SZENT ISTVÁN UNIVERSITY

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

DOCTORAL (Ph.D.) THESIS

NGUYEN THI HUONG

BUDAPEST

2018

Head of Ph.D. School: Prof. Dr. Éva Németh Zámboriné
Doctor of the Hungarian Academy of Sciences
Head of Department of Medicinal and Aromatic Plants
SZENT ISTVÁN UNIVERSITY,
Faculty of Horticultural Sciences

Supervisor: Prof. Dr. Éva Németh Zámboriné
Head of Department of Medicinal and Aromatic Plants
SZENT ISTVÁN UNIVERSITY
Faculty of Horticultural Sciences

The applicant met the requirement of the PhD regulations of the Szent István University and the thesis is accepted for the defence process.

....

Head of Ph.D. School Supervisor

1. BACKGROUND AND OBJECTIVES

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 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.

2. MATERIALS AND RESEARCH METHODS

2.1. Detecting intraspecific variability of *A. absinthium*

2.1.1. Plant materials and cultivation

The basic plant material for the studies included gene bank accessions, market items and seeds collected from wild habitats from different regions with a detailed investigation on 12 accessions (signed: Bel, Eng, Ger0, Ger1, Ger2, Hum, HuW1, HuW2, HuW3, HuW4, Nor, Spa) of *A. absinthium*. Propagation was carried out and the seedlings were planted into open field plots in June 2016 at the Experimental Station of Szent István University in Budapest.

2.1.2. Studying the relationship of wormwood genotypes by morphological traits

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 morphological characters (leaf color, stem color, branch form, growth character, leaf form) modified from the study of Nazar and Mahmood (2011) were observed for each plant individually and for each character a numerical code (0, 1, 2, 3) was defined.

Quantitative morphological characteristics

At the same time as the above, plant height and width 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. The leaves were pressed and a herbarium was prepared following the techniques of DeWolf (1968). The blade thickness 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.

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.

2.1.3. Studying the relationship of wormwood genotypes by molecular markers

Genomic DNA was extracted from fresh leaves of nine accessions (Spa, Nor, Bel, Eng, Ger1, Hum, HuW1, HuW2, HuW3) 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.

2.1.4. 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.

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.

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 and an Agilent Technologies MS 5975 inert mass selective detector. 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).

Total phenolic content (TPC)

Determination of the TPC was carried out by the modified method of Singleton and Rossi (1965).

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).

2.2. Revealing factors influencing the chemosyndroms of wormwood

2.2.1. Ontogenetic and morphogenetic factors

Plant material and growth conditions

The plant material used in this study consisted of both thujone (T) chemotype and *trans*-sabinyl acetate (SA) chemotype. For revealing the role of plant development in the intraspecific variability of wormwood, the second year old plants were used and sampled in 2017. The samples from ten chosen individual plants were harvested at four different developmental phases (vegetative, floral budding, flowering, after flowering) of *A. absinthium.*

Essential oil extraction

Leaves and flowers were divided from stem parts and they were distilled separately in three replicates. The same method as described in chapter 2.4.1.

Gas chromatography-Mass spectrometry analysis

Using the same method mentioned at chapter 2.4.1.

2.2.2. Environmental factors

Treatments

Two phytotron chambers were installed with two growing programs simulating "warm" weather (higher temperature and light intensity) and "cold" weather (lower temperature and light intensity) circumstances. The experiment was conducted from September 2016 to February 2017. The treatments started after an acclimatization period of 14 weeks.

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"). 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).

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. The same method as written in chapter 2.4.1

Gas chromatography-Mass spectrometry analysis

Using the same method mentioned at chapter 2.4.1.

Total phenolic content (TPC) and Antioxidant capacity (AC)

Using the same method mentioned at chapter 2.4.1.

2.3. Optimization of wormwood cultivation

2.3.1. 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. 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). Seed germination was recorded and counted until constant readings were obtained. Germination capacity was defined, the mean germination time (MGT) for each treatment was calculated by the formula given by Ellis and Roberts (1981). Additionally, root length was measured by using a vernier caliper.

2.3.2. Study on vegetative propagation

90 cuttings of Hungarian cultivated material plantation 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).

Layering of 10-10 individuals in both perennial populations ("Hum" and "Spa") was conducted in March 2016 (before the beginning of the vegetation).

2.3.3. Study on allelopathic activity of wormwood

Treatments with different types of plant materials

The shoots of the accession "Hum" 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. Fresh leaves, dried leaves and water extract were used as the treatments and the control treatment 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. The number of germinated seeds and the mean germination time, the number of roots were counted and recorded; additionally, the length of roots of each seedling was also measured by using a vernier caliper.

