0	6	12	d
Hum	18	0	a	18	0	a	6	12	a	0	18	b	18	0	c	16	0	2	a
HuW1	17	0	a	17	0	a	12	5	a	3	14	a	11	6	a	16	0	1	a
HuW2	12	6	b	12	6	a	6	12	b	0	18	b	12	6	a	12	0	6	a
HuW3	18	0	a	18	0	a	14	4	a	0	18	b	7	11	a	17	0	1	a
HuW4	17	0	a	17	0	a	4	13	c	8	9	a	5	12	a	17	0	0	b
Nor	17	0	a	17	0	a	0	17	c	14	3	c	0	17	b	0	17	0	d
Spa	18	0	a	18	0	a	15	3	b	18	0	c	0	18	b	0	0	18	c

For categories of the examined traits see Material and Methods, chapter 3.1.2.1

(*) - *Different letters at column represent significant differences between accessions according Chi-Square Tests*

It was established that except for 3 populations, leaf color was uniform in the majority of the accessions revealing a silvery character (Table 6). Leaf form was also characteristic for the accessions, showing lower intra-population variability. However, concerning the stem color the accessions showed a much more heterogeneous picture with only a single one being uniform from this point of view. Similarly, we found a large diversity in branching form and growth character on each plot.

Based on these differences, the individuals of the studied accessions could be clustered into four groups (Table 7).

Table 7. Clustering of the *A. absinthum* individuals based on the evaluated qualitative morphological traits

Accessions	**Number of individuals in the relevant cluster groups**				**Total**
	1	2	3	4	
Bel	0	0	0	18	18
Eng	0	17	1	0	18
Ger0	0	17	1	0	18
Ger1	0	6	0	12	18
Ger2	6	0	12	0	18
Hum	0	16	2	0	18
HuW1	0	16	1	0	17
HuW2	0	6	6	6	18
HuW3	0	17	1	0	18
HuW4	0	17	0	0	17
Nor	17	0	0	0	17
Spa	15	0	3	0	18

Group 1 consists of the most individuals of accessions “Nor” and “Spa”. This group showed the following main characteristics: silvery leaf color ad axial, light green in leaf color ab axial, purplish color stem, many branches, low height, ovate leaf form (“Spa” and “Ger2”), and flabellate leaf form in the case of accession “Nor”. Most Hungarian accessions, “Eng” and “Ger0” belonged to group 2: silvery leaf color ad axial, light green in leaf color ab axial, few branches, high height development, deltoid leaf form. The third group had primarily the individuals of accession “Ger2” characterised by the following traits: silvery leaf color ad axial,

light green in leaf color ab axial, silvery color stem, many branches, low height development and ovate leaf form. The fourth group had the most individuals of accessions “Bel” and “Ger1” with green leaf color ad axial, grayish in leaf color ab axial, purplish color stem, many branches, high height, ovate leaf form (“Bel”) and deltoid leaf form (“Ger1”).

Nazar and Mahmood (2011) have described *A. absinthium* with silvery-silky hairs on both upper and lower surface of leaves. Another study by Konowalik and Kreitschitz (2012) reported that no significant differences were observed between the investigated wormwood varieties in the micromorphology or anatomy of the leaves. Similarly, our results on the color of wormwood leaves from a taxon in Galow, Poland show dark green color with slight silvery covering and others from other regions had silvery leaves (Konowalik and Kreitschitz, 2012). However, no comprehensive study similar to our one has been fulfilled until now on the distribution of the qualitative morphological traits and their variability.

It could also be established that a considerable intraspecific variability of *A. absinthium* was detected based on qualitative morphological features. Differences in leaf shape of the same individual contribute to the heterogeneity of populations and present additional difficulties in evaluation. The most homogenous accessions were “Nor” and “Spa” which showed uniformity in 5 of the 6 studied characteristics. On the other hand, “HuW2” can be justified as the most heterogeneous as the individuals were uniform here only in a single trait. “Ger 1”, “Ger2” and “HuW1” are also relatively heterogeneous (uniform only from 2 traits).

Based on the qualitative morphological characteristics and their homogeneity it would be difficult to find any connection with the geographical origin of the accessions. Similarly, there does not seem to exist any connection between the manifestation of the evaluated traits and the fact whether the accessions were collected from market or wild growing origin.

4.1.1.2 Quantitative morphological characteristics

Among the accessions, genotype “Eng” exhibited the highest vegetative growth (47.5 cm) and genotype “Nor” had the shortest shoots (18.7 cm). This latter accession was determined as the most homogenous one (CV=11%) from this point of view, too. On the other hand, the largest variability was detected in population “Eng” (CV=31%). Significant differences were ascertained among the twelve accessions. Similarly to this, plant width brought about considerable differences among accessions (Table 8). Two accessions, “Eng” and “HuW2” showed the largest values for plant width (59.4-64.9 cm) while “Nor” developed the narrowest bushes (29.9 cm). We determined a strong correlation between the height and the width of the bushes (r=0.87).

Table 8. Growth characteristics of the studied wormwood accessions

Accessions	Height (cm)		Width (cm)	
	Mean	**SD**	**Mean**	**SD**
Bel	41.1^{bcd}	9.11	42.9^{abc}	10.16
Eng	47.5^{d}	14.80	59.4^{de}	11.78
Ger0	41.9^{bcd}	7.61	50.6^{cd}	6.38
Ger1	40.8^{bcd}	13.46	50.8^{cd}	9.85
Ger2	28.4^{ab}	2.80	42.9^{abc}	4.91
Hum	40.20^{bcd}	9.37	50.3^{bcd}	4.55
HuW1	37.90^{bcd}	14.99	54.0^{cde}	11.08
HuW2	46.2^{cd}	8.84	64.9^{e}	16.74
HuW3	42.7^{bcd}	12.60	56.3^{cde}	8.25
HuW4	32.1^{abc}	9.26	37.0^{ab}	4.55
Nor	18.7^{a}	2.06	29.9^{a}	4.25
Spa	32.7^{abc}	7.53	47.4^{bcd}	5.72

Different letters in the columns represent significant differences between accessions according to Games-Howell test at p=0,05

The results of the quantitative characteristics of the leaves are summarized in Table 9. According to MANOVA Test of between-subjects effects, significant differences were detected in the case of each of the four leaf morphological characters: F*thickness*$_{(11;488)}$=12.00 ($p<0.001$); F*lenghtleaf*$_{(11;488)}$=25.66 ($p<0.001$); F*lengthpetiole*$_{(11;488)}$=18.10 ($p<0.001$); F*ratiobladepetiole*$_{(11;488)}$=6.54 ($p<0.001$). The thickness of blades of the investigated accessions ranged from 0.30 mm to 0.48 mm. The Games-Howell test distinguished 4 subsets at p = 0.05 significance level. Accessions "Ger2" and "Huw1" have the smallest thickness of leaf while "Bel" was characterised by the thickest leaves. Although there is not a study on wormwood that is similar to our findings, an experiment by Witkowski and Lamont (1991) on *Arbutus menziesii* Pursh indicated that leaf thickness of different accessions collected in California and Australia varied greatly among both related populations and even in the same plant individual as well.

Table 9. Leaf characteristics of the studied wormwood accessions

Accessions	Thickness of blade (mm)		Length of leaf (mm)		Length of petiole (mm)		Ratio of blade/petiole	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bel	0.48^{f}	0.09	167.9^{cde}	50.46	83.4^{cde}	37.75	1.12^{d}	0.31
Eng	0.37^{abc}	0.10	185.1^{ef}	42.88	100.3^{ef}	33.06	0.91^{abc}	0.28
Ger0	0.38^{abcd}	0.12	183.2^{def}	30.23	98.7^{ef}	25.98	0.93^{abc}	0.34
Ger1	0.42^{sdef}	0.07	209.2^{f}	56.16	111.3^{f}	33.22	0.90^{ab}	0.18
Ger2	0.30^{a}	0.07	104.4^{a}	18.36	57.8^{a}	15.09	0.85^{ab}	0.23
Hum	0.40^{bcde}	0.10	206.3^{f}	55.18	115.6^{f}	35.30	0.81^{ab}	0.22
HuW1	0.31^{a}	0.08	181.8^{def}	32.06	97.5^{def}	21.49	0.89^{abc}	0.16
HuW2	0.37^{abc}	0.16	148.3^{bc}	42.03	78.8^{abc}	27.35	0.93^{abc}	0.22
HuW3	0.41^{bcdef}	0.13	151.1^{cd}	34.65	79.3^{bcd}	21.34	0.94^{abc}	0.19
HuW4	0.46^{def}	0.20	155.4^{cd}	29.44	81.1^{bcde}	21.49	0.98^{bcd}	0.34
Nor	0.32^{ab}	0.06	124.8^{ab}	12.89	61.6^{ab}	8.73	1.05^{cd}	0.23
Spa	0.47^{ef}	0.10	155.9^{cde}	36.54	90.3^{cde}	24.77	0.76^{a}	0.17
Mean	0.39		165.1		88.3		0.92	

Different letters in the columns represent significant differences between accessions according to Games-Howell test at p=0,05

The length of leaf and the length of petiole seem to be in close connection with each other as the highest values for both of them were found in accessions “Hum”and “Ger1” while the length of leaf and petiole were the shortest in the accession “Ger2”. The marginal values for the leaf length were 209.22 mm (“Ger1”) and the shortest leaf was 104.35 mm (“Ger2”). The ratio between blade and petiole was influenced by the population, too. It varied from 0.76 (“Spa”) to 1.12 (“Bel”). The investigated accessions proved to be significantly different from each other. On the other hand, the four investigated accessions of Hungarian origin revealed similar ratios between 0.81 - 0.98.

Summarizing the data, we can see that accession “Ger2” has the thinnest and shortest leaves, followed by accession “Nor” while “Ger1” was characterised by the longest leaves and “Bel” with the thickest leaves. However, a well established connection among these morphological traits can be excluded.

Concerning the evaluated leaf morphological traits, it was established that the population “Nor” was the most homogenous one (CV: 10% – 22%) while the greatest variability was shown by the

accession “Bel” (CV: 18% – 45%). Similarly to the qualitative leaf morphological traits, no connection could be justified between the geographical origin of the populations and any of the quantitative leaf morphological characteristics.

4.1.1.3 Biomass production

The biomass of twelve studied accessions varied from 63.4 g plant^{-1} (“Nor”) to 322.4 g plant^{-1} (“HuW2”) (Table 10). Additionally, “Eng” and “Ger1” accessions also produced significantly similar high fresh mass: 254.6 g plant^{-1} and 255.2 g plant^{-1} respectively.

Table 10. Biomass production of the *A. absinthium* accessions

Acces-sions	Fresh mass (g/plant)		Dry herb mass (g/plant)		Dry leaf mass (g/plant)	
	Mean	SD	Mean	SD	Mean	SD
Bel	157.0^{ab}	68.24	65.4^{bc}	34.20	29.8^{bcd}	19.24
Eng	254.6^{bc}	60.93	82.6^{c}	24.38	31.4^{cd}	5.93
Ger0	197.4^{abc}	75.46	60.1^{abc}	27.74	26.8^{abcd}	11.05
Ger1	255.2^{bc}	180.63	82.1^{c}	51.46	30.6^{bcd}	17.93
Ger2	130.0^{ab}	56.40	37.0^{ab}	15.27	15.4^{abc}	6.57
Hum	196.2^{abc}	74.90	61.3^{abc}	15.80	26.8^{abcd}	9.99
HuW1	171.8^{abc}	57.61	59.4^{abc}	18.33	25.7^{abcd}	9.57
HuW2	322.4^{c}	211.41	89.0^{c}	48.97	35.5^{d}	14.32
HuW3	190.2^{abc}	62.79	62.1^{abc}	22.95	26.5^{abcd}	8.25
HuW4	88.1^{a}	45.80	32.9^{ab}	19.62	14.1^{ab}	7.29
Nor	63.4^{a}	28.89	21.3^{a}	10.82	12.4^{a}	5.50
Spa	103.8^{a}	35.46	33.3^{ab}	12.14	16.0^{abc}	5.21

Different letters in the columns represent significant differences between accessions according to Games-Howell test at p=0,05

Accession “HuW4” was the one of the four Hungarian accessions which produced the smallest biomass (88.1 g plant^{-1}). Among the twelve accessions a statistical difference was justified for any of them. Similar results were found in the case of total dry mass. As for the leaf dry mass (drug yield), Hungarian plants produced the highest values with 35.50 g plant^{-1} as a mean (HuW2) while the smallest value was 12.40 g plant^{-1} (“Nor”). Similarly to the fresh mass and total dry mass, elevated values of the dry leaf mass were found for accessions “HuW2”, “Eng” and “Ger1”

compared with other accessions such as “Nor”, “Spa”, “HuW4” and “Ger2” which produced less than 16 g plant^{-1}.

In all yield characteristics, a considerable intra-population variability was observed. Especially the high yield accessions like “HuW2” and “Ger1” exhibited a large heterogeneity (CV=66% and 71%, respectively) which may be disadvantageous in cultivation. To the contrary, the lowest variability was found in the accession “Eng” with CV=24%.

There were strong correlations between the height and the biomass (r=0.85), and the width and the biomass as well with r=0.88, while between the biomass and the blade length we found a weak correlation (r=0.46). As a result of this, the height and the width could be used as markers for a potential larger yield before harvesting, but it seems not really true in case of the leaf length.

4.1.2 Studying the relationship of wormwood genotypes by molecular markers

Using 11 RAPD primers, we detected 122 bands which means 11 bands per primer in the average (Table 11). The bands were in the range of 250 - 1500 bp. The primer B10 gave the highest number of bands (16 bands) while the primer OP-A20 was the lowest number of bands (6 bands). A total of 196 scorable bands were generated from 15 ISSR primers. The average number of amplified bands per primer was 13. The Primer ISSR5 and Cag5, composed of trinucleotide repetitions produced as many as 17 bands, while primer ISSR7 amplified the lowest number of bands (7 ones). The size of the bands amplified by the ISSR primers ranged from 120 bp, (by primer ISSR5) to 1500 bp (by ISSR1, ISSR4 and ISSR7). Table 11 shows the number and the size of DNA fragments produced by each of the RAPD and ISSR primers. The proportions of polymorphic bands among wormwood accessions was high, with 81.15% for RAPD and 73.10% for ISSR.

Table 11. The evaluated RAPD and ISSR primers and results of the amplification

RAPD primer name	Sequence	Size of fragment (bp)	Number of fragments
A10	5’-GTGATCGCAG-3’	1450-250	10
B10	5’-CTGCTGGGAC-3’	1500-300	16
E3	5’-CCAGATGCAC-3’	1000-250	11
M2	5’-ACAACGCCTC-3’	1300-300	15
M3	5’-GGGGGATGAG-3’	1300-300	14
OPA-02	5’-TGCCGAGCTG-3’	1100-300	11
OP A20	5’-GTTGCGATCC-3’	1500-400	6
OPB-11	5’-GTAGACCCGT-3’	1200-300	10
OP G18	5’-GGCTCATGTG-3’	1300-350	10
OP G13	5’-CTCTCCGCCA-3’	1300-300	11
Seq-2	5’-GGGTTTAGGG-3’	1300-400	8
Total no. of bands (RADP)			122
Number of polymorphic loci			99
Percentage of polymorphic loci			81.15%
ISSR primer name	**Sequence**	**Size of fragment (bp)**	**Number of fragments**
443	5’-ACACACACACACACACACT-3’	1200-200	14
818	5’-CACACACACACACAG-3’	1100-200	13
825	5’-ACACACACACACACACT-3’	1300-200	16
849	5’-GTGTGTGTGTGTGTGTCG-3’	1100-240	13
A7	5’-AGAGAGAGAGAGAGAGAGAGT-3’	950-200	14
Caa5	5’- CAACAACAACAACAA-3’	1100-200	11
CAg5	5’- CAGCAGCAGCAGCAG-3’	1000-180	17
Ctc4rc	5’-CTCCTCCTCCTCRC-3’	1000-230	16
Issr1	5’-CACACACACACACACAGT-3’	1500-150	14
Issr2	5’-GAGAGAGAGAGAGAGAG-3’	900-220	12
Issr3	5’-GTGTGTGTGTGTGTGTC-3’	850-300	12
Issr4	5’-ACACACACACACACACTG-3’	1500-250	12
Issr5	5’-AGTGAGTGAGTGAGTG-3’	1300-120	17
Issr6	5’-GATAGATAGATAGATAGATA-3’	1100-250	8
Issr7	5’-TCTTCTTCTTCTTCTTCT-3’	1500-530	7
Total no. of bands (ISSR)			196
Number of polymorphic loci			144
Percentage of polymorphic loci			73.10%
Total no. of bands (RADP+ISSR)			318
Number of polymorphic loci			243
Percentage of polymorphic loci			76.18%

There are no references known about molecular markering of intraspecific taxa of *A. absinthium*. Nazar and Mahmood (2011) compared 15 samples of three *Artemisia* species (*A. vulgaris, A. absinthium, A. roxburghiana*) and found a proportion of 68% for polymorphic RAPD bands. Sangwan et. al. (1999) indicated 64% polymorphism for RAPD primers during the investigations on 8 plants of *Artemisia annua* L. in India. Compared to the former findings, our results assure new insight in the molecular differentiation of wormwood.

Nei's genetic distances among the accessions from Hungary are relatively low, between 0.26 and 0.34 (Table 12). The closest similarity was observed between samples collected from wild growing regions in Hungary, "HuW2" and "HuW3" (0.26) while the greatest dissimilarity was observed between the accessions "Bel" and "HuW1" (0.47).

The the largest genetic distance coefficients were determined for accessions "Bel" and "Spa" (>0.4 value detected in 4 cases out of 8). Comparing it to the results of morphological traits of the plants (Chapters 4.1.1.1 - 4.1.1.3), it can be established that the aforementioned two accessions were significantly different from the other ones also in those respects.

Table 12. Genetic distance matrix of the investigated *A. absinthium* accessions based on RAPD and ISSR data

Accessions	HuW1	HuW3	HuW2	Hum	Eng	Nor	Bel	Ger1	Spa
HuW1	1								
HuW3	0.30	1							
HuW2	0.34	0.26	1						
Hum	0.32	0.35	0.3	1					
Eng	0.33	0.27	0.34	0.27	1				
Nor	0.30	0.28	0.35	0.33	0.29	1			
Bel	0.47	0.40	0.37	0.31	0.44	0.42	1		
Ger1	0.43	0.34	0.34	0.34	0.39	0.35	0.38	1	
Spa	0.44	0.39	0.37	0.46	0.40	0.40	0.43	0.38	1

Based on the genetic distance matrix of the 9 accessions, the UPGMA dendogram demonstrates the grouping of the investigated accessions in three main clusters (Figure 3). "Spa" accession seems to form alone a distinct group (group 1), accessions "Nor" and "Bel" were classified into group 2 while all the Hungarian accessions were located in the same group 3 with two further accessions, one from market in England ("Eng") and the other from German seed bank exchange ("Ger1").

