BUDAPESTI CORVINUS EGYETEM

# EVALUATION OF CHEMICAL DIVERSITY AND CULTIVATION POSSIBILITIES OF NATIVE *THYMUS* (THYME) SPECIES

DOCTORAL THESES

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# 1. Introduction and scientific aims

*Thymus pannonicus* All. and *Thymus glabrescens* Willd. are medicinal plants native to Hungary (Király, 2009). Flowering shoots of both species can be collected together under drug name *Serpylli herba* (Ph. Hg. VIII, 2004). Drugs, tea preparations and essential oils obtained from wild thyme species are used for ages to treat the diseases of the upper respiratory tract as anticatarrhal and expectorant agents in colds and flu (Csupor, 2003). Recent studies have shown the antimicrobial activities of their essential oils (Maksimović *et al.,* 2008a,b) as well as the presence of hydroxycinnamic acid derivatives of antioxidant features in their flowering shoots (Boros *et al.,* 2010).

Chemical variability regarding essential oil compounds is already known in the genus *Thymus*, which can be attributed to genetical and ecological factors. If the levels of biological active agents of shoots collected in wild populations are changeable or do not meet the requirements specified by *Pharmacopoeia Hungarica* VIII.(2004), the therapeutical application will be questionable. From this respect the habitat survey and the examination of biological active compounds of wild growing populations of medicinal plants became actual. Based on the description of chemotypes occurring in natural habitats, locations for collection and valuable genotypes for breeding can be recommended. In order to examine the changes in chemical polymorphism and the stability of chemotypes, progenies of wild accessions are suggested to be compared under equal environmental conditions, grown in experimental plots.

Native populations and experimental accessions being of different origin and belonging to two widespread Hungarian thyme species (*Thymus pannonicus* and *Th. glabrescens)* were chosen as objects of our studies. As both species proved to be highly adaptive and frequent in natural habitats we have found further prospects in surveying their chemical patterns, environmental factors as well as cultivation possibilities. According to the literature, *Thymus pannonicus* and *Th. glabrescens* may occure together in plant communities, where essential oil diversity has yet been examined partially. Our research work also involved the evaluation of chemical stability, when comparing the essential oil patterns originating from wild growing populations and established thyme stands.

In the focus of Hungarian *Thymus* research, there are progenies of valuable genotypes having high drug yield, essential oil content and even high thymol ratio. I was also engaged in studying and developing several elements of cultivation technology in the case of both thyme species involved. Furthermore, selection of accessions with upright habit, facilitating harvest procedures was also amongst our purposes.

Although *Th. vulgaris* is indigenous in the Mediterranean area and can be considered as a distant relative, data obtained on its cultivation may serve as a basis for developing growing technics of wild thyme species.

In order to elaborate the most effective propagation methods and to find the most appropriate seed storage conditions for genebank reservation of *Thymus* taxa, optimal germination conditions of seeds were to be examined at first (Palevitch, 1988). In this respect, only reference data for *Th. serpyllum* and *Th. vulgaris* could have been used.

Besides *Th. vulgaris*, wild growing *Thymus* species deserve attention, as the survey of biological active agents of medicinal plants is always actual and important for pharmaceutical industry as well. In this respect thymoquinone is worthy to be mentioned which is an active agent with antitumor and antioxidant properties, detected recently in thyme shoots as well (Radonic & Mastelic, 2008).

**<u>My experimental aims were as follows:</u>**

**I. Evaluation of the chemical diversity of two thyme species involved**

1. To survey the essential oil content, essential oil composition and total hydroxy-cinnamic acid derivatives in shoots of *Thymus pannonicus* and *Th. glabrescens* collected both from wild habitats and from experimentally grown accessions
2. To describe correlations between the essential oil properties and the soil parameters of wild growing *Thymus pannonicus* and *Th. glabrescens* populations

**II. Examination of cultivation possibilities**

3. To survey the growth and phenological features of reproduced *Thymus pannonicus* and *Th. glabrescens* progenies
4. To elaborate the generative and vegetative propagation methods for *Thymus pannonicus* and *Th. glabrescens*
5. To evaluate the efficiency of agrotextile mulching on introduced *Thymus pannonicus* and *Th. glabrescens* accessions

