EXPLORATION OF MORPHOLOGICAL AND CHEMICAL DIVERSITY OF HUNGARIAN WILD MARJORAM (*ORIGANUM VULGARE* L.) POPULATIONS

DOCTORAL THESIS

BEATRIX CSERHÁTI

SUPERVISOR: DR. KRISZTINA SZABÓ (PHD)

BUDAPEST, 2015

PhD School

Name: Doctoral School of Horticultural Sciences

Field: Crop Sciences and Horticulture

Head: Dr. Tóth Magdolna
Academic Professor, DSc
Department of Fruit Sciences, Faculty of Horticultural Science,
Corvinus University of Budapest

Supervisor: Dr. Krisztina Szabó
Associate Professor, PhD
Department of Medicinal and Aromatic Plants,
Faculty of Horticultural Science, Corvinus University of Budapest

The applicant met all of the requirements of the Corvinus University of Budapest PhD regulations. During the revision of the Thesis all remarks and recommendations given by the opponents were taken into consideration, thus the revised Thesis is accepted for the defence process.

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Head of the PhD School Supervisor

SCIENTIFIC BACKGROUND AND AIMS OF THE STUDY

Among the present fields of research in connection with medicinal and aromatic plants special emphasis has been laid on the study of *Origanum* genus (KINTZIOS, 2002). Its actuality originates in its very high, and up to now not fully explored diversity from morphological, chemical and genetic aspects. On the other hand, the increasing rate of consumption of *Origanum* species (oregano and marjoram) worldwide resulted in the fact that oregano is commercially one of the most valued spices on the market (SZABÓ et al., 1998). The need for exploring the diversity has induced many international cooperation programmes. Beforehand IPGRI (International Plant Genetic Resources Institute) devoted special attention to the assessment of European *Origanum* population, later a comprehensive work was realized with the direction of LUKAS et al. (2012) part of which was three wild marjoram populations discovered by us in our country.

The morphological variability of Hungarian *Origanum vulgare* populations - after the processing of SOÓ and BORHIDI (1968) - has not been studied in the past 47 years, and their chemical diversity is hardly/barely known (OSZAGYÁN et al., 1996, OSZAGYÁN, 1999). In the light of the new international scientific results the species is listed not only as a source of essential oil, but also due to other non-volatile components it is a perspective plant raw material in many new areas.

According to the currently accepted taxonomic system of IETSWAART (1980) the Hungarian *Origanum vulgare* taxons can be classified under the category subsp. *vulgare*. Based on researches related to the genus spread of populations with low essential oil content and high degree of morphological diversity is expected.

The main aim of our research is to give an overview of Hungarian wild marjoram (*Origanum vulgare* L.) following the detection of habitat features, morphological characteristics and substance composition.

Our goals can be summarized as follows:

The mapping of the morphologically different Hungarian populations in natural habitats, characterization of chemical diversity according to/in point of view volatile and non-volatile components.

- The characterization and comparing of localities (vegetation, soil analysis, climatic conditions).

- Comparison of the diversity of the surveyed populations.
- Characterization of genotypes in order to test the diversity of all characteristics by the establishing *ex situ* progenies based on the seed material (collected from wild representative quantity of samples) of the analysed populations under the same conditions.
- Analysis of the morphological diversity in the original habitats and *ex situ* production.
- Examination of the chemical profile in the original habitats and *ex situ* production, with particular regard to the mono- and sesquiterpenoids, flavonoids and phenolic components.
- Testing of the biological activity by measuring the antioxidant capacity, furthermore by examining the correlation of the chemical profile and the antioxidant capacity.

A further aim is to draw attention to the fact that considering longer-term plans, it is an emphasized aspect to present the - on an international level - comparable results in case of a genus and species which has been known for a high degree of morphological and chemical diversity. However, with the evaluation of our results beyond the awareness the resolution is not intended.

Our goal of our research was to carry out a comprehensive survey in few *Origanum vulgare* L. (wild marjoram) populations according to the current claims by using the available competence and analytical methods of the 21st century.