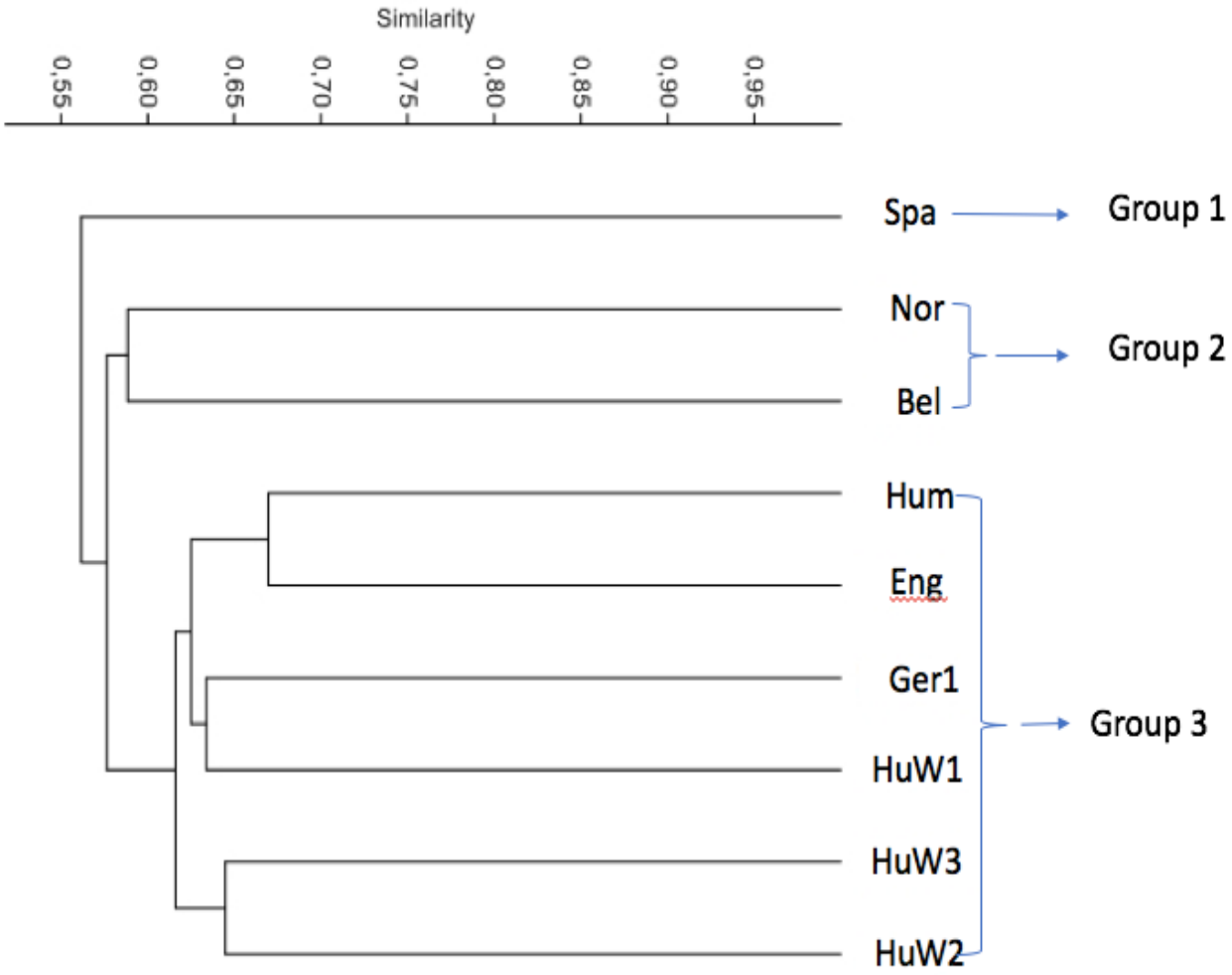

Fig 3. Dendrogram showing the relationship among the studied *A.absinthium* accessions (Generated by combination of RAPD and ISSR primers using UPGMA method)

4.1.3 Studying the relationship of wormwood genotypes by phytochemical characteristics

4.1.3.1 Accumulation level of volatile compounds

The essential oil yield of the investigated accessions was 0.827 ml/100g as a mean, however, it varied on a large scale, between 0.347 ml/100g (HuW4) and 3.215 ml/100g (Spa). According to ANOVA test of between-subjects effects for this trait, significant differences could be detected among the accessions: $F_{(8;81)}=58.707$ ($p<0.001$). The Tukey test provided 3 subsets at $p=0.05$ significance level (Table 13).

Table 13. Essential oil yield (ml/100g DW) of dried leaves of the studied *A. absinthium* accessions grouped according to the Tukey test (p=0.05)

Wormwood accessions	N	Subset		
		1	2	3
HuW4	10	0.3474		
HuW3	10	0.3493		
Hum	10	0.3609		
HuW1	10	0.5205		
HuW2	10	0.5528		
Ger0	10	0.5977		
Eng	10	0.6733		
Ger1	10	0.8306		
Nor	10		1.5675	
Bel	10		1.8916	
Ger2	10		2.0897	
Spa	10			3.2145
Sig.		0.436	0.316	1

Means for groups in homogeneous subsets are displayed. Based on observed means.

Based on this, all accessions from Hungary together with "Eng", "Ger0" and "Ger1" are statistically equal, yielding below 1% essential oil. Among them, the highest mean value was reached by "Ger1" and the lowest one by "HuW4". In general, these values correspond to former data of Orav and co-workers (2006) who obtained 0.1% - 1.1% essential oil from plant material coming from different European regions or to the reference of Basta et al. (2007) about Greek wild plants (0.31%).

Relatively high concentrations of 1.89 ml/100g and 2.09 ml/100g were determined from accessions "Bel" and "Ger2", respectively. These results are similar to the findings of Msaada and co-workers (2015) in Tunisia who described an essential oil content of 1.10% - 1.46% in wild growing plants. Also plant material from Cuba produced similar yield (1.25%), (Pino et al., 1997).

In our study, the accession "Spa" presented an exceptionally high level of volatile compounds which is significantly different from each of the other ones (3.215 ml/100g). Such a high EO level could not be found in the literature.

The accession “Spa” was the most homogenous one in terms of essential oil yield (CV%=12%) while the largest variability was determined in the population “Ger1” from Germany (CV%=64%).

Our results ascertain that the yield of essential oil of wormwood may differ considerably depending on the investigated plant materials (Nguyen and Németh, 2016). At the same time, it can be concluded that the differences are based on genetic variability instead of being the result of diverse ecological conditions as they have manifested themselves in the same environment at our experimental site.

4.1.3.2 Essential oil components and wormwood chemotypes

In the 120 essential oil samples obtained by hydrodistillation of leaves of *A. absinthium* we identified 69 compounds (considering the ones higher than 1% of GC area), among them 30 monoterpenes and 39 sequiterpenes (for detaisl please see Table 28 in the appendix chapter). The widest spectrum was detected in the accession “Eng”, with 48 compounds. On the other hand, the lowest number of components was found in a sample of accession “Ger0” with only 7 components (Table 28). The most frequent components which reached at least 10% in any of the studied accessions are presented in Table 14.

The major components (over 30% of GC area) of these oils were mostly monoterpenes: α-thujone (0%-51.7%) and β-thujone (0%-89.8%); *cis*-epoxy-ocimene (0%-75.7%), *trans*-sabinyl acetate (0%-94.5%), sabinene (0%-33.8%), β-myrcene (0%-68.4%), linalool (0%-52.1%), *cis*-chrysanthenol (0%-37.3%), (Z) iso-citral (0%-49.2%) and some sesquiterpenes: selin- 11- en- 4- α- ol (0%-58%), (*Z*)-nuciferol isobutyrate (0%-37.3%) and (*E*)-nuciferol isobutyrate (0%-33.2%).

Sabinene and ß-myrcene were the most frequent monoterpenes detected in almost each sample of the twelve accessions. On the other hand, α-pinene and α-terpinene were the most rare monoterpenes found in only two samples of two accessions and present in less than 4%. In the case of sesquiterpene compounds, the most widespread, universal component of the analysed oils was β-caryophyllene. Although it was never measured in more than 25%, it was present in 75% of the samples in at least in 1% concentration. As unique components were bornyl acetate, cis-β-farnesene, silphiperfol-6-en-5-one, trans-γ-cadinene and α-cadinene each of them found in only in a single sample.

Table 14. Highest percentages (in GC area %) of some components in the oil of the studied wormwood accessions (Components reaching at least 10% are listed, major ones indicated in bold)

Accesions	**Bel**	**Eng**	**Ger0**	**Ger1**	**Ger2**	**Hum**	**HuW1**	**HuW2**	**HuW3**	**HuW4**	**Nor**	**Spa**
Compound												
Sabinene	7.0	38.1	31.1	11.6		15.1	33.8	18.4	28.9	1.5	7.0	
ß-Myrcene	3.1	26.7	38.3	68.1		44.0	41.6	38.5	68.4	8.0	3.1	
α-Phellandrene		14.5	15.0	10.4		6.7	13.0	4.4	4.2	7.9		
p-Cymol		4.3	10.2	6.1		16.8	14.9	10.0	4.5	10.4		
Linalool		9.7	4.5	27.9		8.7	10.5	18.4	8.7	52.1		
α-Thujone	51.7	1.1		1.8						2.4	51.7	
ß-Thujone	89.8	2.1		85.2		0.7	0.3		2.1	87.6	89.8	
***cis*-epoxyocimene**		65.1					44.9	45.9				75.7
Isocitral (Z)		48.5		49.2								1.0
cis-Chrysanthenol				27.5		37.3						1.0
Terpinene-4-ol		2.7	2.3	1.4		4.5	10.0	22.8	9.8	18.5		
cis-Chrysanthenyl acetate												25.9
Trans-Sabinyl acetate			87.6	36.0	94.5			3.4	75.2			
Thymol							1.2		28.4			
Cyclohexanol acetate		10.9						3.4				11.2
β-Caryophyllene	6.5	11.0	12.2	12.9	1.0	19.6	24.0	25.2	6.9	8.2	6.5	3.8
Germacrene D	1.6	4.7	11.8	11.6	1.1	8.6	11.0	3.0	4.4	12.0	1.6	3.7
α-Curcumene		3.6	6.5	24.4		4.3	9.7		8.2	4.8		
Lavandulyl-isovalerate		2.8	2.5	1.8		8.3	2.1	2.5	1.8	29.0		
Caryophyllene oxide	1.6	5.4	10.4	10.8		9.7	10.6	16.7	9.9	7.4	1.6	
(2R,5E)-Caryophyll-5-en-12-al		4.7	1.5	1.6	1.0	16.9	5.6	4.0	8.8	10.8		1.2
Geranyl-isovalerate		1.4	1.9	3.5		8.3		1.7	2.6	18.5		
selin-11-en-4-α-ol	6.5	15.4	11.1	50.8		21.8	4.9	58.0	27.4	10.4	6.5	1.4
α-Bisabolol				12.5					1.2			
Chamazulene	9.7	1.3	3.9			0.5			3.9		9.7	19.8
Geranyl-p-cymene	1.3	11.8	5.1	7.8		8.5	1.9	2.1	3.7	11.5	1.3	
Unknown	3.1	7.7	15.9	2.0		8.2	6.9	10.9	8.5	3.0	3.1	

(*Z*) Nuciferol isobutyrate	2.5	20.1	22.4	8.9	22.1	9.3	14.1	19.3	37.3	2.5	1.1
(*E*)-Nuciferol isobutyrate		13.2	33.2						4.8		

The order of components based on the retention time

Based on the above mentioned eleven compounds we defined different chemotypes. These are eleven "pure" chemotypes where a single main component represents more than 30% of the total GC area and eleven "mixed" chemotypes in which two – or in a few exceptions, three – major components together make up at least 30% of the total GC area. These chemotypes and their abundance in the examined wormwood populations are demonstrated in Table 14.

"Pure" thujone (both isomers calculated together) chemotype occurred in four accessions out of the twelve we investigated, such as in "Bel" (100% of samples with over 54.5% of the total GC area), "HuW4" (with 40% of its samples in 67.5-82.3%), "Ger1" (with 30% of its samples in 54.7%-86.9%) and 20% of the samples of "Nor". "Pure" *cis*-epoxyocimene chemotypes were found in four accessions and "pure" *trans*-sabinyl acetate chemotype appeared in five ones. Individuals belonging to the ß-myrcene chemotype were also found in five accessions, however in lower quantities, in 10% - 30% of total samples in each accession. Other "pure" chemotypes such as sabinene, linalool, cis-chrysanthenol, (*Z*)-iso-citral and (*E*)-nuciferol isobutyrate were represented only by a few individuals of the total pool.

Mixed chemotypes have been differentiated either as those with a presence of thujone or those without it. Three chemotypes were detected where thujone (both isomers calculated together) and another major compound together represented at least 30% of the oil: thujone (33.4%) + *cis*- epoxyocimene (29.6%), thujone (51.5%) + *trans*-sabinyl acetate (48.4%) and a third one where the proportion of these three compounds was very similar: thujone (34.8-36.5%) + *cis*- epoxy-ocimene (20.3-26.4%) + *trans*-sabinyl acetate (20.3-29.4%). Interestingly, all of these are only present in the accession "Nor".

Besides the above mentioned ones we defined eight "mixed" chemotypes without thujone. Among them sabinene+ß-myrcene and ß-myrcene+β-caryophyllene were found as main types in four accessions for each. The sabinene+ß-myrcene type was the most frequent with ten individuals. The other "mixed" chemotypes without thujone were detected only in 1-2 accessions in lower proportions (10-30%). Twenty one samples did not belong to any of the mentioned wormwood chemotypes as the spectrum of their components was very rich and diverse but each compound was present only in lower concentrations.

4.1.3.3 Evaluation of the accessions based on the essential oil composition

The examined wormwood accessions exhibited a variable picture concerning their chemotype composition (Table 15).

Table 15. Distribution of the identified chemotypes of *A.absinthium* in the examined accessions

Chemotype	Proportion in the accessions (%)											
	Bel	**Eng**	**Ger0**	**Ger1**	**Ger2**	**Hum**	**HuW1**	**HuW2**	**HuW3**	**HuW4**	**Nor**	**Spa**
Pure chemotypes (>30% of total GC area)												
Thujone	**100**			30						40	20	
Cis-epoxyocimene		20					10	30				**100**
Trans-sabinyl acetate			10	10	**100**				10		40	
Sabinene		10										
ß-Myrcene				20		20	10	10	30			
Linalool										10		
Cis-Chrysanthenol						10						
(*Z*)-Isocitral		10		10								
Selin-11-en-4-α-ol								10				
(*Z*)-Nuciferol isobutyrate										10		
(*E*)-Nuciferol isobutyrate			10									
Mixed chemotypes (total components over 30% of total oil)												
Thujone + *cis*-epoxyocimene											10	
Thujone + *cis*-epoxyocimene + *trans*-sabinyl acetate											20	
Thujone + *trans*-sabinyl acetate											10	
Sabinene + ß-Myrcene		20	30				40		10			
ß-Myrcene + β-Caryophyllene				10		10	10	20				
ß-Myrcene + (*Z*)-Nuciferol isobutyrate			10									
Linalool + β-Caryophyllene				10				10				
Linalool + (*Z*)-Nuciferol isobutyrate										30		
β-Caryophyllene + selin-11-en-4-α-ol						20		20				

selin-11-en-4-α-ol + (*Z*)-Isocitral												
selin-11-en-4-α-ol + (*Z*)-Nuciferol isobutyrate						10			10			
Other	**0**	**40**	**40**	**10**	**0**	**30**	**30**	**0**	**40**	**10**	**0**	**0**

The most homogenous accessions were found in wild collections such as "Spa" with 100% of examined oils belonging to *cis*-epoxyocimene chemotype (46.9 - 75.7% of oils), "Bel" with 100% of oils being thujone chemotype, and "Ger2", 100% of whose oils are *trans*-sabinyl acetate chemotype.

A larger proportion of samples (40%) of "Nor" belongs to the *trans*-sabinyl acetate type and 40% of the population "HuW2" in the sabinene + ß-Myrcene type. Similarly, 40% of the plants in the accession "HuW4" represent thujone chemotype.

Most of the Hungarian accessions are rather heterogeneous ones. In four out of the five investigated Hungarian accessions, ß-myrcene type is present in levels of 10% - 20%. Besides, in "HuW1" and "HuW2" *cis*-epoxyocimene type is present in concentrations of 10% and 30%, respectively. Only one Hungarian accession ("HuW4") contained individuals of the thujone type, in relatively large abundance (40%) as mentioned above. The concentration of thujone in these samples is high (67.5% - 89.6% of the oil). Some accessions like the two from market "Eng" and "Ger0" did not represent special chemotype distribution.

Evaluating the accessions by PCA , it could be established that principal components PC1-PC5 are responsible for 15.85%, 10.59%, 9.33%, 7.79%, and 6.68% of the total variance, respectively, and the five PCs explained 50.23% variance altogether. With high positive loading, PC1 is mainly related to *cis*-epoxy-ocimene, *cis*-chrysanthenol, chamazulene, cyclohexanol acetate and *trans*- epoxyocimene while with moderate negative loadings it is related to ß-myrcene, sabinene and ß-thujone. PC2 is highly positively correlated with linalool, geranyl-p-cymene, geranyl- isovalerate, ß-selinene and lavandulyl-isovalerate while it is in moderate negative correlation with sabinene. PC3 is mainly for cedren-13-ol, spathulenol and curcumene (ar-) with positive loadings and for ß-myrcene and sabinene with negative loadings. PC4 is mainly related to ß-myrcene, sabinene, *trans*-sabinyl acetate, α-thujone, ß-thujone, β-caryophyllene, caryophyllene oxide, p-cymol, *trans*-epoxy-ocimene with high positive loadings. PC5 is positively correlated withr *(E)*-nuciferol isobutyrate, selin-11-en-4-α-ol, *(Z)*-nuciferol isobutyrate and *(Z)*- isocitral.

The best differentiating of the accessions was achieved by the first 4 PCs in the coordinate systems below (Figures 4 and 5). By PC1, Spa individuals can effectively be distinguished from the other accessions because of their uniquely high *cis*-epoxyocimene content and PC2 differentiates four

HuW4 individuals from the other accessions because of their special high linalool content (Figure 4).

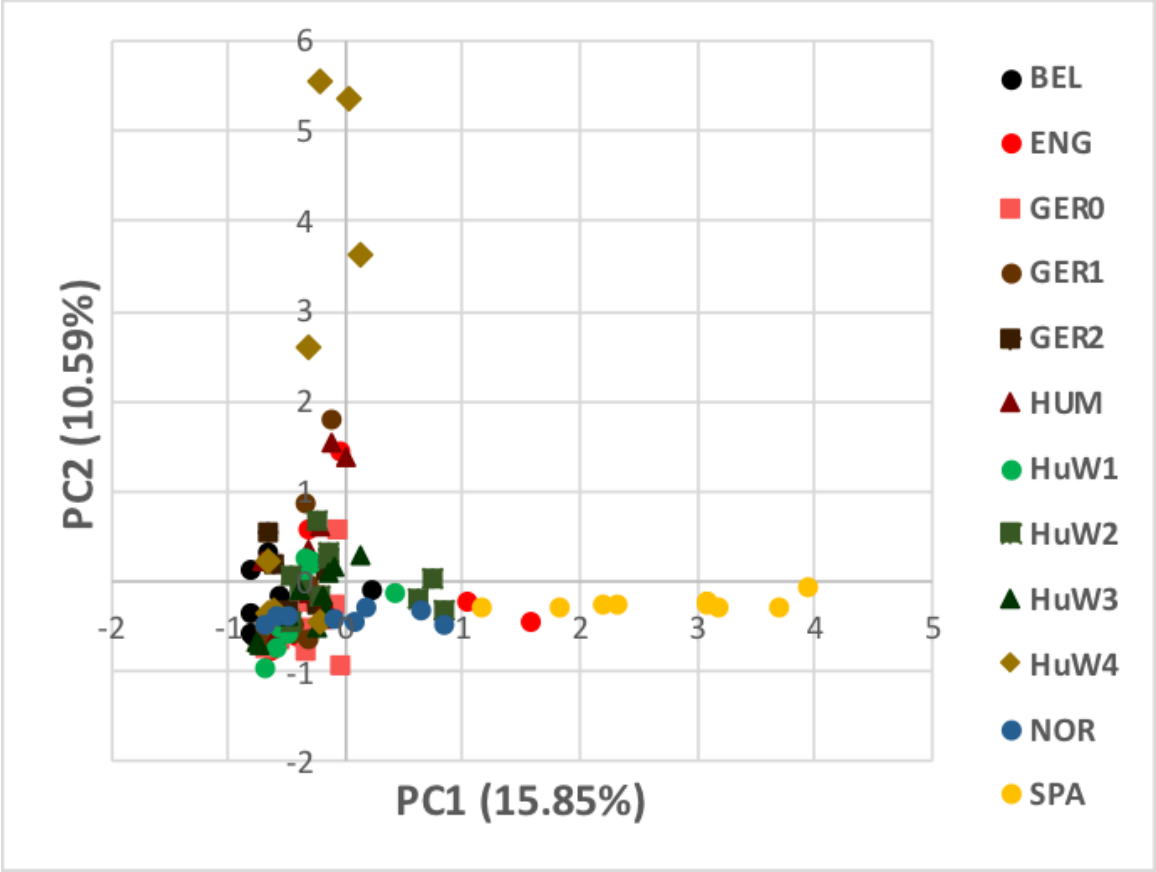

Fig 4. Score plot of 120 individuals of 12 accessions PC1 vs PC2

Based on PC3 scores the homogeneity within the accessions "Bel", "Nor", "Spa" can be concluded, mostly because of their low myrcene and sabinene contents, while all the others, especially "Eng", "Ger0" and "HuW3" are heterogenous from this aspect. By PC4 the accessions with high thujone or *trans*-sabinyl-acetate contents ("Bel", "Ger2", "Nor") are close to each other while some individuals of e.g. "HuW1" and "HuW2" are close to the "Spa" accession because of their *cis*- epoxyocimene content (Figure 5). It can be observed that geographical distances do not play significant role in distinguishing the samples.