**III. Evaluation of progenies concerning gene reservation and breeding**

6. To reserve the genetical resources of valuable chemovariants
7. To evaluate the breeding prospects for chemotypes proven to have stable essential oil features

# 2. Materials and methods

## 2.1. Materials and experimental sites

### 2.1.1. Origin of wild growing *Thymus pannonicus* and *Th. glabrescens* populations

Studies in natural habitats were carried out in 2010 and in 2011 from spring till late autumn, involving **24 locations**. Regarding **North-Transdanubia,** Keszthely Hills (Balatongyörök), Balaton Uplands (Balatonarács, Tapolca), Bakony Hills (Szőc), Vértes Hills (Csákberény), Buda Hills (Érd, Budapest, Nagykovácsi), Pilis Hills (Dorog, Pilisszántó, Pilisszentiván) and Visegrád Hills (Visegrád, Szentendre) were surveyed. Habitats in the **North Hungarian Mountains** were as follows: Bükk Mountains (Cserépváralja, Mónosbél), Bükk Piedmont (Bogács, Noszvaj), Putnok Hills (Sajógalgóc), Aggtelek Karst (Aggtelek, Jósvafő) and Cserehát Hills (Szendrőlád, Rakaca, Rakacaszend).

### 2.1.2 Plant material used at the field experiments

Propagation of selected *Thymus* genotypes and the examination of their cultivated progenies were carried out at the **Soroksár Medicinal Plant Research Station of Corvinus University of Budapest** (Hungary), in the period of 2010-2012. Younger (one and two year old) and older (five, six and seven year old) accessions were propagated via seeds in 2011 and 2005, accordingly. Accessions of *Th. pannonicus* (TPA) originating from Backa region (Ada), Gödöllő Hills (Ceglédbercel) and Bakony Hills (Fenyőfő), while those of *Th. glabrescens* (TGL) from Bakony Hills (Csesznek) and Balaton Upland (Szentbékkálla), respectively.

## 2.2. Methods

### 2.2.1. Studies on natural habitats

During sampling, **flowering and** rarely **vegetative shoots** were collected from *Thymus* populations existing at wild habitats in order to evaluate drug quality and chemical patterns. If both species were found at the same habitat or a species in different coenological status, they were distinguished by using different codes. Samples were dried in natural way before chemical analysis. Seeds were collected in autumn for propagation purposes (from Aggtelek, Balatonarács, Cserépváralja, Érd, Mónosbél, Nagykovácsi).

**Soil samples** were collected from the rhyzosphere (0-20 cm) of wild growing *Thymus* plants. Soil characters were assessed in the Central Laboratory of the Faculty of Food Science, Corvinus University of Budapest, by measuring the following parameters of the soil samples: pH, $K_A$, Ca (%), $CaCO_3$ (%), salt (%), humus (%) as well as contents of $P_2O_5$-, $K_2O$-, $NO_3$- (mg/kg).

### 2.2.2. Experiments in the research field

Seeds were collected in 2010 from wild habitats and in 2010 from the cultivated, five year old accessions for **propagation experiments** and were sown under greenhouse conditions at the Research Station on 17$^{th}$ March, 2011. In September they were planted out to experimental plots covered or uncovered by agrotextile, by plant density of 40x30 cm.

Studies on **harvest optimization** were carried out from May till July in 2011 and 2012 in order to evaluate the accumulation of biological active agents. Plant samples were dried naturally.

When evaluating the efficacy of the **agrotextile mulching**, weed growing dynamics was examined in treated (covered) and control (uncovered) plots in 2011, where 2-2 surveying sectors (each being of 4 $m^2$) were taken into consideration.

Measurements on **growth** were performed on the two year old accessions of *Thymus pannonicus* and *Th. glabrescens* in 2012. Plant height, width and length parameters (cm) were surveyed in three consecutive months (on 21$^{st}$ of March, 20$^{th}$ of April and 18$^{th}$ of May, 2012).