Treatments with wormwood leaf powder

The shoots of the accession "Hum" were cut at about five cm height above the soil surface. 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. The number of germinated seeds and the mean germination time, the number of roots were counted and recorded; additionally, the length of roots of each seedling was also measured by using a vernier caliper.

Treatments with aqueous extracts

The aqueous extracts of leaves of two *A. absinthium* chemotypes - a high thujone chemotype and sabinene + ß-myrcene chemotype 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. 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. The length of roots of each germinated seed was measured by using a vernier caliper.

2.4. 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. 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 and EO content of two accessions growing in climatic chambers as well.

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.

Principal component analysis (PCA): 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.

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. 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).

3. RESULTS

3.1 Intraspecific variability of wormwood

3.1.1 Study the relationship of wormwood genotypes by morphological traits

Qualitative morphological characteristics

Based on these differences, the individuals of the studied accessions could be clustered into four groups. Group 1 consists of the most individuals of accessions “Nor” and “Spa”. Most Hungarian accessions, “Eng” and “Ger0” belonged to group 2. The third group had primarily the individuals of accession “Ger2”. The fourth group had the most individuals of accessions “Bel” and “Ger1”.

Quantitative morphological characteristics

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. 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”).

Biomass production

The biomass of twelve studied accessions varied from 63.4 g plant^{-1} (“Nor”) to 322.4 g plant^{-1} (“HuW2”). 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.

Studying the relationship of wormwood genotypes by molecular markers

A total of 196 scorable bands were generated from 15 ISSR primers. The proportions of polymorphic bands among wormwood accessions was high, with 81.15% for RAPD and 73.10% for ISSR. Based on the genetic distance matrix of the 9 accessions, the UPGMA dendogram demonstrates the grouping of the investigated accessions in three main clusters. Accession "Spa" 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 another accessions.

3.1.2. Studying the relationship of wormwood genotypes by phytochemical characteristics

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.

Essential oil components and wormwood chemotype

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). 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%).

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.

Total phenolic content and antioxidant capacity

The total phenolic content of the investigated accessions ranged from 113.08 (mg GAE/g DW) to 161.84 mg GAE/g DW. Accession "Bel" reached the highest value of TPC while accession "Spa" showed the lowest TPC. 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).

3.2. Factors influencing the chemosyndroms of wormwood

3.2.1 Ontogenetic and morphogenetic factors

Essential oil content

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).

Essential oil composition

The composition of the EO in the sampled phenophases and plant organs in case of the ***thujone chemotype*** was analysed. 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). 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. 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.

The GC results of the ***trans-sabinyl acetate chemotype*** revealed that 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 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. 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. A decreasing tendency was detected in the ratios of neryl-isovalerate and ß-selinene during the plant development.

3.2.2. Environmental factors

Essential oil yield and composition

The essential oil yield varied from 0.188 ml/100mg (Hungarian accession, “warm” chamber) to 1.092 ml/100mg (Spanish accession, “cold” chamber).

The total identified percentage of components varied from 76.3% (Hungarian accession in “cold” treatment) to 87.5% (Spanish accession in “cold” treatment). 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%). The different climatic conditions created in the two chambers have markedly influenced the EO composition. However, the changes are mostly quantitative.

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.

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.

3.3. Optimization of wormwood cultivation

3.3.1. Germination

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). 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.

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. Similar results were observed in the case of Spanish seeds, as well.

3.3.2. Study on vegetative propagation

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. In the case of layering method, 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 (accession “Spa”) to 49.43 cm (accession “Hum”), thus, the young Spanish plants were shorter compared with the Hungarian ones.

3.3.3. Study on allelopathic activity of wormwood

Treatment with different types of wormwood materials

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).

Treatments with wormwood leaf powder

The plant height was reduced by 39.6% as the largest effect (in mustard, at the "50Spa" treatment). 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). 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.

Treatment with aqueous extracts of wormwood

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). 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.

4. 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.

5. CONCLUSIONS

5.1 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.

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. 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.

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 ml/100g and 3.215 ml/100g. The three groups of statistical analysis 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 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.

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.

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.

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.

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.

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.

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.

5.2 Factors influencing the chemosyndroms of wormwood

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: the flowers contain higher ratios of volatiles than do the leaves and the content decreases during the flowering time.

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. Concerning the changes of the ratio of individual components it could be established that they are mostly quantitative ones for both chemotypes.