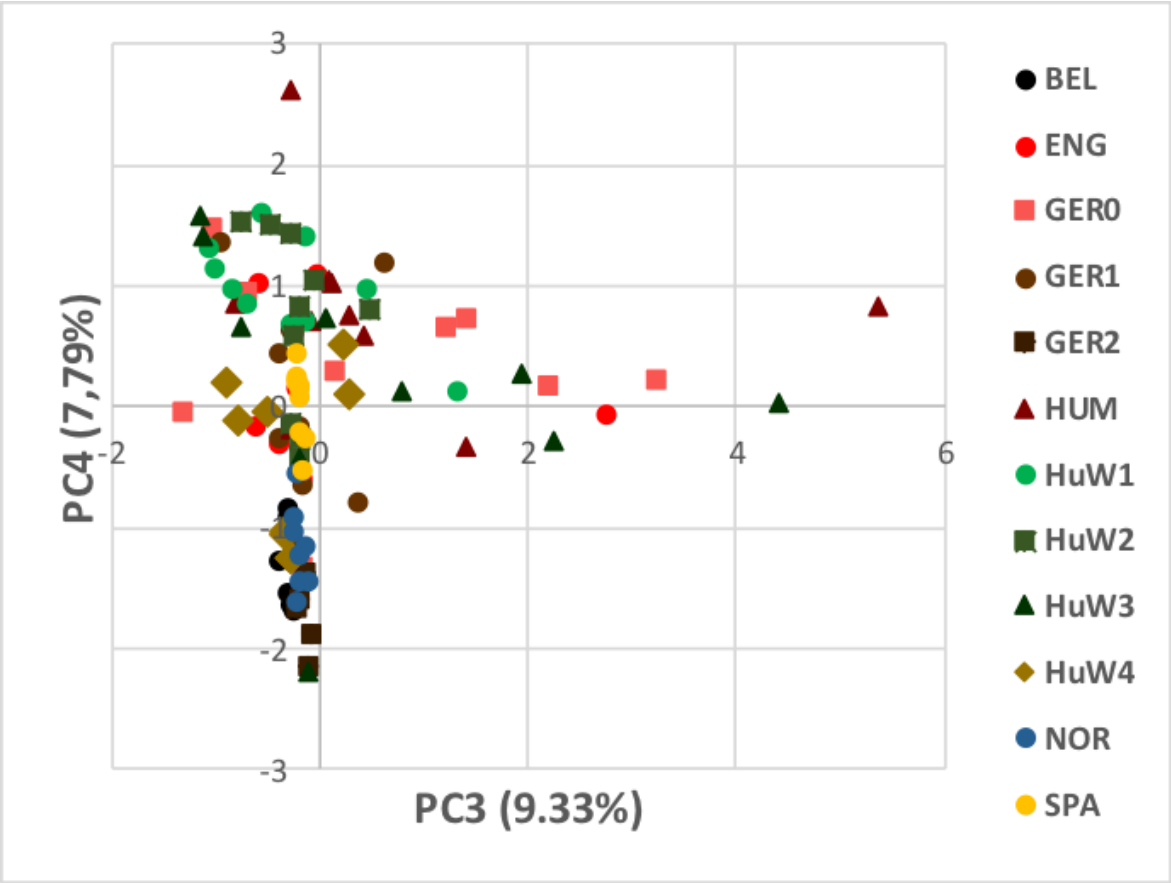

Fig 5. Score plot of 120 individuals of 12 accessions PC3 vs PC4

The large chemical variability of wormwood is well known. However, large and comprehensive studies comparable with our one could hardly be found. Our results showed that thujone is the most characteristic compound only for the accessions originating from Belgium and Norway. However, while in the population "Bel", the ratio between α- and β-thujones showed a large variability, and a balanced ratio of the isomers was demonstrated in "Nor". Both variations can be found in the literature. In Serbian natural populations β-thujone was the absolute major component represented by up to 63.4% in the oil with only 0.4% α-thujone (Blagojević et al., 2006). However, Rezaeinodehi and Khangholi (2008) determined levels of 18.6% and 23.8% for α- and β-thujones respectively in the EO of wild collected Iranian material. A study by Nin et al. (1995) identified samples from the United States (69.7%), Germany (49.8%) and Italy (49.2%) as characteristic β- thujone chemotypes. Pure thujone chemotype (above 42% of the oil) occurred in Croatia (Juteau et al., 2003) and Morocco (Derwich et al., 2009), as well. All these data, together with results obtained in this study, indicate that the occurrence of the thujone chemotype is rather frequent around the Northern hemisphere and cannot be restricted to special areas. In the case of the related species *Tanacetum vulgare* L. which also contains high amounts of thujone, a south to north decline in the frequency of the thujone chemotype has been detected in Europe

(Sorsa et al., 1968). A more detailed study on wormwood could reveal if such a tendency exists in the case of this species.

Cis-epoxyocimene is also a well known component of wormwood oils. It was found as a main compound in samples collected from the Northwest Italian Alps (Mucciarelli et al., 1995). This compound was the basis of determining two chemotypes ((*Z*)-β-epoxyocimene and (*Z)*- β- epoxyocimene + (*Z*)-chrysanthenyl acetate types) in a wild growing population originating from Spain (Llorens-Molinaet al., 2017) and also in France (Juteau et al., 2003). Bailen et al. (2013) reported the presence of *cis*-epoxyocimene, chrysanthenol, and chrysanthenyl acetate as most abundant components in *A. absinthium* oils from Spain, as well. In agreement with this, all individuals of the Spanish material in our study contained high percentages of c*is*-epoxyocimene. Although the presence of this compound was not restricted to this accession, the quantity was much lower and it was never a main compound in the other populations. Based on the above discussion, it seems that *cis*-epoxyocimene is most widespread and a common main component primarily in regions of Southwest Europe (Figure 4).

In parallel with the former references, chrysanthenyl acetate was detected in the majority of our Spanish samples (9% - 26%) while *cis*-chrysanthenol was measured as a main compound only in a single sample from Germany and another one from the "Hum". It can be concluded that chrysanthenyl-type compounds are less connected to special growing areas. This chemotype was reported also from Tajikistan (Sharopov et al., 2012).

The accessions from Germany differed from each other to a large extent (Figure 5). "Ger2" proved to be a homogeneous one consisting only of individuals belonging to the *trans*-sabinyl acetate chemotype. The "Ger1" of wild origin included only a single plant of the former type. All the others represented different chemotypes. The commercial item "Ger0" was also found to be rather heterogenous, with a rich variability in the EO spectrum of its individuals. Until now only a single reference was referring to samples from Germany: Nin et al. (1995) examined five accessions and found variable EO composition.

Data on Hungarian wormwood material are rather scarce, and in this context our study demonstrated new information. Llorens-Molina et al. (2016) established three chemotypes (sabinene+myrcene, β-thujone and a new, sesquiterpene type) in a cultivated population from Hungary. Besides a single individual particularly rich in β-thujone (83.5 %), others contained only lower proportions of this compound. A sample with a relatively high ratio (11.4%) of limonene was also found. Orav and co-workers (2006) reported a Hungarian market sample with 18.1% sabinene as a major compound. Sabinene and β-myrcene were frequent components in our samples, as well: 10% - 50% of the investigated individuals belonged either to the β-myrcene or

to the sabinene + β-myrcene chemotype. Sabinene was detected, although not as a major compound, in 32 out of the 40 analysed samples. However, all of the Hungarian accessions proved to be heterogeneous (Figure 5). Sabinene and β-myrcene seem to be widespread components in wormwood EO in general. Together with sabinyl-acetate they were found in samples originating from Tajikistan, Siberia through Moldova, Scotland and Canada (Nguyen and Németh, 2016). Thus, these compounds are hardly characteristic of a given region and may be considered as universal components of *A. absinthium* EO.

According to our knowledge, the components (*Z*)-*iso*-citral, selin-11-en-4-α-ol, (*Z*)- and (*E*)- nuciferol isobutyrate characterise new chemotypes of wormwood as they have never been mentioned before in its EO. Llorens-Molina et al. (2016) described nuciferol esthers in the root oil of this species.

Sharopov et al. (2012) analysed the data of numerous former reports and mentioned that different chemotypes of wormwood exist depending on geographical origin. However, several samples referred to in former publications originate from commercial activity and thus, geographical origin does not necessarily always mean natural growing habitat. Besides, during sampling of populations, the huge majority of former works analysed bulk samples which are mixtures of several undefined individuals with possibly different EO quality. Consequently, these kind of data are not able to reflect the chemism of the species (Németh-Zámbori, 2015).

4.1.3.4 Evaluation of the accessions based on the total phenolic content and antioxidant capacity

According to ANOVA Test of between-subjects effects, there have been significant differences detected in case of TPC ($F_{(107,11)} = 24.208$, $p < 0.001$) and AC ($F_{(107,11)} = 88.375$, $p < 0.001$) (Table 16).

Table 16. Total phenolic content and antioxidant capacity of twelve *A. absinthium* accessions

Accession	TPC (mg GAE/g DW)		AC (mg AAE/g DW)	
	Mean	**SD**	**Mean**	**SD**
Bel	161.84^{f}	5.044	105.00^{f}	4.473
Eng	120.76^{abc}	4.346	69.42^{ab}	2.435
Ger0	129.80^{cde}	6.533	78.95^{d}	3.342
Ger1	122.00^{abcd}	11.476	70.56^{bc}	4.134
Ger2	137.44^{e}	11.807	87.59^{e}	4.119
Hum	119.32^{abc}	6.485	77.03^{d}	2.026
HuW1	127.59^{bcde}	8.065	66.23^{ab}	3.526
HuW2	125.98^{bcde}	7.269	69.57^{ab}	2.069
HuW3	117.32^{ab}	7.898	64.47^{a}	4.403
HuW4	124.83^{abcd}	3.802	76.19^{cd}	5.624
Nor	134.04^{de}	9.588	78.22^{d}	3.184
Spa	113.08^{a}	6.185	65.24^{ab}	2.766

Different letters in columns represent significant differences between accessions according to the Games-Howell test at p=0,05.

The total phenolic content of the investigated accessions ranged from 113.08 (mg GAE/g DW) to 161.84 mg GAE/g DW. The Games-Howell test distinguished 6 subsets at p = 0.05 significance level. Accession "Bel" reached the highest value of TPC while accession "Spa" showed the lowest TPC. According to Msaada et al. (2015), the total polyphenol contents of wormwood aerial parts harvested at flowering stage from different Tunisian regions varied significantly due to different regions and reached the maximum in the region of Kairouan (99.89 $\pm$ 3.30 mg GAE/g DW). Mohammadi et al. (2015) reported that the total polyphenol content of Iranian wormwood aerial parts from different phenological stages was varied from 31.73 to 48.49 mg GAE/g of EOs which was significantly of smaller value in comparison with our results.

The highest AC value was detected in accession "Bel" (105.00 mg AAE/g DW), while the lowest

one was found in accession “HuW3” (64.47 mg AAE/g DW). Among the twelve accessions a statistical difference was justified for each of them. Hungarian accessions could be divided into two groups: “Hum” and “HuW4” showed higher antioxidant capacity compared to the other Hungarian ones although all of them had significantly similar total phenolic contents.

The TPC and AC seem to be in tight connection with each other as highest values for both of them were found in accessions “Bel”and “Ger2” while the lowest ones found in “HuW3” and “Spa” (R=0.92). However, the AC and TPC are not related to the EO content. We could not find a strong relationship between AC and EO content (r=0.28) as well as TPC and EO content (r=0.23).

4.2. Factors influencing the chemosyndroms of wormwood

4.2.1 Ontogenetic and morphogenetic (organic) factors

4.2.1.1 Essential oil content

The study of composition and content of EOs obtained from different wormwood plant organs provides probably the most important parameter for the characterization of the species. Thus, this research plays an important role in making decisions on the most appropriate organ type and timing of the biomass harvest.

Based on consecutive sampling of individuals of two characteristic chemotypes of wormwood (thujone and trans-sabinyl acetate types) the effect of the ontogenesis on the accumulation of volatile components has been proven (Table 17). The essential oil yield of both investigated accessions varied on a large scale: between 0.13 ml/100 g (from leaves of trans-sabinyl acetate chemotype at after flowering stage) and 1.77 ml/100 g (obtained from flowers of thujone chemotype at floral budding stage). According to two-way ANOVA test of between-subjects effects, significant differences could be detected among different phenological stages in both flowers and leaves of both chemotypes. In the thujone chemotype, $F_{phenological\ stage\ at\ flowers(8;2)} = 54.399$; $p<0.001$ and $F_{phenological\ stage\ at\ leaves(11,3)} = 194.552$; $p<0.001$. In the *trans*-sabinyl acetate chemotype, $F_{phenological\ stage\ at\ flowers(8;2)} = 74.389$; $p<0.001$ and $F_{phenological\ stage\ at\ leaves(11,3)} = 95.396$; $p<0.001$. Significant differences were detected between leaf and flower samples at all phenological phases but only in the case of the trans-sabinyl acetate chemotype. In the thujone chemotype this difference could be demonstrated only at the post-flowering stage ($F_{organs\ at\ post\text{-}flowering\ stage(5,1)} = 64.890$; $p<0.01$).

Table 17. Changes in EO content of two *A. absinthium* chemotypes during different developmental stages

Chemotype	**Phenological stage**	**Essential oil content (ml/100 g)**	
		Leaves	**Flowers**
Thujone	Vegetative	1.49^{a}	-
	Floral budding	1.57^{aA}	1.77^{aA}
	Flowering	0.99^{bA}	1.01^{bA}
	After flowering	0.48^{cB}	0.72^{cA}
Trans-sabinyl acetate	Vegetative	1.20^{a}	-
	Floral budding	0.52^{bB}	1.16^{aA}
	Flowering	0.19^{cB}	1.10^{aA}
	After flowering	0.13^{cB}	0.45^{bA}

Small letters in columns represent significant differences between phenological phases of the same organ and capital letters in rows represent significant differences between leaves and flowers at the same phenological phase, according to the Games-Howell test at p=0,05.

The highest yield of essential oil (ml/100g DW) obtained from leaves of thujone chemotype plants was measured in the budding phenophase (1.57 ml/100 g) and the lowest one after flowering (0.48 ml/100 g). However, the *trans*-sabinyl acetate chemotype showed the highest oil yield at the start of the vegetation period when the leaves were harvested in the vegetative stage (1.20 ml/100 g). The lowest value was obtained after flowering (0.13 ml/100 g), similarly to the other chemotype. The oil content of the flowers was significantly higher compared to the leaves at all developmental stages, but only in samples of the trans-sabinyl acetate chemotype. This difference is only partially true for the other chemotype: at after flowering stage. During the former developmental stages, the oil content of leaves and flowers did not differ significantly. It seems to be a general phenomenon that the accumulation of volatile compounds reaches a peak in the earlier phenophases, usually at the beginning of flowering, and decreases sharply after flowering and during seed ripening (Llorens-Molina et al., 2016; Németh et al., 2007). This dynamic may be in connection with the development of oil glands and an intensive biosynthesis of volatile molecules due to their specific roles in ecological adaptation and other physiological mechanisms of the plants (Guitton et al., 2010; Németh et al., 2001). In the case of wormwood there has not been any detailed background information obtained about these mechanisms until now.

4.2.1.2 Essential oil composition

The composition of the EO in the sampled phenophases and plant organs in case of the ***thujone chemotype*** is presented in Table 18. In total, the number of identified constituents varied from 16 (leaves harvested after flowering) to 24 (flowers harvested during flowering), representing from 86.91% of the GC area (flowers at after flowering stage) up to 98.8% of the total oil (leaves harvested at floral budding).

Thujone is the major component in both organs. The concentration of ß-thujone (31.1% - 63.4%) was higher than that of α-thujone (12.7% - 28.2%) in both leaf and flower samples at all developmental stages. However, leaves always show higher ratios from both isomers than do flowers at the same harvesting time. The thujone chemotype is well known from literature (Juteau et al., 2003; Meschler and Howlett, 1999). There are, however, only limited data on the special composition of the different plant organs, especially those of flowers and leaves separately. Judzentiene and Budiene (2010) determined that the ratio of the two thujone isomers together is 0.0% - 8.9% in leaves and 5.3% - 10.4% of the oil in flowers.

As for other compounds of the thujone chemotype in our study, we found some characteristic ones like geranyl-p-cymene, nuciferol esthers and two unknown compounds present only in the flower oil (Table 18). Beside this qualitative variation, quantitative differences were detected between the organs in the case of neryl-isobutanoate, neryl-isovalerate and caryophyllene oxide, reaching 426% - 880% higher proportions in the flowers.

During the plant development, the ratio of the main compound ß-thujone reached the highest ratio (51.99%) in the flowers at floral budding stage and decreased significantly after that. In the leaves, its proportion fluctuated with significant changes, the highest value (63.4%) being measured at the vegetative stage, at the earliest sampling time. Changes of α-thujone are less characteristic and do not follow the pattern of the other thujone isomer. β-selinene shows a peak value at flowering time in both organs, while for neryl isovalerate, caryophyllene oxide and chamazulene, this peak accumulation was detected only in the flowers. Regarding neryl-isobutanoate and caryophyllene oxide, a significant increasing tendency was found only in the leaves.

Table 18. Oil composition (GC area %) of *A. absinthium* obtained from leaves and flowers of thujone chemotype plants at different phenological stages

Organs			**Leaf**				**Flower**		
Stage			**V**	**FB**	**F**	**AF**	**FB**	**F**	**AF**
BBCH code			**40**	**55**	**65**	**67**	**55**	**65**	**67**
Compound[1]	**LRI**[2]	**LRI**[4]							
Sabinene	969	976	2.57	1.21	1.94	0.38	2.03	1.41	0.92
ß-Myrcene	988	995	1.44	0.20	0.64	0.12	0.29	0.37	0.31
1,8-Cineol	1030[3]	1034	0.96	1.77	1.64	0.13	2.18	0.76	0.13
Linalool	1100[3]	1097	0.36	0.21	0.49	0.18	0.89	1.60	1.19
α-Thujone	1105[3]	1105	28.15[a]	24.86[a]	18.82[b]	26.76[a]	19.80[a]	12.74[b]	20.68[a]
ß-Thujone	1112	1113	**52.54[b]**	**63.41[a]**	**52.24[b]**	**56.82[ab]**	**51.99[a]**	**31.06[b]**	**31.36[b]**
Lavandulol	1165	1166	0.21	0.15	0.18	0.72	-	0.61	0.53
β-Caryophyllene	1417	1420	0.43	1.05	1.03	1.12	1.61	2.14	1.64
Germacrene D	1484	1482	0.40	0.17	0.32	-	0.39	1.04	0.35
ß-Selinene	1489	1486	0.71[b]	0.97[b]	**3.23[a]**	1.48[b]	2.35[a]	**3.89[a]**	1.84[a]
Neryl-isobutanoate	1490	1492	0.61[a]	0.19[b]	0.59[ab]	0.62[a]	2.02[a]	**4.40[a]**	3.43[a]
Lavandulyl-isovalerate	1509	1512	0.05	0.06	0.14	0.22	0.31	1.71	1.27
Lavandulyl-2-methyl-butanoate	1511	1513	0.06	-	0.13	0.24	0.20	1.08	1.16
Himachalene (α-dehydro-ar)	1516	1516	0.23	0.24	0.30	0.12	0.43	0.41	1.16
Neryl-isovalerate	1582	1584	0.28[bc]	0.12[c]	0.66[a]	0.49[ab]	1.99[b]	**5.58[a]**	4.90[ab]
Caryophyllene oxide	1582	1590	0.48[b]	0.23[b]	1.63[a]	1.73[a]	2.56[a]	**5.90[a]**	5.48[a]
10-epi-γ-Eudesmol	1622	1623	0.31	0.17	0.30	-	0.42	1.04	2.08
γ-Eudesmol	1630	1635	0.11	0.19	0.24	-	0.42	0.34	1.26
Selin-11-en-4-α-ol	1658	1661	1.42	1.02	1.49	2.59	2.02	2.17	2.43
Chamazulene	1730	1733	2.52[a]	2.55[a]	2.96[a]	-	3.82[a]	**5.99[a]**	1.77[b]
Geranyl-p-cymene	1946[3]	1939	-	-	-	-	-	1.69	-
Unkown 1		1978	-	-	-	-	2.3	-	1.95
(Z) Nuciferol isobutyrate	1997[3]	1981	-	-	-	-	-	5.40	1.08
(E) Nuciferol isobutyrate	2004[3]	1983	-	-	-	-	-	2.30	-
Unknown 2		2146	-	-	-	-	-	1.56	-
Total monoterpenes (%)			86.23	91.82	79.68	85.11	77.18	48.54	55.12
Total sesquiterpenes (%)			7.59	6.97	11.08	8.62	18.55	46.64	31.79
Total identified percentage			93.83	98.78	90.76	93.73	95.73	95.18	86.91

V: vegetative; FB: floral budding; F: flowering; AF: after flowering

[1] Components reaching 1% of GC area are listed

[2] Linear retention indices according to the literature Adams (2007)

[3] Linear retention indices from the literature, Sharopov et al. (2012) on HP-5MS column

[4] Linear retention indices calculated relative to the elution ranking of n-alkanes (C_9-C_{20}) on the HP-5MS column

Letters in rows represent significant differences between phenological phases of each organ according to the Games-Howell test at p=0,05

Our results are in agreement with the findings of Carnat et al. (1992), who have reported that the content of thujone decreased during the harvesting period from June to November. However, these data refer to the whole aerial parts of wormwood and no reference was found for the plant organs separately. Decreasing ratios of α-thujone during development in the EO of *Salvia officinalis* L. were reported by Santos-Gomes and Fernandes-Ferreira (2001) and Shadi and Saharkhiz (2016).