**Plant division** experiment was carried out in 2011, when shoots of six year old accessions were divided on 11$^{th}$ of May, 2011. To increase rooting of divided shoots, 0.4% β-IBA hormone was applied and shoots were bedded into a mixture of sand and perlite (Probocskai, 1980). During rooting in greenhouse, plants were irrigated daily, then plants were potted on 8$^{th}$ of June, 2011. Bedding out was due on 15$^{th}$ of September, 2011, applying a plant density of 40x30 cm.

### 2.2.3. Methods of laboratory experiments

Investigations on the quantity and quality of **biological active compounds** of dried drugs were carried out in the laboratory of Department of Medicinal and Aromatic Plants, Faculty of Horticultural Science, Corvinus University of Budapest.

Essential oil of dried plant samples of wild growing or cultivated *Thymus* accessions was isolated by hydrodistillation in a Clevenger type apparatus, and the ***essential oil content*** was expressed in mL/100 g, calculated on a dry weight (DW) basis.

***Percentage composition (%) of the essential oil*** samples were analyzed by gas chromatography (GC), by Agilent Technologies 6980 N GC (Agilent Technologies, USA) equipped with HP-5 or HP-5MS capillary coloumns (5% phenyl, 95% dimethyl polysiloxane; length: 30 m, film thickness: 0.25 µm, id. 0.25 mm), the carrier gas was helium (with a constant flow rate of 0.5 mL/min), injector and detector temperature was 250 ˚C. Temperature program of the column was as follows: initial temperature 50 ˚C/5 min, then by a rate of 4 ˚C/min up to 150 ˚C, then by a rate of 12 ˚C/min up to 220 ˚C/10 min. We applied flame ionisation detector (FID) or mass spectrometer (Agilent Technologies MS 5975, USA). In the case of MS, the identification of components were carried out on the basis of mass spectrum by means of data of NIST and home-made libraries, while when applying FID, the standard addition method was used and the standard retention times and indices were observed.

***Chemotypes*** were identified and distinguished according to the principal essential oil compounds (9 %<), and accordingly they were evaluated as known or new for the literature.

We have adapted the test method of ***total hydroxycinnamic acid derivatives*** of *Melissae folium* described in valid *Pharmacopoeia Hungarica* (Ph. Hg. VIII., 2004) to *Serpylli herba*. The absorbancy of the test-solution was measured on λ=505 nm without delay against the compensation solution, using spectrophotometer (Spectro UV-VIS Dual Beam; Laborexport Kft.). The total hydroxycinnamic acid derivative amount was expressed in rosmarinic acid (%) on the basis of specific absorption coefficient ($A_{1\ cm\ 1\%}$ = 400) of rosmarinic acid on λ=505 nm. The total hydroxycinnamic acid derivative content was calculated by the following formula: *(5xA)/m*, in which *A* is the absorbancy of the test solution on *λ=505 nm* and *m* is the weight (grams) of the drug examined.

Before carrying out the **germination tests**, *Thymus* seeds were ***stored*** at room ***temperature*** (at 20-24 $^{o}$C) or in refrigerator (at 4 $^{o}$C). Germination studies were performed in SANYO Versatile Environmental Test Chamber, applying the conditions prescribed in the Hungarian Standard of *Thymus vulgaris (*MSZ 6354-3, 2008) (28 day test, 20/30˚C alternating temperature set with a 16/8h dark/light period). To find the optimal ***germination temperatures*** the low temperature program (10/15˚C) was applied too. At each test, 3 replications were applied with 33 seeds per Petri Dishes. When surveying the ***light*** as germination inducing factor, Petri Dishes of the „dark treated" group were covered by tinfoil. To evaluate the effectiveness of ***germination inducing agents***, we placed seeds in 250 ppm $GA_3$-, 1% $KNO_3$- or 10%, 20% $PEG_{400}$-solutions as pretreatments, lasting for 24 hours.