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. 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.

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.

5.3. Optimization of wormwood cultivation

In the frame of our work we started the elaboration of propagation methods. 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.

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.

Artemisia species are referred to exhibit an inhibitory effect against seeds of other species (Heeger, 1956; Funke, 1943). 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.

LIST OF USED LITERATURE

1. Adams, R.P. (2007). *Identification of Essential Oil Components By Gas Chromatography/Mass Spectrometry, 4th Edition* (4th edition). Carol Stream, Ill: Allured Pub Corp.
2. Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, *239*(1), 70–76
3. Chialva, F., Liddle, P. A. P., & Doglia, G. (1983). Chemotaxonomy of wormwood (*Artemisia absinthum* L.). *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 176(5), 363–366. https://doi.org/10.1007/BF01057728
4. DeWolf, G. P. (1968). Notes on making an herbarium. *Arnoldia*, 28(8/9), 69–111
5. Ellis, R. H., & Roberts, E. H. (Reading U. (UK) D. of A. and H. (1981). The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology (Netherlands)*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=XE8182678
6. Funke, G. L. (1943). The influence of *Artemisia absinthium* on neighbouring plants. (an essay of experimental plant sociology No. III). *Blumea-Biodiversity, Evolution and Biogeography of Plants*, *5*(2), 281–293.
7. Hammer, P., Øyvind, Harper, D. A. T., & Ryan, P. D. (2001). Past: Paleontological statistics software package for education and data analysis. Retrieved from http://citeseerx.ist.psu.edu/viewdoc/citations;jsessionid=F7F97BD571DFF2168BBCD426E9ED1B4D?doi=10.1.1.459.1289
8. Heeger, E. F. (1956). Handbuch des Arznei- und Gewürzpflanzenbaues; Drogengewinnung. Reprintausgabe: Verlag Harri Deutsch Frankfurt am Main 1989, Deutscher Bauernverlag, Berlin, 776 p.
9. Judzentiene, A., & Budiene, J. (2010). Compositional variation in essential oils of wild *Artemisia absinthium* from Lithuania. *Journal of Essential Oil Bearing Plants*, 13(3), 275–285. https://doi.org/10.1080/0972060X.2010.10643822
10. Konowalik, K., & Kreitschitz, A. (2012). Morphological and anatomical characteristics of *Artemisia absinthium* var. absinthium and its Polish endemic variety *A. absinthium* var. calcigena. *Plant Systematics and*

Evolution, *298*(7), 1325–1336. https://doi.org/10.1007/s00606-012-0639-z

11. Lewontin, R. C. (1972). The Apportionment of Human Diversity. In T. Dobzhansky, M. K. Hecht, & W. C. Steere (Eds.), *Evolutionary Biology* (pp. 381–398). Springer US. https://doi.org/10.1007/978-1-4684-9063-3_14
12. Nazar, N., & Mahmood, T. (2011). Morphological and molecular characterization of selected Artemisia species from Rawalakot, Azad Jammu and Kashmir. *Acta Physiologiae Plantarum*, 33(2), 625–633. https://doi.org/10.1007/s11738-010-0545-3
13. Nin, S., Arfaioli, P., & Bosetto, M. (1995). Quantitative determination of some essential oil components of selected *Artemisia absinthium* Plants. *Journal of Essential Oil Research*, 7(3), 271–277. https://doi.org/10.1080/10412905.1995.9698518
14. Rezaeinodehi, A., & Khangholi, S. (2008). Chemical composition of the essential oil of *Artemisia absinthium* growing wild in Iran. *Pakistan Journal of Biological Sciences*, 11(6), 946–949. https://doi.org/10.3923/pjbs.2008.946.949
15. Van Den Dool, H., & Dec. Kratz, P. (1963). A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. *Journal of Chromatography A*, 11, 463–471. https://doi.org/10.1016/S0021-9673(01)80947-X
16. Weisbord, S. D., Soule, J. B., & Kimmel, P. L. (1997). Poison on line — Acute renal failure caused by oil of wormwood purchased through the internet. *New England Journal of Medicine*, 337(12), 825–827. https://doi.org/10.1056/NEJM199709183371205
17. Yeh, F. C., Yang, R.-C., Boyle, T., Ye, Z.-H., & Mao, J. X. (1997). Popgene: the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre. Retrieved from https://www.scienceopen.com/document?vid=5db9fe1d-3632-465d-9e0a-5b2c7f417d08

PUBLICATIONS CONNECTED WITH THE TOPIC OF THE THESIS

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