The ratio of monoterpene compounds together was higher than that of the sesquiterpenes both in leaves and flowers. The highest amount of monoterpene compounds was detected in the oils at floral budding stage in both leaves (91.8%) and in flowers (77.2%) in harmony with the accumulation dynamics of ß-thujone. In parallel with this, the ratio of sesquiterpenes increased during flowering and after flowering phases and reached 31.8% - 46.6% in flowers; however, it was only 8.6% - 11.1% in leaf oils.

The GC results of the ***trans-sabinyl acetate chemotype*** revealed that the major volatile constituent of these oils was *trans*-sabinyl acetate, except for the last phenological stage, when thujone suddenly appeared in higher percentages (Table 19). A total of 31 components representing from 80.1% (in flowers at after flowering stage) to 94.5% (vegetative stage) of the oils were identified. The oil richest in components was that from the flowers harvested at flowering stage, with 31 constituents. On the other hand, the lowest number of components (23) was found in leaf oil at the vegetative phase.

The ratio of *trans*-sabinyl acetate ranged from 10.5% (in flower oil at the last harvesting stage) to 70.8% (in leaf oils at floral budding). The content of this major component was higher by 34% - 188% in leaves compared with flowers in the respective phases. Judzentiene and Budiene (2010) reported this compound as a dominant component of EO varying between 21.8% and 51.3% in fourteen samples collected at the full- flowering stage. In the majority of their samples *trans*- sabinyl acetate content was higher in the leaves compared with flowers. In our study, a characteristic qualitative variability between leaf and flower oils was detected only in 10- epi- γ- eudesmol and geranyl-p-cymene, which were found in flower samples but not in the leaves. Nevertheless, there are obvious quantitative differences in the ratios of some compounds like linalool and neryl-isovalerate, which tend to accumulate mainly in the flowers (2-4 times higher than in the leaves).

Table 19. Oil composition (GC area %) of *A. absinthium* obtained from leaves and flowers of trans-sabinyl acetate chemotype plants at different phenological stages

Organs			**Leaf**				**Flower**		
Phenological stage			**V**	**FB**	**F**	**AF**	**FB**	**F**	**AF**
BBCH code			**40**	**55**	**65**	**67**	**55**	**65**	**67**
Compound[1]	**LRI**[2]	**LRI**[4]							
Sabinene	969	976	**1.56**^{a}	0.13^{ab}	0.02^{b}	0.34^{ab}	0.67^{b}	6.20^{a}	4.06^{ab}
ß-Myrcene	988	995	2.33	0.32	0.13	0.43	0.41	5.81	2.18
α-Phellandrene	1002	1009	0.43	0.02	0.04	0.08	0.02	0.76	0.33
Linalool	1100	1097	0.22	-	0.52	3.56	2.78	5.94	7.56
α-Thujone	1105	1105	**8.35**^{a}	3.31^{ab}	4.63^{ab}	0.53^{b}	2.17^{b}	6.75^{a}	0.45^{b}
ß-Thujone	1112	1113	8.92^{b}	3.38^{c}	2.98^{c}	**26.99**^{a}	2.41^{b}	4.21^{b}	**20.74**^{a}
cis-Epoxyocimene	1137	1130	**11.64**	0.31	5.99	-	0.33	4.20	-
trans-Sabinol	1137	1140	0.67^{ab}	1.40^{a}	0.84^{ab}	0.17^{b}	1.13^{a}	0.31^{b}	0.28^{b}
Trans-Sabinyl acetate	1289	1289	**60.70**^{ab}	**70.84**^{a}	**48.92**^{b}	**23.48**^{c}	**52.81**^{a}	**16.98**^{b}	**10.50**^{c}
β-Caryophyllene	1417	1420	0.23^{d}	1.03^{c}	3.13^{b}	3.71^{a}	1.41^{b}	2.76^{a}	2.27^{a}
Curcumene (ar-)	1479	1480	-	-	-	0.84	-	2.10	1.89
Germacrene D	1484	1482	0.29	0.64	0.77	-	1.91	-	-
α-Curcumene	1479	1483	0.15	0.18	0.91	2.2	0.27	0.92	1.22
ß-Selinene	1489	1486	-	2.52^{a}	1.50^{ab}	1.18^{ab}	2.65^{a}	1.07^{b}	0.86^{b}
Neryl-isobutanoate	1490	1492	0.27	0.60	1.09	0.56	1.91	1.98	0.97
Lavandulyl-isovalerate	1509	1512	0.22^{c}	0.50^{c}	0.99^{b}	1.82^{a}	0.65^{b}	1.03^{b}	2.01^{a}
Lavandulyl-2-methyl-butanoate	1511	1513	0.27^{c}	0.37^{bc}	0.60^{ab}	0.84^{a}	0.44^{b}	0.64^{b}	1.06^{a}
Himachalene (α-dehydro-ar)	1516	1516	-	-	0.10	-	-	0.64	0.94
Neryl-isovalerate	1582	1584	0.23^{b}	1.66^{a}	1.90^{a}	1.02^{ab}	5.11^{a}	3.29^{b}	2.80^{b}
Spathulenol	1577	1584	0.12	0.34	0.92	2.38	0.34	0.72	1.23
Caryophyllene oxide	1582	1590	0.26^{c}	1.62^{b}	2.11^{ab}	2.75^{a}	2.72^{a}	2.25^{a}	2.78^{a}
(2R,5E)-Caryophyll-5-en-12-al	1638[3]	1606	0.14	1.46	1.94	5.51	1.82	1.18	1.89
10-epi-γ-Eudesmol	1622	1623	-	-	-	-	0.04^{b}	0.93^{a}	1.15^{a}
Caryophylla-4(12),8(13)-dien-5-beta-ol	1639	1643	0.05	0.28	0.20	0.73	0.45	0.42	1.51
Selin-11-en-4-α-ol	1658	1661	0.28	0.46	**3.10**	1.16	0.25	**2.07**	0.82
epi-α-Bisabolol	1683	1690	0.03	0.09	0.15	0.53	0.12	0.14	1.20
α-Bisabolol	1685	1686	0.06	0.23	0.59	1.66	0.26	0.50	1.09
Geranyl-p-cymene	1946[3]	1939	-	-	-	-	-	1.83	1.46
Unknown 1		1978	-	-	-	0.99	-	2.91	1.93
(Z)-Nuciferol isobutyrate	1997[3]	1981	-	-	1.47	1.94	1.06	1.11	2.79
(E)-Nuciferol isobutyrate	2004[3]	1983	-	1.20	2.04	-	1.30	3.86	2.15
Unknown 2		2146	-	-	2.02	-	1.02	1.47	-
Total monoterpenes (%)			94.82	79.79	64.07	55.58	62.75	51.17	46.11
Total sesquiterpenes (%)			2.61	13.17	25.51	29.82	23.74	33.82	34.00
Total identified percentage			97.42	92.95	89.58	85.40	86.49	84.99	80.11

V: vegetative; FB: floral budding; F: flowering; AF: after flowering

[1] Components reaching 1% of GC area are listed

[2] Linear retention indices according to the literature Adams (2007)

[3] Linear retention indices from the literature, Sharopov et al. (2012) on the HP-5MS column

[4] Linear retention indices calculated relative to the elution ranking of n-alkanes (C_9-C_{20}) on the HP-5MS column

Letters in rows represent significant difference between phenological phases of each organ according to the Games-Howell test at p=0,05.

During plant development, the majority of observed compositional changes are similar both for leaf and flower oils (Table 19). The main component, *trans*-sabinyl acetate, showed a characteristic decreasing tendency with significant changes during developmental phases, except the first (vegetative) phases of the leaves. The same tendency could be observed also in the proportions of the related compound, *trans*-sabinol, but this is not the case for sabinene, whose ratio is increasing.

The ratio of the thujones fluctuates only moderately. However, a sharp and significant increase of ß-thujone at the last phenophase (after flowering) can be observed compared with former phases. This phenomenon is, however, not detectable for the isomer α-thujone, the ratio of which dropped at the end of vegetation.

As for the other compounds, a decreasing tendency was detected in the ratios of neryl-isovalerate and ß-selinene during the plant development. On the other hand, the proportion of several components, like lavandulol- esthers (lavandulyl-isovalerate, lavandulyl-2-methyl-butanoate), β- caryophyllene and its derivatives (caryophyllene oxide, caryophylla-4(12), 8(13)-dien-5-beta-ol), showed increasing ratios during the development both in leaves and flower parts. Similarly, the characteristic compound of the flowers, 10-epi-γ-eudesmol displayed a growing ratio during the flowering phase.

Monoterpenes were found in higher abundance (46.1% - 94.8%) than sesquiterpenes (2.6% - 34.0%) both in leaf and flower oils. The percentage of sesquiterpene compounds increased from the vegetative stage to the subsequent developmental phases, reaching the highest area percentages at post-flowering stage, found both in leaves (29.8%) and flowers (34.0%).

Based on the data above it can be established that the accumulation level of volatile compounds in the two examined chemotypes of wormwood shows the same organic and developmental characteristics as many other EO accumulating species according to the literature references. The flowers contain higher ratios of volatiles than the leaves do, and the content decreases during the flowering time. Differences between the thujone and *trans*-sabinyl acetate chemotypes were found only in the magnitude of the values.

The ratio of monoterpene compounds was higher than that of the sesquiterpenes in both organs, and in each growth stage with slight quantitative differences between chemotypes.

The qualitative composition of the flowers and leaves is largely similar, with the exception of one and five compounds in the cases of thujone and *trans*-sabinyl acetate chemotypes, respectively. The compositional changes are mostly quantitative for both chemotypes. However, the dynamics and tendency of these changes are different in the two wormwood chemotypes, except the

tendencies of linalool, the lavandulol esthers and caryophyllene oxide. Variations in the ratios of the main components are lower in the thujone type; therefore, the chemotype can be identified from any samples independent of organic composition and harvest time, while this is not the case for the *trans*-sabinyl-acetate chemotype.

4.2.2. Environmental factors

4.2.2.1 Essential oil yield and composition

Evaluating the phytotron experiment and the samples of the studied two chemotypes it is obvious that the essential oil yield varied from 0.188 ml/100mg (Hungarian accession, "warm" chamber) to 1.092 ml/100mg (Spanish accession, "cold" chamber) (Fig 6).

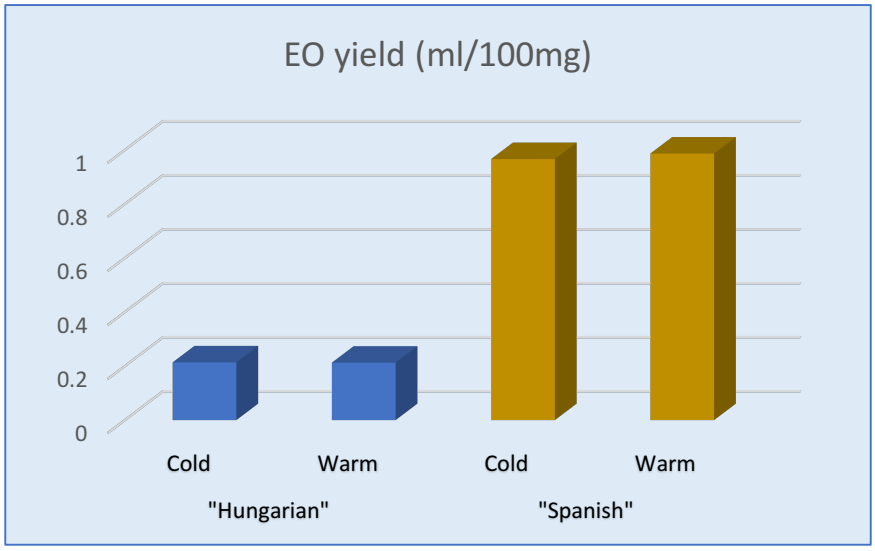

Fig 6. The effect of climatic conditions on essential oil yields of wormwood from two accessions

According to the two-way ANOVA test of between-subjects effects for this trait, significant differences could be detected among the accessions: $F_{(1;10)}=233.681$ and $p<0.001$; however, weather programs had no effect on EO yield of any of the accessions ($F_{(1;10)}=0.036$; $p=0.854$). Similar to the findings here, in our investigation of the EO yield of the Spanish accession (results shown in chapter 4.1.3.1) was 6 times higher than that of the Hungarian accession (HuW1) from Csór (Nguyen et al., 2017). Numerous references have indicated that the EO content of *A. absinthium* L. shows a large variability depending on the origin of the sample (EO yield of plant materials from different European regions fluctuated between 0.1% - 1.1% DW (Orav et al.,

2006). Tunisian wormwood gave relatively high yields: 1.10% - 1.46% (Msaada et al., 2015). A study by Riahi et al. (2015) indicated that the oil content of wormwood increased from 1.24% (arid region) to 2.22% (humid region).

The influence of climatic factors such as temperature, light intensity, air humidity, etc. on accumulation of volatile compounds in wormwood has not been adequately documented untill now. Similar to our findings, Kindlovits et al. (2014) reported that the EO content of yarrow (*Achillea collina* Becker) was not affected by temperature and light intensity; however, hyssop (*Hyssopus officinalis* L.) in the "hot" environment produced 150% higher EO content (1.22 ml/100g) than it did in the "cold" climatic chamber.

Fig 7. Hungarian accession showing morphological differences after 3 months' treatment. On the left side: "warm" treatment, on the right side: "cold" treatment

Fig 8. Spanish accession showing morphological differences after 3 months' treatment. On the left side: "warm" treatment, on the right side: "cold" treatment

The total identified percentage of components varied from 76.3% (Hungarian accession in "cold" treatment) to 87.5% (Spanish accession in "cold" treatment) (Table 20). During evaluation of the components higher than 1% of GC area, 33 compounds were identified, among which, in each treatment, sesquiterpenes were found in a higher abundance (61% - 70%) than monoterpenes (30% - 39%). Major components of the oils were sabinene (0% - 10.8%), ß-myrcene (1.7% - 16.5%), *cis*-epoxy-ocimene (1.2% - 57.7%), *cis*-chrysanthenyl acetate (0% - 13.8%) and (Z) nuciferol-isobutyrate (1.7% - 10%).

Table 20. The effect of climatic conditions on chemical composition of wormwood essential oils (GC area percentages)

Accessions			**Spanish**		**Hungarian**	
Treatment			**Cold treatment**	**Warm treatment**	**Cold treatment**	**Warm treatment**
Compound[1]	LRI[2]	LRI[4]				
Sabinene	969	976			**2.3**	**10.8**
ß-Myrcene	988	995	**2.2**	**1.7**	**8**	**16.5**
α-Phellandrene	1002	1009			3	7
p-Cymene	1020	1026			1.7	3.9
Linalool	1100	1097			1.5	2.8
ß-Thujone	1112	1113			**3.3**	**0**
***cis*-Epoxyocimene**	1137	1130	**57.7**	**54.1**	**6.8**	**1.2**
Camphor	1141	1144	3.5	2.8		
Terpinene-4-ol	1174	1175				1
***cis*-Chrysanthenyl acetate**	1261	1254	**8**	**13.8**	**6.6**	**0**
Bornyl acetate	1284	1284			1.1	
β-Caryophyllene	1417	1420	1.6		1.4	2.7
Germacrene D	1484	1482	2	1.3	2.6	5
α-Curcumene	1479	1483			2.1	4.9
Neryl-isobutanoate	1490	1492			5.5	4.3
Lavandulyl-isovalerate	1509	1512				1.6
Geranyl-isobutanoate	1514	1516			1.1	1.2
Neryl-isovalerate	1582	1584			4.9	4.1
Caryophyllene oxide	1582	1590	1.1		5.3	4.2
(2R,5E)-Caryophyll-5-en-12-al	1638[3]	1606			1.9	2.1
Selin-11-en-4-α-ol	1658	1661	1.5	1.5	3	2.2
Chamazulene	1730	1733	5	4.6		
Geranyl-p-cymene	1946[3]	1939	1.3	1.4	2.1	
Unknown 1		1978	3.4	1.5	2.9	1
Unknown 2		1980		2.2	6.9	1.8
***(Z)*-Nuciferol isobutyrate**	**1997[3]**	**1981**	**5.8**	**1.7**	**9.3**	**10**
(E)-Nuciferol isobutyrate	2004[3]	1983	2.5		1.2	
Unknown 3		2146	3.3		2.7	2
Total identified percentage:			85.8	85.3	76.3	87.59
Unknown presented			7.7	3.8	12.5	4.8

[1] Components reaching 1% of GC area are listed
[2] Linear retention indices according to the literature Adams (2007)
[3] Linear retention indices from the literature, Sharopov et al. (2012) on the HP-5MS column
[4] Linear retention indices calculated relative to the elution ranking of n-alkanes (C_9-C_{20}) on the HP-5MS column

In this experiment we could establish the formerly identified chemical differences between the two accessions (Chapter 4.1.3.2). The different climatic conditions created in the two chambers have markedly influenced the EO composition. However, the changes are mostly quantitative. Thus, the percentage of *cis*-chrysanthenyl acetate rose from 8.0% ("cold" chamber) to 13.8% ("warm" chamber) in the Spanish plants, while sabinene increased from 2.3% to 10.8% and β- myrcene rose from 8.0% to 16.5% in the "warm" and "cold" chambers, respectively, in the Hungarian samples. Interestingly, the main compound of the Spanish accession, *cis*- epoxy- ocimene, showed only a slight change (decrease from 57.7% to 54.1%) due to the "warm" weather condition.

Considerable (at least 50%) changes were registered also for some minor compounds. Specifically, the ratio of sesquiterpenes decreased, e.g. that of caryophyllene, nuciferol esthers, unknown 1 and 3 in the Spanish accession as well as the ratios of unknown components 1 and 2 in the Hungarian accession. In this latter chemotype the proportions of ß-thujone, *cis*-epoxy-ocimene and *cis*-chrysanthenyl acetate also dropped with an increase parallel to some minor monoterpene components.

4.2.2.2 Total phenolic content and antioxidant capacity

TPC of Spanish plants growing in the "warm" chamber reached the highest value (40.339 mg GAE/g DW) while the Hungarian accession growing in the "cold" environment showed the lowest TPC with 25.982 mg GAE/g DW (Table 21). As a mean, the Spanish accession showed significantly higher TPC than the other one in the "warm" chamber ($F_{(1,16)}$=377.923, $p<0.001$), but no significant difference was found between the two accessions in the "cold" chamber ($F_{(1,16)}$=2.629, $p=0.124$). The direction of changes due to the variable environmental conditions was similar for both accessions. However, while the increase was a significant one ($F_{(1,16)}$=111.592, $p<0.001$) in the Spanish samples (from 28.0351 mg GAE/g DW in the "cold" chamber to 40.339 mg GAE/g DW in the "warm" chamber) , only a 6% elevation was measured ($F_{(1,16)}$=3.559, $p=0.077$) in the Hungarian accession.

The highest AC value was detected in Spanish samples in the "warm" chamber (24.7673 mg AAE/g DW), while the lowest one was found in Hungarian samples in the "cold" chamber (13.251 mg AAE/g DW). Differences between the two accessions were statistically justified only under the "warm" circumstances ($F_{(1,16)}$=126.951, $p<0.001$), similar to the results of the TPC. As for the effect of weather, a significant increase of AC was detected in the "warm" chamber for both accessions ($F_{(1,16)}$=18.701, $p<0.01$ in the case of the Hungarian one and $F_{(1,16)}$=68.457, $p<0.001$ for the Spanish one.)

Table 21. The effect of climatic conditions on total phenolic content and antioxidant capacity of wormwood accessions

Accession	Treatment	TPC (mg GAE/g DW)		AC (mg AAE/g DW)	
		Mean	SD	Mean	SD
Hungarian	Warm	27.538^{Ab}	1.613	15.578^{Ab}	0.985
	Cold	25.982^{Ab}	1.878	13.251^{Ba}	1.279
Spanish	Warm	**40.339^{Aa}**	1.141	**24.767^{Aa}**	2.240
	Cold	**28.035^{Bb}**	3.303	**14.013^{Ba}**	3.192

Capital letters in the columns show the significant differences of climatic treatment for each accession. Lower case letters in the columns show the significant differences of accessions for each climatic treatment, $p<0.05\%$.

According to the the Pearson product-moment correlation coefficient between TPC and AC, there was a strong correlation ($r=0.96$, $p<0,05$) between total phenolic content and antioxidant activity of wormwood samples from both accessions. Thus, the findings of several other studies (Canadanovic-Brunet et al., 2005; Riahi et al., 2013) on other species have been ascertained. No previous data are available; however, regarding the effect of weather conditions on the accumulation of TPC and the AC of wormwood. Although phenolic compounds are usually considered as defense molecules and their accumulation is frequently stimulated by stress conditions (e.g. Msaada et al., 2015), this is not obvious in the case of *A. absinthium*, and it would require further investigation. In any case, it could be demonstrated that the TPC and FRAP AC of the wormwood leaf extracts highly depend on the weather conditions, as a significant increase under the "warm" conditions was demonstrated.