### 2.2.4. Methods of statistical analysis

Statistical analyses were carried out by using *PASW Statistics 18, Statistica 11* and *SPSS 20* program packages. Pairwise t-tests, Cluster analysis, correlation analysis or analysis of variance (ANOVA) tests were applied. Differences measured at variable treatments were considered as significant in the case of $p<0.05$.

## 3. Results and conclusions

### 3.1. Occurence and habitat preferences of the examined *Thymus* populations

When examining the wild populations and the available literature, it was found that both species can be considered as *tolerant with respect to the soil type and base rock*. *Th. glabrescens* (TGL) could have been found on base rocks of limestone, dolomite, rhyolite and sand while *Th. pannonicus* (TPA) could have developed on base rocks of limestone, dolomite, rhyolite, loess, marble, vulcanic tuff, claystone and mudstone. Soil samples of TPA possessed higher phosphate, potassium and humus content, than those of TGL. TPA was capable of tolerating soils of lower pH values as well, however, in average, both species preferred neutral soils. In this respect, *our findings complement the existing literature* concerning the two species involved (Mártonfi *et al.*, 1996; Pluhár *et al.*, 2011).

### 3.2. The chemotaxonomical evaluation of natural *Thymus* populations

It was recorded that the investigated samples of *Th. pannonicus* (TPA) and *Th. glabrescens* (TGL) originating from wild populations of Hungary, were proven to show different quality, variable essential oil content and distinct composition. The biosynthesis of the essential oil compounds is genetically determined, which is proven scientifically and can be confirmed by my findings as well. It was ascertained on the basis of plant samples gathered from wild habitats, that *TPA is more able to provide higher essential oil levels*, than TGL. According to the literature and my results, collection of TPA is suggested from selected natural populations. Concerning *the influence of the year*, I observed that shoots originating from wild growing populations of TPA, gathered in 2010 showed much higher and less extreme results concerning essential oil content (0.270-1.079 ml/100g) than those obtained in 2011.

Furthermore, it was also found that *samples of Th. pannonicus collected from wild populations, a thymol rich essential oil can be obtained* very frequently, which is favourable in respect of therapeutical use. Meanwhile, essential oil samples of *Th. glabrescens* originating from wild habitats were rich in - so far therapeutically not thoroughly examined - sesquiterpenes. Publications regarding the reasons of this phenomenon have not been published yet.

Several new chemotypes of the two species could have been described and the demonstration of it can be seen below, based on the species and the main terpene compounds.

<u>17 new *Thymus pannonicus* chemotypes have been found as follows:</u>

***Essential oils with monoterpene main compounds***

- **carvacrol (40.7%)/p-cymene (15.9%)/γ-terpinene (13.7%)-** Bükk Mountains (Mónosbél)
- **linalool (24.6%)/p-cymene (14.1%)/thymol (10.8%)/thymol methyl ether (9.9%)/carvacrol (10.3%)-** Bükk piedmont (Noszvaj)
- **linalool (26.6%)/thymol (22.3%)/p-cymene (14.6%)/γ-terpinene (11.1%)-** Szendrő Hills (Rakacaszend)
- **linalool (47.1%)/p-cymene (15.1%)-** Bükk piedmont (Bogács), and **linalool (61.6%)/p-cymene (12.5%)-** Pilis Hills (Szentendre)
- **linalool (61.5%)/thymol (11.1%)-** Aggtelek Karst (Jósvafő)
- **linalool (68.7%)-** Bükk piedmont (Bogács)
- **p-cymene (28.0%)/thymol (11.7%)/thymol methyl ether(11.7%)/linalool (10.6%)-** Bükk piedmont (Noszvaj)

- **p-cymene (31.6%)/thymol (17.3%)/thymol methyl ether (17.2%)-** Bükk piedmont (Noszvaj)
- **thymol (30.2%)/p-cymene (25.9%)/thymol methyl ether (13.4%)/γ-terpinene (9.5%)-** Vértes Hills (Csákberény)
- **thymol (40.7%)/p-cymene (19.8%)/carvacrol methyl ether (11.9%)/thymol methyl ether (10.2%)-** Vértes Hills (Csákberény)