4.3. Optimization of wormwood cultivation

4.3.1. Germination

Duration and temperature of storage had significant effects on seed germination capacity and mean germination time of both investigated accessions (Table 22). The initial germination

capacity before storage was very high, both in case of the Hungarian origin (98.00%) and the Spanish accession (98.66%).

Table 22. Effect of storage temperature and storage period on seed germination and mean germination time of the two wormwood accessions

Accession	**Hungarian**				**Spanish**			
Treatment	**4°C**		**Room**		**4°C**		**Room**	
Date	**Germination capacity (%)**	**Time (days)**	**Germination capacity (%)**	**Time (days)**	**Germination capacity (%)**	**Time (days)**	**Germination capacity (%)**	**Time (days)**
18.10.2015	98.00^{c}	4.67^{a}	98.00^{b}	4.67^{a}	98.66^{c}	4.33^{a}	98.66^{c}	4.33^{a}
19.11.2015	98.00^{c}	4.67^{a}	93.34^{b}	5.33^{ab}	98.66^{c}	4.33^{a}	94.00^{bc}	4.67^{ab}
20.2.2016	97.34^{c}	5.00^{a}	97.34^{b}	4.33^{a}	98.66^{c}	4.33^{a}	96.66^{c}	4.33^{a}
22.5.2016	90.66^{bc}	4.67^{a}	92.00^{b}	5.33^{ab}	88.00^{bc}	4.33^{a}	90.66^{abc}	4.33^{a}
21.8.2016	84.66^{ab}	5.00^{a}	79.34^{a}	5.67^{ab}	78.66^{ab}	5.67^{a}	82.00^{abc}	5.00^{b}
19.11.2016	82.66^{ab}	5.33^{a}	78.00^{a}	5.00^{ab}	78.00^{ab}	5.00^{a}	82.00^{abc}	5.67^{b}
19.2.2017	82.00^{a}	5.00^{a}	76.66^{a}	6.00^{ab}	75.34^{ab}	4.33^{a}	80.66^{abc}	5.67^{b}
20.5.2017	78.00^{a}	5.33^{a}	76.66^{a}	6.33^{b}	74.66^{a}	5.00^{a}	80.00^{abc}	6.00^{b}
21.8.2017	80.00^{a}	5.00^{a}	76.00^{a}	5.33^{ab}	74.00^{a}	5.00^{a}	78.00^{ab}	5.67^{b}
19.12.2017	78.66^{a}	6.00^{a}	74.66^{a}	6.33^{b}	72.66^{a}	5.33^{a}	78.00^{ab}	5.67^{b}
20.3.2018	76.66^{a}	6.00^{a}	74.00^{a}	6.33^{b}	70.66^{a}	5.33^{a}	75.34^{a}	5.67^{b}

Different letters in columns represent significant differences between duration of seed storage to the Games-Howell test at p=0.05.

When stored at 4°C the germination capacity of the Hungarian wormwood seeds decreased significantly ($F_{(9,30)}$=22.97; p<0.001) from 98.00% (initial value) to 82.66% (after 1 year of storage) and 76.66% (at the last test after storage for 29 months). Mean germination time increased slightly during storage and ranged from 4.67 days (initial test) to 6 days (during the last test). However, the duration of storage had no significant effect on germination time ($F_{(9,30)}$=0.82; p = 0.607). In the same storage conditions, Spanish seeds reached the highest germination capacity (98.66%) at the first test and the lowest germination rate (70.66%) after 29 months followed by 78% (after 1 year of storage) and 72.66% (after 2 years of storage). It is a significant reduction ($F_{(9,30)}$=16.64; p<0.001) with the increasing storage duration, similar to the other

accession. Already after a year a decrease of 10% germination capacity was recorded. At the same time a slight elongation of the germination time - but statistically no significant difference - during storage duration could be detected ($F_{(9,30)}$=1.74; p = 0.144)..

Storage at room temperature resulted in 92%, 78% and 74% seed germination after 6 months, 1 year and 29 months of storage respectively, which was about a 20% decrease after 1 year compared with the first test in the case of Hungarian seeds. It represents a significant reduction after storage in total 29 months ($F_{(9,30)}$= 15.28; p <0.001). The mean germination time was also significantly influenced by storage duration ($F_{(9,30)}$=2.83; p <0.05) and showed an elongation from 5.33 days (at the initial test) to 6.33 days (after 29 months storage). Similar results were observed in the case of Spanish seeds, as well. Significant ($F_{(9,30)}$=4.81; p <0.001) reduction in germination capacity with about 12% after 1 year of storage and 20% decrease after two year to 29 months. In parallel with this, a significant ($F_{(9,30)}$=3.19; p <0.05) increase in mean germination time with one day increase were found during increasing storage duration.

Cumulatively for both populations, seeds stored at 4°C and room conditions showed no difference in term of germination percentage and mean germination time at the same period of storage. For both accession, the strongest decrease of germination capacity was recorded in the first 10 months storage followed by a smaller reduction after that independently from the storage temperature (Figure 9).

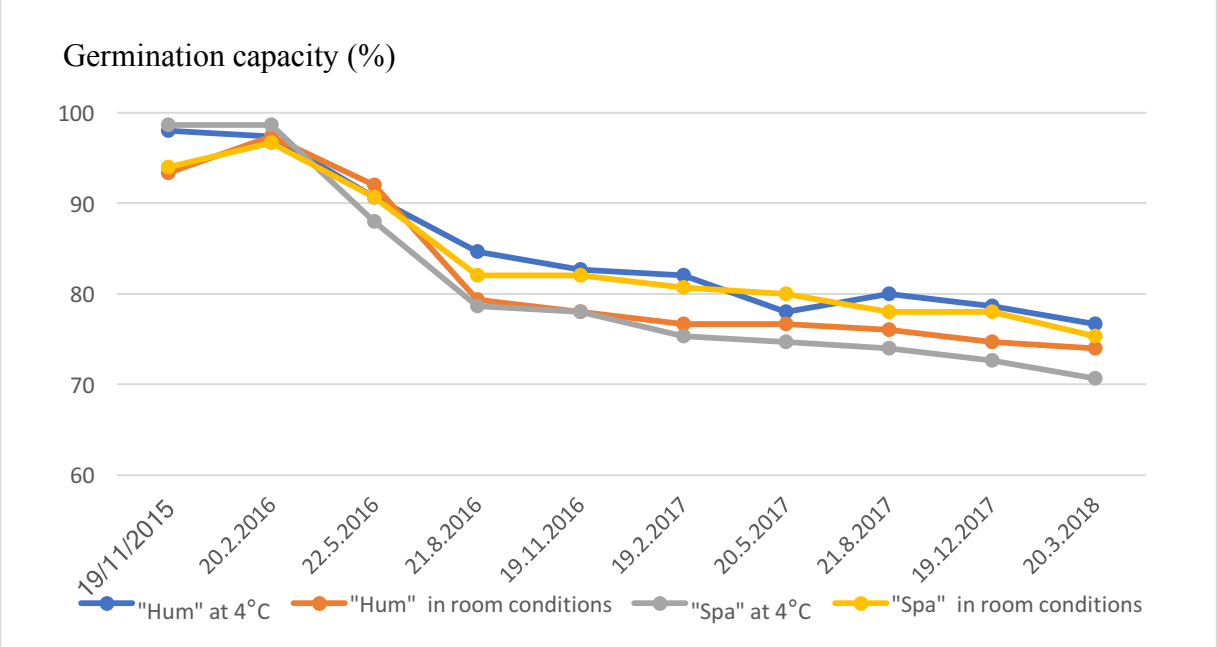

Fig 9. Effect of storage condition and storage period on germination capacity of two wormwood accessions “Hum” and “Spa”

4.3.2. Study on vegetative propagation

Applying the cutting method, the ratio of living plants was relatively high in both treatments with only a slight advantage of the hormone application: control (85%), IBA (90%). However, the development of the young plantlets has definitely been stimulated by the IBA application. According to one-way ANOVA tests of between-subjects effects, significant differences could be detected among the treatments in the root length ($F_{(1;18)} = 303.53$; $p<0.001$) and the number of roots ($F_{(1,18)} = 77.79$; $p<0.001$) (Table 23).

Table 23. Results of the cutting propagation of two *A. absinthium* accessions "Spa" and "Hum"

Treatment	Root length (cm)		Nr. of roots/plant	
	Mean	Std. D	Mean	Std. D
Control	6.30[a]	1.16	2.60[a]	0.97
IBA	15.70[b]	1.25	7.00[b]	1.25
Total	11.00		4.80	

Different letters in columns represent significant differences between treatments according to the Games-Howell test at p=0.05.

The root length of cuttings varied from 6.30 cm (control treatment) to 15.70 cm (IBA treatment). The mean of root number at control treatment was 2.6 pcs/plant while it was 7.0 pcs/plant in the IBA treatment. These difference may be of benefit in large scale propagation or in case of valuable genotypes.

The results of the layering experiment also demonstrated that this method may provide vigorous new plantlets. According to ANOVA Tests of between-subjects effects, there have been significant differences detected between the investigated Hungarian and Spanish accessions in case of all of the measured parameters: $F_{no.plant(1;8)}=20.513$ ($p<0.01$); $F_{plantheight(1;12)}=409.77$ ($p<0.001$); $F_{rootlength(1;12)}=230.342$ ($p<0.001$). The results are summarized in Table 24.

Table 24. Results of the layering propagation of two *A. absinthium* accessions "Spa" and "Hum"

	Height (cm)		Rootlength (cm)		Number of plants	
Accession	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Hungarian	49.43[b]	1.17	23.94[b]	1.17	5.8[b]	1.48
Spanish	24.04[a]	1.11	14.70[a]	1.11	1.8[a]	1.30
Total	36.74		19.32		3.8	

Different letters in columns represent significant differences between accessions according to the Games-Howell test at p=0,05.

The Hungarian accession produced a significantly higher number of new plants (5.8 plantlets/mother plant) compared to the Spanish accession (1.8 plantlets/mother plant). Plant height varied from 24.04 cm (Spanish accession) to 49.43 cm (Hungarian accession), thus, the young Spanish plants were shorter compared with the Hungarian ones. The mean length of the developed new roots during layering was 23.94 cm in the Hungarian accession and significantly shorter in the Spanish one (14.70 cm).

The results showed that a long layering of perennial mother plants might be an effective method of propagating wormwood on a smaller scale. Nevertheless, the actual efficacy seems to be dependent on the accession, too.

4.3.3. Study on allelopathic activity of wormwood

4.3.3.1 Treatment with different types of wormwood materials

Our experiments showed that wormwood may exhibit a definite allelopathic effect on the germination and early development of other species but the results depended on many factors. The results of the effects of fresh and dry leaf powder and aqueous extracts of leaves on the germination and early growth of lettuce and basil are summarized in Table 25.

Table 25. Effect of different type of *A. absinthium* materials from Hungarian accession on germination and seedlings of lettuce and mustard

Treatment	Species	Height (cm)	Nr. of leaves (pc/seedling)	Root length (cm)	Nr. of germinated seeds (pcs)
control	Lettuce	5.65[a]	1.87 [a]	3.37 [a]	42.67[c]
fresh leaf powder		4.80[b]	1.80 [a]	2.87 [a]	35.67[b]
dry leaf powder		3.80[b]	1.40 [a]	2.96 [a]	25.33[a]
water extract		4.29[b]	1.53 [a]	3.20 [a]	29.67[a]
control	Mustard	10.49[a]	3.53 [a]	3.30 [a]	47.33[b]
fresh leaf powder		9.52[b]	3.60 [a]	3.38 [a]	45.00[b]
dry leaf powder		7.51[c]	3.33 [a]	3.23 [a]	34.33[a]
water extract		9.41[b]	3.20 [a]	3.37 [a]	43.33[b]

Different letters in columns represent significant differences between treatments according to the Games-Howell test at p=0.05.

In the case of lettuce, both the number of germinated seeds ($F_{(3,8)}$=39.231, p<0.001) and height of seedlings ($F_{(3,1)}$=10.443, p<0.001) were significantly reduced in each treatment compared to the control. Among the treatments, the only one difference was found in the number of germinated seeds where the treatment with fresh leaves had lower inhibition effect compared to the other treatments. We may observe that the lowest survival rate (germinated seeds) and the smallest size of seedlings were found in the dry leaf treatment (40.64% and 32.74% inhibition compared to the control, respectively). However, the treatments had no significant effects on the number of leaves ($F_{(3,1)}$=3.554; p=0.06) and the length of roots ($F_{(3,1)}$=3.592, p=0.057).

Similar to lettuce, in mustard significant effects were found in the case of the number of germinated seeds ($F_{(3,8)}$=14.37, p<0.01) and that of plant height ($F_{(3,1)}$=21.827, p<0.001). Plant height varied from 7.51 cm (powder treatment) to 10.49 cm (control treatment) which showed a 28.41% inhibition. No significant difference was detected for the number of leaves ($F_{(3,1)}$=1.879, p=0.431) and the length of roots ($F_{(3,1)}$ = 1.879, p=0.431).

Based on the result we concluded that powdered, dry leaves of wormwood mixed into the soil had the strongest inhibitory effect on both species manifesting primarily in lower ratio of germinated seeds and plant height.

4.3.3.2 Treatments with wormwood leaf powder

Based on the our results, we intended to clear up the effect of the intraspecific taxon and the concentration of dry leaf powder in the soil on germination and plant development.

A significant difference among the treatments was only found in the case of the number of germinated seeds and for mustard in the height of the seedlings (Table 26).

Table 26. Effect of wormwood powder from two Hungarian and Spanish accessions on germination and seedlings of three test species

Treatment	Species	Height (cm)	Root length (cm)	Number of leaves (psc)	Germination time (days)	Nr. germinated seeds (pcs)
Control	Basil	12.95^{a}	3.23^{a}	4.00^{a}	6.27^{a}	43.67^{d}
50Spa		7.27^{a}	2.95^{a}	3.33^{a}	6.27^{a}	32.33^{bc}
100Spa		7.02^{a}	2.97^{a}	3.73^{a}	6.53^{a}	26.67^{a}
50Hu		7.95^{a}	3.06^{a}	3.87^{a}	6.27^{a}	36.33^{c}
100Hu		8.13^{a}	3.18^{a}	3.87^{a}	6.53^{a}	28.33^{ab}
Control	Mustard	19.97^{c}	3.47^{a}	4.00^{a}	7.40^{a}	46.67^{c}
50Spa		14.30^{a}	2.90^{a}	4.00^{a}	7.40^{a}	36.67^{b}
100Spa		14.50^{a}	2.75^{a}	4.00^{a}	8.00^{a}	31.00^{a}
50Hu		17.77^{bc}	3.31^{a}	4.00^{a}	7.20^{a}	36.67^{b}
100Hu		15.30^{ab}	2.94^{a}	4.00^{a}	7.40^{a}	32.33^{ab}
Control	Lettuce	8.88	4.24	3.40	6.80	40.00
50Spa		-	-	-	-	-
100Spa		-	-	-	-	-
50Hu		6.58	3.47	2.33	6.50	15.00
100Hu		-	-	-	-	-

Different letters in column represent significant differences between treatments according to the Games-Howell test at p=0,05.

In each combination we found a lower number of successfully germinated seeds when the higher dosage of the wormwood was applied. Based on this, it may be anticipated that a further increase of dosage would lead an (almost) total inhibition on germination for each of the three test species. Plant height was reduced by 39.6% as the largest effect (in mustard, at the "50Spa" treatment).
It could also be established that the intraspecific variability of *A. absinthium* is reflected also in the tested allelopathic effect. Between the tested accessions the Spanish one showed stronger inhibition on germination compared to the Hungarian one. This result could be demonstrated in three tested species in both doses, especially in the case of lettuce at a dosage of 50g. In basil and mustard the height of seedlings treated with materials of the Hungarian accession was by 15.8% (in basil at 50g dosage) and by 24.3% (in mustard at 100g dosage) higher than in the case of plants treated by the material of the Spanish genotype.
Concerning the root-length, number of leaves and germination time of the test species no significant differences were established either between the different intraspecific taxa or between the applied dosages.
As for the individual test species, basil was statistically justified with $F_{(4,15)}= 35.492$, $p<0.001$ for the number of germinated plants and the values were highest in the control one. In the case of mustard, the plant height varied from 14.30 cm (in the "50Spa" treatment) to 19.97 cm (in the "control" treatment). For this characteristic, the difference among the treatments was significant ($F_{(4,70)}=18.877$, $p<0.001$). Similarly, there was a significant difference found for the number of germinated seeds ($F_{(4,15)}= 42.438$, $p<0.001$), too. In comparison, lettuce was the most sensitive species when applying wormwood powder into the soil. No lettuce plants could survive in the treatments 50Spa, 100Spa and 100Hu. Only 15 plants could develop in the "50Hu" treatment with 6.58 cm in height and germination a time of 6.5 days while lettuce could grow in the "control" treatment with 40 plants survived as mean.

4.3.3.3 Treatment with aqueous extracts of wormwood

Different concentrations of leaf extract had significant effects on number of germinated seeds and the rooot length of the test species but no significant effects on mean germination time in the case of both chemotypes of wormwood. The results are summarized in Table 27.

Table 27. Effects of aqueous extracts obtained from two different wormwood chemotypes (thujone chemotype "Bel" and non-thujone chemotype "Hu") on germination of the test species

Treatment	Species	Germination time (days)	Nr. germinated seeds (pcs)	Root length (cm)
Control	Basil	5.67^{a}	39.67^{f}	2.04^{f}
Non-thujone 0.1 mg/ml		7.33^{a}	17.33^{bc}	1.28^{e}
Non-thujone 0.2 mg/ml		6.67^{a}	15.67^{b}	0.85^{d}
Non-thujone 0.3 mg/ml		6.67^{a}	10.33^{a}	0.52^{d}
Non-thujone 0.4 mg/ml		6.67^{a}	7.00^{a}	0.33^{abc}
Thujone 0.1 mg/ml		6.67^{a}	26.33^{e}	0.85^{d}
Thujone 0.2 mg/ml		7.33^{a}	21.67^{d}	0.43^{bc}
Thujone 0.3 mg/ml		6.67^{a}	20.00^{de}	0.24^{ab}
Thujone 0.4 mg/ml		6.00^{a}	10.33^{a}	0.17^{a}
Total		6.63	18.70	0.77
Control	Lettuce	5.33	38.33	2.90
Non-thujone 0.1 mg/ml		6.33	11.33	1.24
Non-thujone 0.2 mg/ml		6.00	6.33	0.57
Non-thujone 0.3 mg/ml		-	-	-
Non-thujone 0.4 mg/ml		-	-	-
Thujone 0.1 mg/ml		5.67	16.67	2.74
Thujone 0.2 mg/ml		6.00	12.67	1.73
Thujone 0.3 mg/ml		5.67	11.33	1.37
Thujone 0.4 mg/ml		-	-	-
Total		3.89	12.08	2.38

Different letters in columns (basil species) represent significant differences between accesions according to the Games-Howell test at p=0,05.

Lettuce plants were died mostly so that we could not run statistical analysis in the case of lettuce

No germination of lettuce seeds was observed when using the plant extracts at concentrations higher than 0.3 mg/ml (non-thujone chemotype) and at the highest concentration of 0.4 mg/ml (thujone chemotype) while the highest number of germinated seeds (38.33 seeds) was detected in the control treatment (only distilled water). Significant differences between extracts

concentrations was observed in basil with $F_{(8;18)} = 155.053$; $p<0.001$. It ranged from a lowest number of 7.00 germinated seeds (non-thujone chemotype in concentration of 0.4 mg/ml) to the highest number of 39.67 germinated seeds (control). The largest inhibition (0.4 mg/ml from non-thujone chemotype) reduced the number of germinated seeds by 82.35%.). Different extract concentrations had significant influence also on the root length of basil ($F_{(8,75)} = 177.872$; $p <0.001$). The shortest root (0.17 cm) was found when using the concentration of 0.4 mg/ml extract from thujone chemotype.

Among the chemotypes of wormwood, the non-thujone chemotype showed stronger inhibition of germination: at a concentration of 0.3 mg/ml no seeds could germinate while 22.7% germination capacity was detected when using plant extracts of the thujone chemotype. In the case of basil, there was a two times higher rate (40.0%) of germination with 0.3 mg/ml extract from the thujone chemotype compared with the non-thujone one (20.7%).

Investigating the two test species, lettuce was more sensitive to the wormwood extracts compared to basil.

As a summary it can be established that aqueous extracts of both wormwood chemotypes inhibited the germination of basil and lettuce. However, our research was focusing on the early stage of seedlings growth. New experiments seem to be worthwhile to further clarify the allelopathic effect (plant material, chemotype, treatment, duration, etc.) of wormwood and the possibilities of regulating and optimising it, in order to establish an effective, ecologically friendly agricultural technology of *A. absinthium*.

CHAPTER 5. NEW SCIENTIFIC RESULTS

1. For the identification of morphological diversity of *A. absinthium* species qualitative and quantitative traits of the 12 accessions were analyzed. Based on the result the qualitative traits (leaf and stem color, leaf form) as well as quantitative traits (thickness of blade, length of leaf and petiole) are suitable for morphological distinction. The method which evaluates parallel both qualitative and quantitative traits, will be suitable in the future for characterization of different gene bank accessions and endogenous populations, as well.
2. For the first time we introduced the application of molecular markers into the genetical distinction of *A. absinthium* accessions. It was proved that using 11 RAPD and 15 ISSR primers the distinction of the accessions is possible. In the case of RAPD primers B10, while in the case of ISSR primers Cag5 and Issr5 gave the highest number of bands. The method in the future can be generalized for the other gene bank and endogenous populations.
3. Based on the essential oil analysis of the *A. absinthium* accessions the chemical diversity of species shows much more variability than was expected from the literature references. We proved that the accumulation of thujone is not an overall phenomenon. In 3 accessions, the biosynthetic pathway leading to the thujone formation is absolutely lacking. In 4 accessions, only traces of thujone accumulates. We proved the presence of three different chemotaxa: 4 accession show only a thujone character, while 1 accession accumulates mainly *trans*-sabinyl acetate and 1 accession with selin-11-en-alpha-ol. The other accessions can be characterized by the presence of different compounds characterized as "mixd chemotype". The chemical characterization of "mixed chemotypes" need further investigations.
4. We proved that the essential oil composition of the different chemotaxa changes during the ontogenesis as well as the effect of environmental conditions. However, the amplitude of changes is smaller as it could have modified the chemotaxonomical ranking.
5. For the future introduction of *A. absinthium* in to the agrarian-system the different aspects of optimal propagation method (germination capacity, gene bang storage, vegetative propagation etc) have been analyzed. Both generative and vegetative propagation may be sufficient. However, the advantage of vegetative propagation is that it may contribute to the chemical homogeneity of the cultivated stand.

6. Based on our results remarkable allelopathic activity of different products made of *A. absinthium* (fresh- and dry leaf powder, aqueous extract) has been proved. In some treatments (for instance in the lettuce germination test) total inhibition of germination was measured. This results may help us in the construction of a chemical free cultivation method, or methods with restricted amount of chemicals.

CHAPTER 6. CONCLUSIONS

6.1 Intraspecific variability of wormwood

Our experiments demonstrated the large intraspecific variability of wormwood (*Artemisia absinthium* L.).

In the same growing habitat, under the conditions of our experiment, characteristic differences in growth, width of the bushes, size and form of the leaves were registered.

Accessions can be well distinguished by the leaf color and form while stem color, growth characteristics and branching type are less adequate for identification of them, presumably also due to the large intra-population variability of these traits. Differences in leaf shape of the same individual contribute to the heterogeneity of populations and present additional difficulties in evaluation. There are no similar studies found in literatures, thus, our data represent new findings.

In the 12 experimental populations morphological measurements of the first-year-old plants revealed that the accession "Nor" presented the shortest shoots (18.7 cm height) and smallest bushes (29.9 cm in diameter), on the other side "Eng" demonstrated the highest vegetative growth (47.5 cm height) and "HuW2" was recorded with the largest bushes (64.9 cm diameter). Accessions "Spa" and "Bel" developed the thickest leaves (0.47 mm and 0.48 mm, respectively), "Ger1" and "Hum" provided the largest leaves while "Ger2" presented the thinnest leaf blade (0.30 mm) and the smallest leaves (104.4 cm). The coefficient of variation for the morphological traits ranged from 10% to 45% and was the highest value in the case of the length of petiole.

It would be difficult to find any connection with the geographical origin of the accessions based either on the qualitative or the quantitative morphological characteristics of the leaves or their homogeneity. Similarly, there does not seem to exist any connection between the manifestation of the evaluated traits and whether the accessions were collected from market or wild growing habitats.

Our findings for the biomass production of the first-year-old plants as a considerable intra-population variability was also observed. The biomass of the twelve studied accessions varied from 63.4 g ("Nor") to 322.4 g ("HuW2"). The highest yield (accession "HuW2") exhibited a large intra-population heterogeneity (CV=71%) while the lowest variability was found in the accession "Eng" (CV=24%).

It can be established that our data are based on a systematic evaluation of twelve wormwood accessions and therefore are much more comprehensive than any of the formerly published ones (e.g. Konowalik and Kreitschitz, 2012; Nazar and Mahmood, 2011). Moreover, our investigations were achieved under the same ecological conditions and the populations maintained by the same

agricultural methods, thus the described differences may reflect the intraspecific genetic manifestation of *A. absinthium* in all respects.

We determined a strong correlation between the height and the width of the bushes (r=0.87); the height and the biomass (r=0.85), followed by the width and the biomass as well with r=0.88, while between the biomass and the blade length we found a weak correlation (r=0.46). It means that the yield of wormwood could be predicted based on the appearance of height and width, but not on the basis of the leaf parameters.

The large intraspecific variability of wormwood has been demonstrated also in the accumulation of the volatile compounds.

Marginal values for the yield (content) of the essential oil were 0.349 and 3.215 ml/100g. additionally, intra-accession differences were also considerable, even higher than in the case of the morphological traits: CV for essential oil yield ranged between 18% and 64%. The three groups of statistical analysis representing significantly different levels of oil yield (Table 13) do not seem to be in connection with the geographical origin of the accession. The genotypes showing significantly similar values such as e.g. “Nor” and “Bel” are both genebank accessions presumably of wild origin, thus in no obvious relationship with each other. The largest group of relatively low essential oil yielding accessions consisted all the Hungarian wild growing populations. However, they are grouped together with other, geographically very distinct origins such as the “English” and two other “German” ones. Unfortunately, one of the former accessions had been obtained as a market item, thus may not represent a natural habitat. Based on our investigations further systematic studies are necessary to determine any possible tendency between the yield of essential oil and the geographical habitat of the populations.

As for the composition of the EOs we found that the number of mono- and sesquiterpene fractions is similar in studied accessions. The total number of the monoterpenes components and that of sequiterpenes in the oils were 30 and 39 (which were presented higher than 1% of GC area), respectively.

Thujone was the major compound with high percentages (accumulating up to 89.8% of GC area) in several investigated samples originating from Belgium and Norway. Sabinene and ß-myrcene were the most frequent monoterpenes detected in almost each sample while α-pinene and α-terpinene were the most rare monoterpenes found in only two samples from the total 120 investigated ones as minor components (below 4%). In case of sesquiterpene compounds the most widespread, universal component was β-caryophyllene. As unique components bornyl acetate, *cis*-β-farnesene, silphiperfol-6-en-5-one, *trans*-γ-cadinene and α-cadinene were identified, each of them found only in a single sample.

The bicyclic monoterpene thujone found in the essential oil of *A. absinthium* may be considered

as the most characteristic constituent of wormwood oil (Juteau et al., 2003; Meschler and Howlett, 1999). Other major compounds of *A. absinthium* volatile oil observed in several studies are myrcene, sabinene, linalool, *cis*-epoxyocimene, chrysanthenyl acetate and *trans*-sabinyl acetate (Orav et al. 2006; Martín et al. 2011; Sharopov et al. 2012 etc.) . Beside these main components, β-pinene was the second most important constituent of Iranian wormwood essential oil (Rezaeinodehi and Khangholi, 2008).

It is worth to mention that *A. absinthium* has been most frequently defined as a thujone containing species and thus the object of special food safety regulations (Weisbord et al. 1997 and others, see in Introduction chapter). However, it has now been demonstrated that due to the large chemical variability of the EO compounds this statement is not entirely correct. Out of the 120 analysed EO samples in our study, 92 (76.7%) of them contained no or only traces (below 1% of GC area) of thujones and the lack of this compound has also been demonstrated in many scientific works as mentioned above. This fact and the need for further research would be important to emphasize when dealing with new regulations.

It can be established that the spectrum of our samples was comparable with the known references and our data ascertained the large chemical diversity and compositional heterogeneity of wormwood EOs. At the same time it proved that our sample pool represented a reliable base for the investigations.

Based on the detected composition of the EOs in our accessions, we determined eleven "pure" chemotypes where a single main component represents more than 30% of the total GC area and eleven "mixed" chemotypes in which two – or in a few exceptions, three– major components together make up at least 30% of the total GC area. Four accessions ("Bel","HuW4", "Ger1" and "Nor") out of the twelve investigated contained "pure" thujone (both isomers calculated together) type individuals. Three "pure" chemotype individuals, the *cis*-epoxyocimene, *trans*-sabinyl acetate, ß-myrcene ones were found in 4-5 accessions. Other "pure" chemotypes such as sabinene, linalool, *cis*-chrysanthenol, (*Z*)-iso-citral, (*Z*)-nuciferol isobutyrate and (*E*)-nuciferol isobutyrate were represented only by a few individuals of the total pool therefore can hardly be connected with the accession. Eleven mixed chemotypes have been identified either as those with the presence of thujone or those without it. Three chemotypes were detected where thujone (both isomers calculated together) and another major compound together represented at least 30% of the oil.

Some "pure" and "mixed" chemotypes detected in our examined wormwood EOs were found in agreement with other authors. According to numerous publications (Chialva et al., 1983; Nin et al., 1995; Judzentiene and Budiene, 2010; Rezaeinodehi and Khangholi, 2008 etc), both "pure" chemotypes and "mixed" chemotypes (where the plants contain two or more components in

higher proportions, however no exact value of percentage was given by these authors) have been defined. As "pure" chemotypes *cis*-epoxyocimene, sabinyl acetate and β-thujone types and as "mixed" ones β-thujone + *cis*-epoxyocimene, β-thujone + sabinyl acetate, *cis*-epoxyocimene + chrysanthenyl acetate + sabinyl acetate ones, etc. were mentioned. These and others mentioned in the papers are partly overlapping with the chemotypes determined by us however, as the "mixed" chemotype has never been defined quantitatively in these papers, our data seem to be more exact and the comparison with them is difficult.

In our investigated wormwood samples four chemotypes ((*Z*)-*iso*-citral, selin-11-en-4-α-ol, (*Z*)- and (*E*)-nuciferol isobutyrate detected as main components) turned out to be new ones which have never been mentioned before in scientific papers.

Our results are the first which are based on investigation of wormwood accessions in the same field in the same environment, thus may represent the genetic determination of the accumulation patterns of these volatile components. Besides, our data are based on investigating plant individuals and not bulk samples of populations. This latter approach is likely to hide the real chemism as mixed samples can not demonstrate the genetic background. The huge majority of former works (e.g Orav et al., 2006) analysed bulk samples which are mixtures of several undefined individuals with possibly different EO quality. Consequently, these kind of data are not able to reflect the chemism of the species (Németh-Zámbori, 2015). In this context our data are the first, revealing the intraspecific chemical variability at individual level from several accessions.

Through our results the existence of numerous chemotypes has been ascertained, but the connection between chemotype and geographical origin cannot be justified in most cases. Besides, it was established that accessions from natural populations may display similar heterogeneity to those purchased as market items. This fact is not obvious from former references as several ones e.g. Orav et al. (2006) and Tucker et al. (1993) examined commercial samples and thus the mentioned geographical origin does not necessarily always mean natural growing habitat.

The total phenolic content and antioxidant capacity of the investigated accessions distinguished 6 subsets at $p = 0.05$ significance level based on the Games-Howell test. Accessions "Bel" reached the highest values of TPC and AC (161.84 mg GAE/g DW and 105.00 mg AAE/g DW, respectively) while accession "Spa" showed the lowest TPC and AC as well. TPC and AC is in tight connection with each other ($r=0.92$); on the other hand, no relationship between AC and EO content ($r=0.28$) as well as TPC and EO content ($r=0.23$) could be found in our study.

It can be concluded that our data obtained by different methods such as morphological measurements, molecular genetic analysis and phytochemical investigations may effectively

demonstrate the wide intraspecific variability of wormwood. Based on the study, it may be concluded that the quantitative morphological traits such as growth and leaf shape could not be connected to the essential oil accumulation potential of the accessions. Similarly, no characteristic quantitative morphological marker trait has been found for essential oil composition or chemotype, although it would be an advantageous phenomenon for breeding and cultivation.

However, the grouping of the accessions based on the qualitative morphological traits, on production, EO yield, TPC and AC coincides with the groups based on the applied RAPD and ISSR molecular markers. Accessions "Nor", "Bel" and "Spa" are considered the most homogenous ones based on our results of all aspects; at the same time it also could be concluded that these accessions are the most divergent from all the other accessions. According to the large variability of wormwood and the considerable divergence of certain accessions from the other ones, it seems to be advisable to think about a possible taxonomic division of this species. Nevertheless, this approach needs a large amount of further data.

In case there is a need for controlled raw material for the industry from cultivation, we suggest for introduction the homogenous accessions "Bel" and "Nor" as producing thujone containing EO (in case it is required) or the accessions "Ger2" and "Spa" for production of non thujone raw material which produced high EO yield. However low biomass were obtained.

6.2 Factors influencing the chemosyndroms of wormwood

During our systematic experiments, it has been detected that each of the ontogenesis (developmental phase), the examined plant organ (flowers, leaves) and the environmental factors (temperature, light) may contribute to the phenotypic appearance of the chemism.

Essential oil (volatile compounds)

Based on the investigations of two typical chemotypes of wormwood (thujone and trans-sabinyl acetate chemotypes) we found that the accumulation level of volatile compounds showed the same **organic and developmental** characteristics as many essential oil species according to the literature references (e.g. Carnat et al. 1992; Judzentiene and Budiene, 2010; Llorens-Molina et al. 2016; Németh, 2005): the flowers contain higher ratios of volatiles than do the leaves and the content decreases during the flowering time. Differences between the thujone and *trans*-sabinyl acetate chemotypes were found only in the magnitude of the accumulation of essential oil but not in its dynamics.

The ratio of monoterpene compounds was higher than that of the sesquiterpenes in both organs, and at developmental stage (from the vegetative phase to the end of flowering) with slight quantitative differences between the chemotypes. As for the individual volatile compounds, the

qualitative composition of the flowers and leaves is to a large extent similar, with the exception of one and five compounds in the cases of thujone and *trans*-sabinyl acetate chemotypes, respectively. For example, in the thujone chemotype oils the ratio of neryl-isobutanoate and caryophyllene oxide reached 426% - 880% higher proportions in the flowers than it did in the leaves and their dynamics during development are also different in the two organs. In parallel, the major component in the *trans*-sabinyl acetate type, *trans*-sabinyl acetate was higher in the leaves compared with flowers; however, other components such as linalool and neryl-isovalerate tend to accumulate mainly in the flowers (by higher than 210% - 405% ratios compared to the leaves).

Concerning the changes of the ratio of individual components it could be established that they are mostly quantitative ones for both chemotypes. The dynamics and tendency of these changes during the studied ontogenetic phases are different however, for the specific compounds in the two wormwood chemotypes, except the tendencies of linalool, the lavandulol esthers (lavandulyl-isovalerate and lavandulyl-2-methyl-butanoate) and caryophyllene oxide.

Variations in the ratios of the main components are lower in the thujone chemotype; therefore, it can be identified from any samples independently from organic composition and harvest time, while this is not the case in *trans*-sabinyl acetate chemotype. Although the biochemical processes behind these compositional changes still await explanation, it seems that neither the yield nor the quality of the essential oil of wormwood is stable and in practice they should be optimized separately for the required chemotype. Similar findings have been described formerly in the case of the related and typically thujone containing species *Tanacetum vulgare* L. where the examined six chemotypes revealed specific changes of their main compounds during ontogenesis (Németh et al., 1994).

The results showed that under our experimental conditions **termperature and light** differences did not result in significantly different levels of EO accumulation in either of the two examined accessions. Genetic differences (accession) were presumably determinant in this respect. The qualitative composition of the EO was not significantly influenced, either but showed the characteristic spectrum of the chemotype. However, the different weather conditions induced quantitative changes in the essential oil profile of both chemotypes. The ratio of *cis*-chrysanthenyl acetate rose from 8.0% (in the “cold” chamber) to 13.8% (in the “warm” chamber) in the “Spa” plants while sabinene increased from 2.3% to 10.8% and β-myrcene rose from 8.0% to 16.5% in the “cold” and “warm” chambers respectively, in the “Hum” samples. Thus, the shift in the composition depends on the chemotype.

Data on chemosyndroms of wormwood due to environmental factors can hardly be found. In other species’ accumulating volatiles, variable results have been reported. A significantly higher accumulation of EO together with qualitative changes in its composition due to warmer climatic

conditions was detected in hyssop (*Hyssopus officinalis* L.), while in the same experiment the active ingredients of yarrow (*Achillea collina* Becker) changed only moderately (Kindlovits et al., 2014). Bernáth et al. (1991) have indicated that biomass and EO production of *Salvia* species changed significantly due to different temperature and light regimes.

It was established that the characteristic intraspecific genetic difference between the studied accessions in terms of accumulation of volatiles and the spectrum of EO compounds is a principal one, manifesting itself under variable ecological conditions. However, under different temperature and light conditions considerable quantitative changes in the EO composition may occur, leading to an uncertain drug quality in the production practice. Further studies would be needed to reveal the possible background of the changes observed by us, at the level of enzyme activity and/or gene expression arising from variable weather conditions.

Phenolics

In our *in vitro* investigations it could be demonstrated that the chemical parameters of leaf extracts highly depend on the **weather conditions**, as they showed a significant increase (44%) under the "warm" conditions. Elevated temperatures stimulated the accumulation especially in the examined Spanish accession compared with the Hungarian one, thus although the tendencies are the same, the sensitivity to temperature concerning the accumulation of the phenols is also specific for the genotype, similarly to the volatile components. According to our knowledge, no previous data are available regarding the effect of weather conditions on the accumulation of TPC and AC in the case of wormwood. In other species there are numerous references to the flexibility of phenolic compounds under different weather conditions or in different vegetation years (Dou et al. 2017; Selmar and Kleinwächter, 2013; Radácsi et al. 2016 etc.). At the same time, no general behaviour of the phenolic accumulation can be established but it depends on the species and the magnitude of the studied factors as well as from other circumstances. Our results demonstrated that not only the species but even the intraspecific taxon (population/accession) may react specifically to the abiotic conditions.

6.3 Optimization of wormwood cultivation

Based on our results above, it could be ascertained that wormwood possess an extraordinarily high intraspecific and intra-population variability especially concerning the EO composition. Additionally, the actual quality may be influenced by the temperature and harvest time, showing also chemotype-specific reactions. All these findings call attention to the fact that the required drug quality could not be assured from wild collected populations but introduction of this species to the cultivation is necessary.

In the frame of our work we started the elaboration of propagation methods. Seed germination behaviour (germination capacity and mean germination time) was not significantly influenced by the accession. A 20% - 22% significant decrease of germination capacity was recorded after 29 months of storage, independently from the storage temperature (+4°C or room temperature) for both experimental samples. The decrease appeared in the first 10 months of storage followed by a slight reduction after that. In parallel, an increasing mean germination time of the seeds could be experienced as storage period was longer. However, the differences in the germination time between the storage periods were slight (1.0-1.3 days) and not justified statistically.

Among vegetative propagation methods, both cutting (with or without IBA pre-treatment) and layering may provide vigorous new plantlets usable for clonal propagation and establishment of new plantations from valuable genotypes. By half woody cutting in May 85% - 90% rooting could be achieved and by layering in early spring 1.8 – 5.8 new plantlets can be produced after two months. The data show that the differences in the potential of the intraspecific genotypes of wormwood should be taken into account first of all at the vegetative propagation methods (cutting, layering) while their reaction is similar to each other in case of seed germination.

Artemisia species are referred to exhibit an inhibitory effect against seeds of other species (Heeger, 1956; Funke, 1943). This phenomenon may be of significance in cultivation. Besides, these approach might open even new directions for utilization as environmentally friendly herbicide. Since data on the allelopathic activity of wormwood are mostly old and rather restricted, in our study we wanted to obtain additional information.

Based on the results on three test species (basil, lettuce and mustard) we concluded that powdered, dry leaves of wormwood mixed into the soil had stronger germination and growth inhibition activity than fresh leaves. Application of leaf water extract is also effective, however, under the conditions of our *in vitro* experiment the minimum inhibitory concentration was 0.3 mg/ml dry leaf. The inhibition was reflected in each of the ratios of germinated seeds, plant height and root length of the seedlings however, but not in the germination time. It could also be established that the intraspecific chemical variability of *A. absinthium* is also influencing the allelopathic effect - although the responsible molecules have not been clarified untill now. It seems that the accessions which can be considered as different chemotypes based on their EO composition may be different also from the point of view of other, water soluble secondary compounds that might be responsible for the allelopathic effect.