***Essential oils with sesquiterpene main compounds***

- **β-cadinene (28.8%)/germacrene D (13.2%)-** Bükk Mountains (Cserépváralja)
- **germacrene D (26.4%)/caryophyllene oxide (10.4%)-** Aggtelek Karst (Aggtelek)
- **tau-cadinol (36.5%)/germacrene D (13.6%)-** Aggtelek Karst (Aggtelek)

***Essential oils with mixed (mono- and sesquiterpene) main compounds***

- **geranyl acetate (24.1%)/β-bisabolene (16.3%)/geranial (12.0%)-** Aggtelek Karst (Jósvafő)
- **linalool (38.5%)/trans-sabinene hydrate (13.7%)/β-cadinene (12.9%)-** Aggtelek Karst (Jósvafő)
- **thymol (26.1%)/geraniol (23.5%)/geranyl acetate (12.7%)/β-bisabolene (10.5%)-** Bükk Mountains (Mónosbél)
- **thymol (38.2%)/γ-terpinene(12.1%)/β-bisabolene (11.3%)**- Tapolca Valley (Tapolca)

12 chemotypes of *Thymus glabrescens* proved to be new for the literature:

***Essential oils with sesquiterpene main compounds***

- **β-caryophyllene (15.8%)/germacrene D (15.3%)-** Buda Hills (Budapest)
- **β-caryophyllene (29.8%)/germacrene D (23.8%)/β-cadinene(11.9%)-** Pilis Hills (Pilisszentiván)
- **β-caryophyllene (36.7%)/caryophyllene oxide (27.7%)/germacrene D (15.8%)/E,E-pharnesol (11.6%) –** Keszthely Hills (Balatongyörök)
- **germacrene D (17.8%)/nerolidol (12.9%)/β-cadinene(12.9%)/ β-bisabolene(9.2%)-** Pilis Hills (Dorog)
- **germacrene D (29.5%)/biciclogermacrene (17.2%)/β-caryophyllene (10.8%)/tau-cadinol (9.1%)-** Buda Hills (Nagykovácsi)
- **germacrene D (32.1%)/β-caryophyllene (27.3%)/δ-cadinene (13.9%)-** Buda Hills (Budapest)
- **germacrene D (44.7%)/β-caryophyllene (13.9%)/ biciclogermacrene (10.5%)-** Buda Hills (Nagykovácsi)
- **germacrene D (49.4%)-** Balaton Uplands (Szőc)
- **germacrene D (56.9%)/β-pharnesene (9.0%)-** Buda Hills (Érd)
- **caryophyllene oxide (35.8%)/β-caryophyllene (26.5%)/α-humulene (9.5%)-** Pilis Hills (Pilisszentiván)
- **tau-cadinol (43.2%)/germacrene D (15.5%)/cis-γ-cadinene (10.4%)-** Vértes Hills(Csákberény)

***Essential oils with mixed (mono- and sesquiterpene) main compounds***

- **germacrene D (49.4%)-** Balaton Uplands (Szőc)

### 3.3. Correlations between the essential oil and soil parameters

On the basis of correlation analysis of essential oil features and soil parameters of populations of *Th. pannonicus* and *Th. glabrescens,* I proved that strong positive correlation could have been found between the essential oil amount and the salt, nitrate, calcium ion, calcium carbonate content of the soils examined, while it showed weaker correlation with soil phosphate content. Between the calcium carbonate content and the essential oil content, strong positive correlation was found. The humus content of the soils and the essential oil levels measured in the plants were in direct relation in the case of *Th. pannonicus* while inverse relation could have been observed at *Th. glabrescens* in this respect.