SUMMARY

Artemisia absinthium L. is a perennial herb, growing to 40 cm -150 cm in height and developing abundantly branching shoots. Wormwood oil has been used for centuries as anthelmintic, anti-cold, anti-inflammatory, antimicrobial, antidepressant, digestive, carminative, choleretic drug, curing insect and spider bites, herpes and parasitic worm infections. The main secondary metabolites of wormwood are volatiles in the leaves and flowers which lend the plant a characteristic and strong aromatic smell. The essential oil of *A. absinthium* is usually known and reported to be rich in bicyclic monoterpene thujone but several other main compounds have also been detected, e.g. myrcene, sabinene, linalool, *cis*-epoxyocimene, chrysanthenyl acetate and *trans*-sabinyl acetate.

Quantitative evaluation of phytochemical diversity of wormwood populations from different natural geographic areas supports the existence of distinct natural chemotypes within the species. The species exhibits a very large intraspecific variability concerning its morphological traits and active ingredients. Systematic research is, however, rather scarce on this aspect, except for references on essential oil composition.

The main goal of the research was to provide basic knowledge and information about the biology and chemistry of the species and to acquire practical information for the introduction and elaboration of cultivation. Furthermore, we wanted to obtain and characterize prosperous genotypes for domestication and further breeding.

In the study we started with a detailed investigation on 12 accessions (signed: Bel, Eng, Ger0, Ger1, Ger2, Hum, HuW1, HuW2, HuW3, HuW4, Nor, Spa) of *A. absinthium*. The source of the plant material for the experiments included gene bank accessions, market items and seeds collected from wild habitats. Unlike the huge majority of former studies, in this trial all the accessions were grown at the same place under uniform circumstances to exclude the influence of the environment or technology.

Experiments were conducted on open field and under controlled condition (climatic chamber) between 2016 and 2018. Morphological assessments were carried out in the year of open field plantation, in vegetative stage. Individuals of each accession with both qualitative and quantitative morphological characteristics as well as the biomass production of each accession were evaluated. Additionally, studying the relationship of wormwood genotypes by molecular markers was conducted with nine accesions involved. Out of the primarily screened 13 RAPD

and 15 ISSR primers only 11 RAPD and all the 15 ISSR primers produced clear, reproducible and scorable bands, thus, the investigations have been carried out using these ones.

The laboratory works for studying the relationship of wormwood genotypes by phytochemical characteristics were carried out at the Department of MAPs, SZIU. Essential oils were produced from 50 g of dried materials by hydro-distillation (500 ml water) for 2.5 hours. Composition of the essential oils was analysed with GC–MS. Total phenolic content (TPC) was determined by the modified method of Singleton and Rossi (1965) and antioxidant capacity (AC) was measured using the FRAP (ferric reducing antioxidant power) method according to the modified method of Benzie and Strain (1996).

To reveal ontogenetic and morphogenetic factors influencing the chemosyndroms of wormwood, we used both thujone (T) chemotype and *trans*-sabinyl acetate (SA) chemotype which were harvested at 4 different developmental stages. EO compositions obtained from leaves and flowers separately were analysed with GC-MS. To reveal the effect of environment, two wormwood accessions from Spain and Hungary were planted in two phytotron chambers which were installed with two growing programs simulating "warm" weather (higher temperature and light intensity) and "cold" weather (lower temperature and light intensity) circumstances. EO yield and composition, TPC and AC were measured to evaluate the influence of environmental factors on chemical features of wormwood.

In order to optimize effective methods of wormwood cultivation, a study on generative propagation (effect of storage on seed germination) and a study on vegetative propagation (layering and cutting methods) were carried out both on open field and in plastic house. The number of germinated seeds, the mean germination time and the number of roots were counted and recorded, additionally, the length of roots of each seedling/new plant was also measured. In addition, a study on allelopathic activity of wormwood with different treatments (wormwood leaf powder, aqueous extracts, different concentrations) was also conducted in a climatic chamber and greenhouse.

In the 12 experimental populations, morphological measurements of the first-year-old plants revealed that the accession "Nor" presented the shortest shoots (18.7 cm height) and smallest bushes (29.9 cm in diameter), on the other hand, "Eng" demonstrated the highest vegetative growth (47.5 cm height) and "HuW2" was recorded with the largest bushes (64.9 cm diameter). Accessions "Spa" and "Bel" developed the thickest leaves (0.47 mm and 0.48 mm, respectively), "Ger1" and "Hum" provided the largest leaves while "Ger2" presented the thinnest leaf blade (0.30 mm) and smallest leaves (104.4 cm). As for the biomass production of the first-year-old plants, a considerable intra-population variability was observed. Our investigations were achieved under the same ecological conditions and the populations maintained by the same

agricultural methods thus the described differences may reflect the intraspecific genetic manifestation of *A. absinthium* in all respects.

The essential oil content of twelve accessions varied from 0.349 to 3.215 ml/100g. The distinguished three groups representing significantly different levels of oil yield do not seem to be in connection with the geographical origin of the accession. The total number of the monoterpene components and that of sequiterpenes in the oils were 30 and 39 (which were present in higher than 1% of GC area), respectively. Thujone was the major compound with high percentages (accumulating up to 89.8% of GC area) in several investigated samples originating from Belgium and Norway. Sabinene and ß-myrcene were the most frequent monoterpenes detected in almost each sample while α-pinene and α-terpinene were the rarest monoterpenes found in only two samples from the total 120 investigated ones as minor components (below 4%). Based on the detected composition of the EOs in our accessions, we determined eleven "pure" chemotypes and eleven "mixed" chemotypes. The latter ones were characterised by two – or in a few exceptions, three– major components together. The components (*Z*)-*iso*-citral, selin-11-en-4-α-ol, (*Z*)- and (*E*)-nuciferol isobutyrate as major constituents represent new chemotypes of wormwood as they have never been mentioned before in its EO. Our results are the first which are based on investigation of wormwood accessions in the same field in the same environment, thus, they may refer to the genetic determination of the accumulation patterns of these volatile components.

The total phenolic content and antioxidant capacity of the investigated accessions were significantly different. Accessions "Bel" reached the highest values of TPC and AC (161.84 mg GAE/g DW and 105.00 mg AAE/g DW, respectively) while "Spa" accession showed the lowest TPC and AC as well. Accessions "Nor", "Bel" and "Spa" are considered as the most homogenous ones based on our results of all aspects; at the same time it also could be concluded that these accessions are the most divergent from all the other accessions.

Based on the investigations of two chemotypes of wormwood (thujone and trans-sabinyl acetate chemotypes) we found that the accumulation level of volatile compounds showed similar organic and developmental characteristics. Concerning the changes of the ratio of individual components it could be established that they are mostly quantitative ones for both chemotypes. The dynamics and tendency of these changes during the studied ontogenetic phases are different for the specific compounds in the two wormwood chemotypes except the tendencies of linalool, the lavandulol esthers (lavandulyl-isovalerate and lavandulyl-2-methyl-butanoate) and caryophyllene oxide.

Under the *in vitro* experimental conditions, temperature and light differences did not result in significantly different levels of EO accumulation in either of the two examined accessions. The qualitative composition of the EO was not significantly influenced, either, but showed the

characteristic spectrum of the chemotype. However, the different weather conditions induced quantitative changes in the essential oil profile of both chemotypes. The ratio of *cis*-chrysanthenyl acetate grew from 8.0% (in "cold" chamber) to 13.8% (in "warm" chamber) in the "Spa" plants while sabinene increased from 2.3% to 10.8% and β-myrcene rose from 8.0% to 16.5% in the "cold" and "warm" chambers, respectively in the "Hum" samples.

The direction of changes of TPC due to the variable environmental conditions was similar for both accessions. However, while the increase was a significant one in the Spanish samples (from 28.035 mg GAE/g DW in the "cold" chamber to 40.339 mg GAE/g DW in the "warm" chamber), only a 6% elevation was measured in the Hungarian accession. The highest AC value was detected in Spanish samples in the "warm" chamber (24.767 mg AAE/g DW), while the lowest one was found in Hungarian samples in the "cold" chamber (13.251 mg AAE/g DW). In our *in vitro* investigation, it could be demonstrated that the TPC and AC of the wormwood leaf extracts highly depend on the weather conditions, as they showed a significant increase under the "warm" conditions.

In the frame of our work we started the elaboration of plant propagation methods for cultivation. A 20% - 22% significant decrease of germination capacity was recorded after 29 months of storage, independently from the storage temperature (+4°C or room temperature) for both experimental samples. Based on vegetative propagation methods, 85% - 90% rooting could be achieved by half woody cutting in May, and 1.8 – 5.8 new plantlets could be produced from each mother plant by layering in early spring in two months.

The results of allelopathic investigations showed that the powdered, dry leaves of wormwood mixed into the soil had stronger germination and growth inhibition activity than fresh leaves. The leaf water extract is also effective: the minimum inhibitory concentration was 0.3 mg dry leaf /ml water where the inhibition manifested in each of the ratio of germinated seeds, plant height and root length of the seedlings.

It can be concluded that our data obtained by different methods such as morphological measurements, molecular genetic analysis, phytochemical investigations as well as the allelopathic effect may effectively demonstrate the wide intraspecific variability of wormwood. Due to this, a revision of the uniform taxonomical inclusion might be considered. Based on our results, it was demonstrated that homogeneous raw plant material can only be produced by cultivation for which clonal propagation methods may be suggested and a harvest optimization for the required chemotype.

ÖSSZEFOGLALÁS

Az *Artemisia absinthium* L. 40-150 cm magasságot elérő, évelő, lágyszárú faj, amely dúsan elágazó bokrot képez. A fehér ürmöt évszázadok óta elterjedten használják féregűző, megfázás elleni, gyulladáscsökkentő, antimikrobiális, antidepresszáns, emésztést serkentő, szélhajtó és epehajtó tulajdonságaiért, továbbá hatásosnak tartják rovar- és pókcsípés valamint herpesz kezelésére. A növény legfontosabb hatóanyagai a levelek és a virágzatok illó komponensei, amelyek jellegzetes, erős aromás illatot is kölcsönöznek. Az *A. absinthium* illóolaját általában tujontartalmúnak ismerik, -amely egy biciklusos monoterpén-, de emellett gyakran leírtak más fő komponenseket is, pl. myrcén, sabinén, linalool, *cis*-epoxyocimén, chrysanthenil-acetát, *trans*-sabinil-acetát.

Különböző földrajzi régiókból származó minták fitokémiai elemzése alapján úgy tűnik, hogy ez a faj széleskörű kémiai intraspecifikus diverzitással rendelkezik. Ugyancsak nagy variabilitás tapasztaltak a morfológiai bélyegekben is, de ezzel kapcsolatban a szisztematikus kutatások hiányoznak.

Kutatásunk fő célja az volt, hogy szélesebb körű ismerteket nyerjünk e faj biológiai és kémiai tulajdonságaival kapcsolatban valamint olyan gyakorlati információkat, amelyek a termesztésbevételt alapozzák meg. Mindemellett célunk volt, hogy ígéretes genotípusokat tudjjunk kiválogatni az esetleges jövőbeni nemesítés számára.

A munkát 12 különböző származású *A. absinthium* populáció (a továbbiakban a következő jelzésekkel: Bel, Eng, Ger0, Ger1, Ger2, Hum, HuW1, HuW2, HuW3, HuW4, Nor, Spa) részletes vizsgálatával kezdtük. A kísérletekhez felhasznált maganyag részben génbanki tételekből, részben kereskedelmi mintákból illetve általunk vadon termő állományokban gyűjtött szaporítóanyagból állt. Kísérletünkben valamennyi anyagot azonos termőhelyen, egységes termesztési feltételek között állítottuk kísérletbe, azért, hogy a környezeti vagy technológiai hatásokat kiszűrhessük, s ez alapvetően eltér a korábbi publikációkban közölt vizsgálatoktól.

A kísérletek szabadföldön illetve klímakamrában, szabályozott körülmények között folytak, 2016 és 2018 között. A morfológiai felméréseket a szabadföldi parcellákban végeztük, vegetatív fejlődési állapotban. Minden populációban egyedi növényeken mértük fel a kvantitatív és kvalitatív morfológiai tulajdonságokat, valamint a biomassza produkciót. Emellett kilenc anyag kiemelésével a tételek közötti genetikai rokonsági kapcsolatokat molekuláris markerezéssel is értékeltük. Az eredetileg tesztelt 13 RAPD és 15 ISSR primer közül 11 RAPD és valamennyi

15 ISSR primer adott tiszta, reprodukálható és értékelhető sávokat, így ezekkel végeztük el a vizsgálatot.

A fitokémiai és molekuláris genetikai munkák a Szent István Egyetem Gyógy- és Aromanövények Tanszékének laboratóriumában folytak. Az illóolajat 50 g szárított növényanyagból vízdesztillációval (500 ml víz, 2.5 óra) állítottuk elő. Az illóolajok összetételét GC–MS módszerrel elemeztük. Az összfenol tartalmat (TPC) Singleton and Rossi (1965) módosított módszerével határoztuk meg, az antioxidáns kapacitást (AC) pedig a FRAP (ferric reducing antioxidant power) módszerrel, Benzie and Strain (1996) alapján.

Az onogenetikus és szervi összetétel kemoszindrómákat befolyásoló hatásának vizsgálata során tujonos (T) chemotype és *trans*-sabinil-acetátos (SA) kemotípussal dolgoztunk, amelyeket 4 különböző fejlődési fázisban mintáztunk. Az illóolaj mérése és elemzése során a levelek és a virágzati részek jellemzőit különválasztva értékeltük. A környezeti hatások vizsgálata során egy spanyol és egy Magyar vadon termő populációt vontunk vizsgálatba. Két fitotron kamrában, "meleg" (magasabb hőmérséklet és fényintenzitás) illetve "hideg" (alacsonyabb hőmérséklet és fényintenzitás) körülményeket szimuláltunk. Ez utóbbi vizsgálatban az illóolaj kihozatal és összetétel mellett a TPC-t és AC-t is mértük.

Annak érdekében, hogy a fehér üröm termesztésbe vonását is kidolgozhassuk, tanulmányoztuk mind a generatív (a tárolás hatása a magvak csírázására) mind a vegetatív (dugványozás és bújtás) szaporítás lehetőségét szabadföldben illetve üvegházi körülmények között. Meghatároztuk a csírázóképességet, a csírázási időt, a járulékos gyökerek számát és hosszát. Mindemellett a fehér üröm allelopátiás hatásának vizsgálatára is állítottunk be kísérleteket, amelyekben különböző nyersanyagok (aprított levél,vizes kivonatok) hatását teszteltük klímakamrában és üvegházban.

A 12 kísérleti populáció első éves egyedeinek mérése alapján megállapítottuk, hogy a "Nor" anyag fejlesztette a legkisebb bokrot (18.7 cm magas és 29.9 cm átmérőjű), míg az "Eng" taxon növekedése volt a legerőteljesebb (47.5 cm magasság) illetve a "HuW2" fejlesztette a legszélesebb bokrokat (64.9 cm átmérő). A "Spa" és a "Bel" nevű anyagok levelei voltak a legvastagabbak (0.47 mm és 0.48 mm), a "Ger1" és a "Hum" levelei a legnagyobbak, és a "Ger2" levéllemezei bizonyultak a legvékonyabbnak (0.30 mm) és legkisebbnek (104.4 cm).

Az első éves növények biomassza produkciójában jelentős populáción belüli heterogenitást állapítottunk meg. Mivel vizsgálataink azonos környezeti feltételrendszerben és agrotechnikai eljárások között folytak, a tapasztalt különbségek minden tekintetben feltehetően az *A. absinthium* faj genotípusosan rögzített intraspecifikus változékonyságát tükrözik.

A vizsgált 12 taxon illóolajtartalma 0.349 és 3.215 ml/100g között változott. Az illóolajtartalom alapján statisztikailag elkülönülő három csoport nem áll összefüggésben a populációk földrajzi eredetével. Az illóolaj mintákban azonosított monoterpén komponensek száma 30, a

szeszkviterpéneké 39 volt (a GC area százalékban kifejezve 1%-nál nagyobb komponenseket tekintve). A Belgiumból és a Norvégiából származó anyag mintáinak nagy részében tujon volt az olaj fő komponense, amely esetenként igen magas arányt is elért (89.8% -ig, GC area %-ban kifejezve). Sabinén és ß-mircén volt az illóolajok leggyakrabban detektált komponense, míg α-pinén és α-terpinén igen ritkán, az analizált 120 minta közül csak két esetben fordultak elő minor összetevőként (4% alatt).

A minták illóolaj összetétele alapján 11 „tiszta" kemotípust és 11 „vegyes" kemotípust írtunk le, az utóbbiakban általában két vegyület adta az olaj fő komponenseit, illetve néhány esetben három vegyület. Az (*Z*)-*iso*-citrál, selin-11-en-4-α-ol, (*Z*)- és (*E*)-nuciferol isobutirát főkomponensű egyedek új kemotípusokat reprezentálnak, mivel ezeket még sosem írták le fő komponensként a fehér üröm illóolajában. Eredményeink az első olyan adatok, amelyek a sokféle populáció illóolaj összetételét azonos körülmények között kitermesztett növények mintái alapján születtek, így e komponensek felhalmozódása valószínűleg genetikailag rögzített különbségeket tükröz.

A vizsgált származéksorok összfenol tartalma és antioxidáns kapacitása szignifikánsan eltérő volt. A "Bel" nevű populációban mértük a legmagasabb értékeket, (TPC: 161.84 mg GAE/g DW és AC: 105.00 mg AAE/g DW), míg a "Spa" populációban a legalacsonyabbakat. Eredményeink alapján elmondható, hogy a "Nor", "Bel" és "Spa" populációk voltak a leginkább homogének minden tekintetben, s ugyanakkor ezek térnek el leginkább az összes többitől.

A vizsgálatba vont két kemotípus (thujonos és trans-sabinil acetátos) esetében megállapítottuk, hogy az illó komponensek felhalmozódása mindkettőben hasonlóan alakult az ontogenetikai fázisok során és a szervi megoszlás tekintetében. A komponens összetételt illetően azt tapasztaltuk, hogy mindkét kemotípusban alapvetően kvantitatív változások voltak a fenti tényezők hatására. Ugyanakkor e változások dinamikája és tendenciája az egyedfejlődés során azonban egymástól eltérő, kivéve a következő komponenseket: linalool, lavandulol észterek (lavandulil-isovalerát, lavandulil-2-methil-butanoát) és kariofillén oxid.

Az *in vitro* kísérletben az eltérő hőmérsékleti és fényviszonyok nem indukáltak szignifikáns eltérést egyik növényanyagban sem az illó anyagok felhalmozódását tekintve. Az illóolaj kvalitatív összetétele sem változott szignifikánsan, hanem a kemotípusra jellemző spektrumot mutatta. Ugyanakkor a különböző időjárási körülmények az illóolaj összetételében kvantitatív változásokat idéztek elő. A "Spa" poulációban a *cis*-chrysanthenil acetát aránya 8.0% (a „hideg" kamrában) 13.8%-ra nőtt (a „meleg" kamrában), míg a "Hum" mintákban a szabinén aránya 2.3%-ról 10.8%-ra, a β-mircén aránya pedig 8.0%-ról 16.5%-ra nőtt a „meleg" kamrában a „hideg" paraméterekhez képest.

Mindkét növényanyagban hasonlóan változott a TPC az időjárási körülmények függvényében. Ugyanakkor amíg a növekedés csak a spanyol populáció esetében volt szignifikáns, (28.035 mg

GAE/g DW a “hideg” kamrában és 40.339 mg GAE/g DW a “meleg” kamrában), addig a magyar anyagban csak 6% emelkedést mértünk. A legmagasabb AC értékeket a spanyol populáció növényegyedeiben, a “meleg” kamrában (24.767 mg AAE/g DW) kaptuk, míg a legalacsonyabbakat a magyar tétel növényeiben a „hideg” kamrában (13.251 mg AAE/g DW). I*n vitro* vizsgálatokkal tehát bizonyítottuk, hogy a levelek kivonatának TPC és AC jellemzői nagymértékben függenek az időjárási tényezőktől, és a „meleg” feltételrendszerben mutattak szignifikánsan kedvezőbb értékeket.

Munkánk keretében megkezdtük a termesztéshez a szaporítási módszerek kidolgozását is. A 29 hónapos tárolási időszak végén a magvak csírázóképessége 20-22%-os csökkenést mutatott, függetlenül a tárolási hőmérséklettől (+4°C vagy szobahőmérséklet) mindkét tételünk esetében. A vegetatív szaporítási kísérletek eredménye azt mutatta, hogy májusi félfás dugványozással 85 - 90% gyökeresedés érhető el és kora tavaszi bújtással 1.8 – 5.8 új növényt nyerhetünk egy anyatőről két hónap alatt.