## 3.4. Evaluation of the results obtained in cultivation experiments

### 3.4.1. Improvement of propagation methods and the possibilities of gene reservation

Concerning the two plant species involved, the first germination tests were carried out by us. The examined seeds showed low germination capacity among laboratory circumstances, after a half year of storage, both in control and treated groups. According to our results, germination of seeds of both species is *independent of light*. *The applying of the higher alternating temperature program (30/20 ˚C) resulted in higher germination rates* than the lower set (15/10 ˚C). *Significantly higher germination capacity* was found at seeds stored in paper+nylon bags *at the temperature of +4 ˚C* than seeds stored at 20-24 ˚C. Germinating-power could not have been improved by applying any of the germination stimulating agents. Seeds may have afterripening tendency and *endogenous dormancy* as well. Annual gathering and reservation of their seeds is proposed.

Thyme species can be propagated by plant division or seed sowing. The average rooting rate of *Th. pannonicus* propagated via slipping at the research station was 58.2%, while that of *Th. glabrescens* was 46.5%. Highest rooting ratios were found at the Ada accession of TPA and Szentbékkálla progeny of TGL. Our data correspond to the literature described for other *Thymus* species concerning the adequacy of 0.4% β-indole-3-butyric acid used as rooting hormone and the timing of the procedure. According to our results plant division is proposed to begin in mid May and bedding-out is appropriate in September (Driemeier-Kreimeier & Barros, 2006; Fodor, 1991; Gyöngyösi *et al*., 2008).

### 3.4.2. Assessment of agrotextile mulching

The usefulness of applying agrotextile was that even if the shoots grew together, they were not able to root down and consequently, they could have been harvested more easily in contrary to progenies grown on control field without mulching. Agrotextile mulching was effective in *reducing the manual labour* as in the mulched field much less weed species could have been found with lower abundance. Agrotextile mulching *increased the puritiy of the drug and the essential oil accumulation at some progenies* of *Th. glabrescens*. To observe further advantages of agrotextile mulching on the two examined *Thymus* species, multiyear experiments are needed.

### 3.4.3. Evaluation of growth dynamics and phenology of experimental accessions

Concerning plant habit, *Th. pannonicus* is valuable for cultivation as it has more advantageous, upright shoots and its flower/shoot ratio is also higher than that of *Th. glabrescens*. Agrotextile mulching did not influence significantly the growing rate and parameters of shoots of cultivated accessions. According to our results, *a plant density of 50x40 cm is recommended for both species in cultivation.*

One year old individuals of *Th. pannonicus* and *Th. glabrescens*, propagated via seeds in March, bloomed first in mid August, while earlier flowering periods (from May till July) could have been observed at older (5-7 year old) accessions. A *second blooming phase* was detected at younger (2 year old) progenies, while older ones have generally no tendency for second flowering. The *optimal time for harvest* of both species can be planned from the end of May till mid June, which includes the beginning of full blooming.

### 3.4.4. Assessment of the active substance content of experimental *Thymus* accessions

***Evaluation of the factors influencing the essential oil content***

*Accessions grown* under the same environmental conditions at the Research Station, showed higher accumulation levels of essential oil and more balanced values on the average than wild populations sampled in natural habitats. At both species, the *younger* accessions accumulated higher essential oil levels than older ones being of same origin in the experimental field. However, significant differences couldn't have been found between the 1and 6 year old (gathered both in 2011) or the 2 and 7 year old (harvested both in 2012) progenies.

According to the statistical analysis, the *age* (one, two, five, six and seven year-old) does not affect the essential oil content, while the *time of harvest* (end of May or September) and the *phenophase* influences essential oil accumulation. Vegetative shoots of both species, sampled after first cutting, contained significantly less essential oil. I have found that samples obtained during first flowering (May-July) showed much better results both qualitatively and quantitatively than those of further harvests (August-September), especially in the case of *Th. pannonicus*.

According to my results, the *older* (5-7 year old) cultivated accessions of *Th. glabrescens* were not able to provide the required essential oil content (0.300 mL/100g) specified in the valid *Hungarian Pharmacopoeia* (Ph. Hg. VIII., 2004). In contrary to accessions of *Thymus glabrescens* and *Th. vulgaris* (Pank & Krüger, 2003; Pluhár *et al*., 2003), cultivated accessions of *Th. pannonicus* were capable to accumulate high amount of essential oil, even above 0.900 ml/100g on the average, always meeting the minimum limit of Ph. Hg. VIII (2004).

After evaluating the effect of propagation, I ascertained that progenies of plant division had lower essential oil levels than younger and older progenies propagated via seeds, though, our results are based only on studies of one year (2011).

### *Evaluation of the factors influencing the essential oil composition*

*Stability of chemotypes* of experimental *Thymus pannonicus* progenies was found to be independent of cutting time (1 and 2 year old accessions) and of age as well (1, 2, 5, 6, 7 year old stands). Most of these accessions possessed thymol dominated essential oil (30%<thymol ratio<60%) with the exception of some progenies of Ada (Vajdaság) and Mónosbél, which maintained their original chemotype at the experimental field, with main compounds of geraniol and geranyl acetate.

One year old accessions of TPA, propagated from seeds, have been proven to retain the original essential oil pattern of wild growing populations or their essential oil spectrum tended to be very similar to the original one. This phenomenon was characteristic for the 5-7 year old progenies as well. On the basis of these results, *Th. pannonicus* is suitable for introduction, its accessions have stable and valuable chemotype patterns which proved to be perspective for breeding and cultivation. Several kinds of dominant components were detectable from essential oils of TGL while from those of TPA only a few compounds could have been identified. Therefore the former possesses of more variable, while the latter more homogenous essential oil composition.

When examining the effect of cutting time, it was established that the essential oils of first flowering *Th. pannonicus* progenies contained thymol and γ-terpinene in highest ratio, while during second flowering, the most frequent components were thymol and p-cymene. At second blooming of *Th. pannonicus*, the ratio of thymol methyl ether, carvacrol methyl ether and β-bisabolene have increased. The essential oil compounds of *Th. glabrescens* accessions were more variable during second flowering.

### *Evaluation of results of total hydroxycinnamic acid derivatives*

As I applied and adapted for the first time the method of determination of total hydroxycinnamic acid derivatives (%) for thyme species, in this respect our results are new for the literature. It was proven that *habitat* and *genotype* are significant factors influencing the accumulation of total hydroxycinnamic acid derivatives in the examined *Thymus* shoots but neither *age* nor *species* or *ontogenetical phase* have considerable influence on levels of these agents. Independently of age, the total hydroxycinnamic acid derivative content values of cultivated accessions were very similar to those of wild growing populations. One year old, cultivated accessions showed a much balanced accumulation level (1.75-2.42 %), than shoots of older, wild growing populations (0.89-2.35 %). The latter phenomenon can be accributed to the uniform, less extreme circumstances acting at the research station and the lack of competitors. In contrary to data obtained at the analysis of essential oil content, the two species accumulate hydroxycinnamic acid derivatives in the same amount.

### 3.5. Summary and proposals

According to our results <u>*Th. pannonicus is worth collecting and cultivation*</u> owing to its upright habit, higher average drug yield, flower/plant ratio and essential oil content (ml/100g) with adequate, high thymol ratio (%) in it and its stable essential oil composition (chemotype).

Extremely high accumulation levels of essential oil and thymol ratio could have been identified at progenies of *Ceglédbercel* and *Fenyőfő* and at genotypes originating from wild habitats such as *Balatonarács, Balatongyörök, Csákberény, Dorog, Tapolca* and *Visegrád* all of which represented *Th. pannonicus*. Their *habitats can be proposed to be collection sites* in consideration of regulations prevailing over protected areas.

Concerning both species a remarkable *in vivo collection* has been established at the Research Station of Soroksár. Seed materials of each accessions have been reserved in the genebank of the Department annually. The Research Station can be considered as an adequate place for introduction and cultivation of *Th. pannonicus* and *Th. glabrescens* possessing of good permeable sand soil, high amount of sunshine and periodical irrigation. In the future the task will be to further evaluate the productivity of selected and valuable genotypes and to work out and optimize the elements of their cultivation technology.

On the basis of the previous literature and my findings, *Th. pannonicus* and *Th. glabrescens* are valuable genetical resources and they have outstanding accessions for further examinations.