Az allelopátis vizsgálatok tapasztalatai szerint a fehér üröm száraz, porított levelét a talajba keverve erősebb csírázást gátló hatást fejt ki, mint a friss levelek ugyanígy. A levelek vizes kivonata is hatásos, a minimális gátló koncentráció 0.3 mg száraz levél /ml víz, és ez a hatás megnyilvánult a kicsírázott magvak számában, a csíranövények nagyságában és gyökérhosszában is.

A különböző módszerekkel – morfológiai mérések, molekuláris genetikai elemzés, növénykémiai analízisek illetve allelopátia tanulmányozása – nyert adataink alapján arra a következtetésre jutottunk, hogy az *Artemisia absinthium* széleskörű intraspecifikus variabilitással rendelkező faj. Ebből kifolyólag a taxonómiailag egységes faj besorolást is érdemes lehet újragondolni. Eredményeink rámutatnak, hogy homogén növényanyag csak termesztéssel érhető el. Ehhez a vegetatív szaporítás javasolható és a betakarítást mindenképpen kemotípusra specifikusan érdemes optimalizálni.

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LIST OF PUBLICATION RELATED PhD TOPIC

Journal articles with IF

1. **Nguyen, H. T.,** Inotai, K., Radácsi, P., Tavaszi-Sárosi, S., Ladányi, M., & Zámboriné-Németh, É. (2017). Morphological, phytochemical and molecular characterization of intraspecific variability of wormwood (*Artemisia absinthium* L.). *Journal of Applied Botany and Food Quality*, *90*(0), 238–245. https://doi.org/10.5073/JABFQ.2017.090.030 **(IF: 1.2)**
2. **Nguyen, H.T.,** Radácsi, P., Gosztola, B., Németh, É.Z., 2018. Effects of temperature and light intensity on morphological and phytochemical characters and antioxidant potential of wormwood (*Artemisia absinthium* L.). *Biochem. Syst. Ecol.* 79, 1–7. https://doi.org/10.1016/j.bse.2018.03.005 **(IF: 1.21)**
3. **Nguyen, H. T**., Radácsi, P., Gosztola, B., Rajhárt, P., & Németh, É. Z. (2018). Accumulation and composition of essential oil due to plant development and organs in wormwood (*Artemisia absinthium* L.). *Industrial Crops and Products*, *123*, 232–237. https://doi.org/10.1016/j.indcrop.2018.06.076 **(IF: 3.48)**
4. **Nguyen, H. T.,** Sárosi, S. T., Llorens-Molina, J. A., Ladányi, M., & Zámborine-Németh, É. (2018). Compositional variability in essential oils of twelve wormwood (*Artemisia absinthium* L.) accessions grown in the same environment. *Journal of Essential Oil Research*, 1–10. https://doi.org/10.1080/10412905.2018.1496856 **(IF: 1.05)**

Peer-reviewed journal (MTA list) publications

1. **Nguyen, H. T**., & Németh, Z. É. (2016). Sources of variability of wormwood (*Artemisia absinthium* L.) essential oil. *Journal of Applied Research on Medicinal and Aromatic Plants*. 1–10. https://doi.org/10.1016/j.jarmap.2016.07.005

Conference proceedings

1. **Nguyen, H.T.,** Llorens Molina J.A., Zámboriné Németh É. (2018) Intraspecifische Variabilität and Drogenqualität des Wermuths (*Artemisia absinthium* L.), Julius Kühn Archives, (ISSN 1868-9892), Heenemann Druckerei, Berlin. 460, p. 57-61.

International conferences

1. Title: “The fact of organic farming in Vietnam”, Organic Agriculture for agro biodiversity preservation, Novi Sad, Serbia, 31st May - 3rd June 2017. Abstract book tittle: Organic Agriculture for agro biodiversity. Page of topic: 22
2. Title: “Phytochemical and molecular characterization of intraspecific variability of wormwood (*Artemisia absinthium* L.)”, 48th ISEO Pécs, Hungary, 11st – 13rd September 2017. Abstract book tittle: Natural volatiles and essential oils. Page of topic: 52
3. Title: “Phytotoxic activity of aqueous extracts from different *A.absinthium* chemotypes on seed germination of lettuce and basil”, 10th Conference on Medicinal and Aromatic Plants of Southeast European Countries in Split Croatia, 20st May - 24th May 2018. Abstract book tittle: 10th Conference on Medicinal and Aromatic Plants of Southeast European Countries, Page of topic: 98
4. Title: “Changes of volatile compounds of two wormwood (*Artemisia absinthium* L.) accessions under controlled weather conditions”, 49th ISEO 2018, 13rd-16th September 2018, Nis - Serbia. Abstract book tittle: Physics, Chemistry and technology Vol. 16, No 1, Special Issue 2018. Page of topic: 36

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APPENDIX

PICTURES OF EXPERIMENTS

Planted out in 2016

Plantation in 2016

After overwinter in 2017

Flowering in 2017

EO distilation

Essential oils of wormwood

Table 28. Chemical composition of the investigated *Artemisia absinthium* L. accessions (components reaching 1% of GC area are listed)

Accesions	RT	LRI[a]	Bel			Eng			Ger0			Ger1			Ger2			Hum		
Compound			Max	Min	St.D	Max	Min	St.D	Max	Min	St.D	Max	Min	St.D	Max	Min	St.D	Max	Min	St.D
α-Pinene	5.56	938				1.6	1.6											3.9	3.9	
Sabinene	6.52	976	7.0	1.0	2.2	**38.1**	1.2	13.6	**31.1**	2.4	11.0	11.6	2.0	3.6				15.1	3.1	5.0
1-Octen-3-ol	6.81	987				1.2	1.2		2.0	2.0										
ß-Myrcene	6.99	995	3.1	1.2	0.9	26.7	3.1	8.6	**38.3**	2.5	13.2	**68.1**	3.6	22.8				**44.0**	1.4	16.4
α-Phellandrene	7.43	1009				14.5	1.4	9.3	15.0	15.0		10.4	4.6	4.1				6.7	1.2	2.2
α-Terpinene	7.79	1018																3.6	3.6	
p-Cymol	8.09	1026				4.3	4.3		10.2	10.2		6.1	1.3	2.1				16.8	1.1	6.1
(*Z*)-Ocimene	8.50	1037				2.1	1.7	0.3												
γ-Terpinene	9.20	1056				1.2	1.0	0.2												
Linalool	10.76	1097				9.7	1.3	3.2	4.5	1.1	1.4	27.9	1.2	9.8				8.7	1.4	3.8
α-Thujone	11.07	1105	**51.7**	1.2	24.9	1.1	1.1					1.8	1.0	0.4						
ß-Thujone	11.41	1113	**89.8**	24.7	22.7	2.1	2.1					**85.2**	2.1	34.9				0.7	0.1	0.3
***cis*-Epoxyocimene**	12.12	1130				**65.1**	**34.5**	21.6												
iso-3-Thujanol	12.24	1133	1.7	1.3	0.3															
***trans*-Epoxyocimene**	12.45	1138				3.2	1.7	1.1							4.4	3.4	0.7			
***trans*-Sabinol**	12.51	1140							3.2	3.2					3.9	2.4	0.8			
(*Z*)-*Iso*-citral	13.39	1161				**48.5**	10.4	27.0				**49.2**	**43.2**	4.3						
***cis*-Chrysanthenol**	13.51	1164										27.5	27.5					**37.3**	**37.3**	
Lavandulol	13.58	1166	6.1	1.1	2.8													3.1	3.1	
Terpinene-4-ol	13.96	1175				2.7	1.1	0.7	2.3	1.2	0.4	1.4	1.2	0.1				4.5	1.5	1.2
***cis*-Chrysanthenyl acetate**	17.29	1254																		
Perilla aldehyde	17.89	1268				1.5	1.1	0.2										1.1	1.1	
Bornyl acetate	18.54	1284							1.9	1.9										
Lavandulyl acetate	18.59	1285										2.7	2.7							
***trans*-Sabinyl acetate**	18.77	1289							**87.6**	**87.6**		**36.0**	1.2	17.7	**94.5**	**81.8**	6.0			
Cyclohexanol acetate	18.88	1292				10.9	10.9													
β-Caryophyllene	23.68	1420	6.5	1.0	1.8	11.0	2.2	3.1	12.2	2.2	3.2	12.9	1.0	5.4	1.0	1.0		19.6	2.6	5.7
Linalyl butanoate	23.85	1424				1.2	1.2											1.9	1.2	0.5
***cis*-β-Farnesene**	24.32	1436							1.2	1.0	0.1									
α-Humulene	25.07	1454				1.1	1.1		3.4	1.2	1.6	1.4	1.4					1.3	1.3	0.0
Curcumene (ar-)	26.10	1480				12.4	1.2	4.1	7.7	1.5	3.1	3.9	1.2	1.3				13.7	1.1	4.9
Germacrene D	26.18	1482	1.6	1.3	0.2	4.7	4.7		11.8	1.0	4.6	11.6	2.6	6.3	1.1	1.1		8.6	1.2	3.9
α-Curcumene	26.26	1483				3.6	1.2	1.2	6.5	3.2	1.4	24.4	1.6	12.9				4.3	1.2	1.3
ß-Selinene	26.38	1486	3.5	1.8	0.8	3.2	1.2	0.9	1.5	1.1	0.3	2.4	1.9	0.3	4.0	2.3	0.9	3.9	1.2	1.1

Neryl-isobutanoate	26.62	1492				2.5	1.2	0.7	4.4	1.6	1.4	1.9	1.9					4.3	1.1	1.6
Lavandulyl-isovalerate	27.35	1512				2.8	1.0	0.9	2.5	1.6	0.5	1.8	1.8					8.3	1.1	3.0
Lavandulyl-2--methyl-butanoate	27.41	1513				1.0	1.0											2.0	1.4	0.4
Himachalene (α-dehydro-ar)	27.52	1516	1.5	1.5		1.3	1.0	0.2	1.5	1.5										
Neryl-isovalerate	29.98	1584				4.0	2.4	0.9	5.9	1.1	2.2	2.9	2.0	0.5				5.3	1.2	1.6
Thujopsan-2-β-ol	29.96	1583				1.1	1.1		3.6	3.6		1.1	1.1					2.3	1.9	0.3
Spathulenol	29.98	1584				2.9	1.1	0.8	6.4	1.5	2.2	2.2	1.4	0.6				6.9	1.9	2.3
Caryophyllene oxide	30.20	1590	1.6	1.6		5.4	1.1	1.9	10.4	1.4	3.3	10.8	1.8	4.5				9.7	4.0	2.0
Geranyl-2-methyl-butanoate	30.44	1597				3.0	3.0													
(2R,5E)-Caryophyll-5-en-12-al	30.81	1606				4.7	1.2	1.5	1.5	1.5		1.6	1.2	0.3	1.0	1.0		16.9	2.9	7.6
Geranyl-isovalerate	30.89	1609				1.4	1.4	0.0	1.9	1.1	0.6	3.5	3.5					8.3	2.6	2.6
10-epi-γ-Eudesmol	31.45	1623	3.2	3.2		2.4	1.6	0.6				2.1	2.1					1.3	1.3	
γ-Eudesmol	31.92	1635				1.1	1.1					1.3	1.3							
Caryophylla-4(12),8(13) -dien-5-beta-ol	32.21	1643				1.6	1.1	0.3	1.3	1.3		2.0	2.0					2.6	1.0	0.6
α-Eudesmol	32.71	1656	1.4	1.4								3.4	3.4					1.3	1.3	
Selin-11-en-4-α-ol	32.90	1661	6.5	1.1	2.1	15.4	1.3	4.7	11.1	2.7	3.5	**50.8**	1.6	26.2				21.8	2.3	6.6
α-Bisabolol	33.84	1686										12.5	12.5							
Cedren-13-ol (8)	33.95	1688				2.8	1.0	0.8	5.1	1.1	1.5	1.4	1.3	0.1				4.8	1.2	1.4
epi-α-Bisabolol	34.00	1690				3.0	1.4	1.1	1.9	1.2	0.4									
Muurol-5-en-4-one (cis-14-nor)	34.07	1692				1.6	1.6													
(*Z*)-α-*trans*-Bergamotol	34.09	1692				1.1	1.1		1.0	1.0										
Chamazulene	35.48	1733	9.7	1.7	5.7	1.3	0.6	0.5	3.9	3.9								0.5	0.5	
Geranyl-p-cymene	42.69	1939	1.3	1.3		11.8	1.2	4.6	5.1	1.0	1.8	7.8	3.7	2.9				8.5	1.2	4.1
Unknown	44.34	1978	3.1	3.1		7.7	1.5	2.6	15.9	1.8	6.0	2.0	1.3	0.4				8.2	1.1	2.8
(*Z*) Nuciferol isobutyrate	44.46	1981	2.5	1.8	0.5	20.1	1.0	6.7	22.4	1.2	9.5	8.9	2.8	4.3				22.1	3.5	6.6
(*E*)-Nuciferol isobutyrate	44.54	1983				13.2	13.2		**33.2**	**33.2**										

Continued

Table 28 (continued)

Accesions	RT	LRI[a]	HuW1			HuW2			HuW3			HuW4			Nor			Spa		
Compound			**Max**	**Min**	**St.D**	**Max**	**Min**	**St.D**	**Max**	**Min**	**St.D**	**Max**	**Min**	**St.D**	**Max**	**Min**	**St.D**	**Max**	**Min**	**St.D**
Sabinene	6.52	976	**33.8**	2.4	11.1	18.4	1.9	5.3	28.9	1.3	10.3	1.5	1.2	0.2	6.6	1.0	2.4			
1-Octen-3-ol	6.81	987	1.7	1.5	0.1	1.5	1.5					2.1	2.1							
ß-Myrcene	6.99	995	**41.6**	1.9	12.5	**38.5**	1.8	12.9	**68.4**	1.1	25.4	8.0	2.8	1.9						
α-Phellandrene	7.43	1009	13.0	1.3	5.3	4.4	4.4		4.2	1.5	1.9	7.9	7.9		3.5	1.0	1.7			
α-Terpinene	7.79	1018							1.0	1.0										
p-Cymol	8.09	1026	14.9	1.1	5.9	10.0	1.3	4.4	4.5	1.3	1.7	10.4	10.4		2.5	2.5				
(Z)-Ocimene	8.50	1037				6.8	2.3	2.3												
γ-Terpinene	9.20	1056	1.1	1.1																
Linalool	10.76	1097	10.5	1.5	3.6	18.4	1.1	6.7	8.7	5.1	1.7	**52.1**	1.0	18.0						
α-Thujone	11.07	1105										2.4	1.2	0.5	24.8	14.5	3.8			
ß-Thujone	11.41	1113	0.3	0.3					2.1	1.2	0.7	**87.6**	**66.4**	9.1	26.8	0.2	8.7			
***cis*-Epoxyocimene**	**12.12**	**1130**	**44.9**	**44.9**		**45.9**	**38.7**	4.1							29.6	16.6	5.1	**75.7**	**46.9**	8.3
iso-3-Thujanol	12.24	1133				1.1	1.1					3.3	1.5	1.0						
***trans*-Epoxyocimene**	12.45	1138				2.3	1.6	0.4	4.9	4.9					3.1	1.4	0.8	2.9	1.1	0.7
Camphor	12.68	1144													3.3	1.1	1.1	5.6	3.5	1.5
(*Z*)-Iso-citral	13.39	1161																1.0	1.0	
***cis*-Chrysanthenol**	13.51	1164																1.0	1.0	
Lavandulol	13.58	1166	3.8	1.8	1.4				2.0	1.7	0.2				1.3	1.3				
Terpinene-4-ol	13.96	1175	10.0	1.1	3.4	22.8	1.1	9.3	9.8	1.0	4.2	18.5	18.5							
***cis*-Chrysanthenyl acetate**	17.29	1254																25.9	9.4	5.4
Lavandulyl acetate	18.59	1285				2.5	1.6	0.6												
***trans*-Sabinyl acetate**	18.77	1289				3.4	3.4		**75.2**	**75.2**					77.8	20.3	21.0			
Thymol	18.81	1290	1.2	1.2					28.4	28.4										
Cyclohexanol acetate (cis-2-tert-butyl)	18.88	1292				3.4	3.1	0.2							7.5	3.9	1.9	11.2	1.3	3.7
Carvacrol	19.20	1300	2.1	2.1		1.3	1.3		1.9	1.9										
α-Terpinyl acetate	21.00	1349							1.3	1.3										
β-Caryophyllene	23.68	1420	24.0	1.5	7.3	25.2	4.3	7.3	6.9	1.5	2.1	8.2	2.1	2.6	4.6	1.0	1.5	3.8	1.1	0.9
Linalyl butanoate	23.85	1424										1.1	1.1							
α-Humulene	25.07	1454	1.4	1.4		2.1	2.1													
Curcumene (ar-)	26.10	1480	1.8	1.8		1.5	1.5		8.1	1.1	3.4									
Germacrene D	26.18	1482	11.0	2.7	3.7	3.0	3.0		4.4	4.4		12.0	12.0		1.1	1.0	0.1	3.7	1.1	0.9
α-Curcumene	26.26	1483	9.7	1.5	3.1				8.2	3.9	2.0	4.8	1.8	2.2						
ß-Selinene	26.38	1486	2.2	1.5	0.3	2.0	1.4	0.3	2.9	1.6	0.9	4.2	3.1	0.5				1.5	1.5	
Neryl-isobutanoate	26.62	1492	1.4	1.1	0.2	2.2	2.0	0.2				1.8	1.1	0.5						
Lavandulyl-isovalerate	27.35	1512	2.1	1.2	0.7	2.5	1.1	0.7	1.8	1.4	0.2	29.0	1.7	11.0						

Lavandulyl-2--methyl-butanoate	27.41	1513										4.5	1.1	2.4						
Geranyl-isobutanoate	27.52	1516				1.6	1.3	0.2												
Himachalene (α-dehydro-ar)	27.52	1516																		
***trans*-γ-Cadinene**	27.52	1516				1.3	1.3													
α-Cadinene	28.40	1540	1.7	1.7																
Neryl-isovalerate	29.98	1584	2.2	1.6	0.4	4.0	1.1	1.2	1.2	1.0	0.1	2.8	1.2	0.7						
Spathulenol	29.98	1584	4.1	1.5	1.3				6.5	2.1	1.8	2.7	2.1	0.4						
Caryophyllene oxide	30.2	1590	10.6	1.2	3.9	16.7	1.1	5.2	9.9	1.7	3.9	7.4	1.3	2.7						
Geranyl-2-methyl-butanoate	30.44	1597																		
(2R,5E)-Caryophyll-5-en-12-al	30.81	1606	5.6	1.2	1.8	4.0	1.1	1.0	8.8	1.1	4.1	10.8	4.7	4.4				1.2	1.2	
Geranyl-isovalerate	30.89	1609				1.7	1.7		2.6	2.6		18.5	11.7	4.8						
10-epi-γ-Eudesmol	31.45	1623	3.0	3.0		2.1	1.5	0.4	1.2	1.2								1.8	1.6	0.1
Silphiperfol-6-en-5-one	31.63	1628				1.7	1.7													
γ-Eudesmol	31.92	1635																2.2	1.2	0.5
Caryophylla-4(12),8(13)-dien-5-beta-ol	32.21	1643	1.5	1.1	0.3	4.7	1.2	1.6	1.7	1.4	0.1	2.2	2.2							
α-Eudesmol	32.71	1656																1.0	1.0	
Selin-11-en-4-α-ol (γ-Selinene?)	32.9	1661	4.9	1.1	2.7	**58.0**	1.3	18.0	27.4	1.3	10.2	10.4	3.3	5.0	2.1	2.1		1.4	1.1	0.2
α-Bisabolol	33.84	1686							1.2	1.2										
Cedren-13-ol (8)	33.95	1688	2.9	1.3	1.2				5.7	1.7	1.7	1.3	1.1	0.1						
epi-α-Bisabolol	34.00	1690				1.7	1.7					4.5	4.5							
Muurol-5-en-4-one (cis-14-nor)	34.07	1692										1.4	1.4							
(Z)- α-*trans*-Bergamotol	34.09	1692				1.2	1.2													
Chamazulene	35.48	1733							3.9	0.3	2.6							19.8	4.1	5.1
Geranyl-p-cymene	42.69	1939	1.9	1.1	0.5	2.1	1.5	0.3	3.7	1.3	1.1	11.5	7.0	2.4						
Unknown	44.34	1978	6.9	1.3	3.0	10.9	2.1	5.0	8.5	1.1	4.2	3.0	2.0	0.7						
(*Z*)-Nuciferol isobutyrate	44.46	1981	9.3	1.0	3.3	14.1	1.4	6.2	19.3	1.2	6.7	**37.3**	1.1	13.2				1.1	1.1	
***(E)*-Nuciferol isobutyrate**	44.54	1983										4.8	4.8							

[a]Linear retention indices (LRI) calculated relative to the elution ranking of *n*-alkanes on HP-5 column

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