# 4. New scientific findings

Investigations on *Thymus pannonicus* and *Th. glabrescens* were carried out in populations of new natural habitats and on accessions cultivated under the same experimental conditions in the years of 2010-2012, where the effects of origin, phenophase, propagation methods, growing conditions and age were taken into consideration.

**1. New findings on the essential oil features of the two *Thymus* species are the following:**

- Several new chemotypes have been found in wild growing populations examined in 2010-2011.
- The most frequent essential oil compounds for samples of native *Th. pannonicus* populations were thymol, p-cymene, γ-terpinene, linalool and thymol methyl ether.
- Collection sites can be proposed concerning *Th. pannonicus* showing appropriate essential oil quantity and quality with high thymol levels.
- Essential oil properties of samples belonging to *Th .glabrescens* proved to be variable and rich in sesquiterpene compounds.
- On the basis of correlation analysis concerning soil parameters and essential oil properties I proved that salt, nitrate, Ca and $CaCO_3$ contents of the soil samples had strong influence on the essential oil content of the two species.
- Experimental progenies of the accessions had more balanced and higher essential oil levels than samples collected from the respective wild habitats.
- It was ascertained for the first time that plant age can not be considered as an influencing factor on the essential oil properties of *Thymus pannonicus* and *Th. glabrescens* progenies.
- I documented that essential oil properties of both species were influenced by the taxa (genotype), phenophase, cutting time and agrotextile mulching, respectively.
- It was established that *Th. pannonicus* samples obtained from the phenophase of first flowering provided higher essential oil content and thymol % than those of the second flowering.
- It was found in my studies for the first time that the essential oil features of *Th. pannonicus* progenies were stable, predictable and reliable under all experimental conditions, meeting the requirements of the *Hungarian Pharmacopoeia* VIII. (Ph. Hg. VIII., 2004) as well.

**2. New findings regarding the accumulation of hydroxycinnamic acid derivatives are as follows:**

- Total hydroxycinnamic acid derivative content in shoots of the examined *Thymus* species was measured first in my experiments
- Almost the same total hydroxycinnamic acid derivative content (%) could have been detected from samples gathered from wild habitats and from the experimental fields of both species.
- According to my results, both habitat conditions and genotype are significant factors influencing the amount of these compounds in shoots of *Th. pannonicus* and *Th. glabrescens*.
- I proved that the accumulation of total hydroxycinnamic acid derivatives was not affected either by plant age or phenophase.

**3. New results concerning germination capacity of the two thyme species are the following:**

- I have found that germination of seeds (nutlets) of the two *Thymus* taxa was independent of light as it could be induced in light and in dark as well resulting in similar capacity.

- Furthermore I proved that higher germination capacity can be reached by applying the alternating high temperature setting 20/30˚C (with 16/8 h dark/light period) and by storing their seeds in refrigerator at +4 ˚C prior to germination tests.
- Germination capacity could not have been improved by applying any of the following agents and pretreatments: 250 ppm $GA_3$-, 1% $KNO_3$-, 10% or 20% $PEG_{400}$-solutions.
- It was also established that seeds of both species possess almost the same germination capacities but the results proved to be dependent on the origin.

**4. New data in connection with the introduction of *Thymus pannonicus* and *Th. glabrescens* into cultivation are as follows:**

- Plant division experiments were carried out regarding the two species for the first time
- Although the applied 0.4% β-IBA rooting hormone, the timing of plant division (mid May) and bedding-out (September) of young plants were proved to be relatively effective, the procedure resulted in low propagation rates, which should be improved in the future.
- Experiments on agrotextile mulching were carried out for the first time by me concerning the accessions of *Th. pannonicus* and *Th. glabrescens*.
- I documented that agrotexile mulching increased the essential oil content at some *Th. glabrescens* progenies and decreased the manual labour, facilitated the harvest and increased the puritiy of the processed drugs at both species
- Plant density of 50x40 cm is recommended in field growing of the species involved.

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## Publications in connection with the dissertation

**Journal article with IF:**

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