MATERIALS AND METHODS

The investigated *Origanum vulgare* L. populations

The mapping of the populations in natural habitats

We collected information about the current localities of *Origanum vulgare* L. in Hungary based on the taxonomical description of SOÓ and BORHIDI (1968). The local names and descriptions of geographic locations and mountains, hills, plateaus, etc. SOÓ and BORHIDI (1968) are given. This allowed us today to monitor their taxonomic survey in the Carpathian, using the methods available to us. In addition, other plant cover is used as an aid in literatures (eg.: BORHIDI, 2003), taking into account possible changes of the habitat during the last 42 (→ 2010) years. The discovery of Hungarian *Origanum vulgare* L. subsp. *vulgare* populations started in summer of 2010 by the work of SOÓ and BORHIDI (1968).

The 11 appointed populations and locations can be found from east to west within a 370 kilometer-long of track, in 5 shires of Hungary, in the area or in the surroundings of 7 cities as follows: Fertőrákos in Győr-Moson-Sopron county; Diszel in Veszprém county; Budapest, Nagykovácsi and Visegrád in Pest county; Felsőtárkány in Heves county and Kisgyőr in Borsod-Abaúj-Zemplén county.

The *ex situ* production locality of progeny populations

The production locality of the progenies of the examined native wild marjoram populations can be found on the plot of the Medicinal plant sector of Experimental and Research Farm of the Faculty of Horticultural Science in XXIII. district (Soroksár) of Budapest.

Characterisation of the natural localities and the field experimental area

Our investigations were carried out during full flowering phenophase of wild marjoram in July and August. The habitat and morphological characteristics were documented by photos and notes, moreover we collected plant samples and made herbarium sheets in order to help the data collection.

The habitat characterisation includes the following:

- description of some main features of the plant associations
- soil characterisation with soil sampling, based on their analysis

- description of specific geographic locations using databases of the appropriate meteorological stations

In each natural production sites we made soil sampling from tilth, then the analysis was carried out by the Central Laboratory of Corvinus University of Budapest (Faculty of Food Science, Department of Food Chemistry and Nutritional Science).

We obtained the data of the appropriate county meteorological stations with the help of the Department of Soil Science and Water management of Faculty of Horticultural Sciences of Corvinus University of Budapest. The soil characteristics descriptions of the Experimental and Research Farm of Soroksár were realized in cooperation with the Central Laboratory and the weather data provided by the Department of Entomology.

Establishment of *ex situ* plantations under the same conditions

Collection of nutlets from native populations

Nutlets of representative amount of individuals of each wild/native population (9) were collected (in practical terms: seeds) during the ripening phenophase in autumn of 2010 (September and October) so that there was cut off a fruiting shoot per individual (each/min 20 ind.). During the summer sample collections we marked the sampled individuals and in Autumn those marked shoots were cut at first and then in order to the appropriate quantity of propagating material we complemented them by cutting of further growing shoots as far as possible from the sampled individuals. Than these were dried naturally, to enhance the process of after-ripening. After drying the nutlets were cleaned and separated with hands and sieves, thereafter were stored in a cool, dry place until sowing.

Establishment of the ex situ plantations

In 2011, 9 populations from 11 were selected to further propagation. We worked representative amounts of these populations (from 20 individuals hundreds of seed can be collected). The seeds of remnant stocks placed in gene bank. Based on the earlier results born in the Department of Medicinal and Aromatic Plants (Corvinus University of Budapest, Faculty of Horticultural Sciences) (HORVATH et al., SZABÓ, 2000, SZABÓ and HALÁSZNÉ, 2000) optimum conditions were used for sowing and cultivation in unheated greenhouse. Then the seedlings were grown in a greenhouse till planting. The plants were irrigated during planting and then longer rainless period and days with high mean temperature.

Morphological characterisation of the populations

The morphological characterisation in the native populations we performed with survey sheets, which were edited based on the taxonomical descriptions of *Origanum vulgare* L. subsp. *vulgare*, subsp. *barcense* and subsp. *prismaticum* (IETSWAART, 1980, SOÓ és BORHIDI, 1968). Moreover we collected herbarium samples to the checking and to help the comparing with the progenies. In order to confirmation we documented our experiences with/by photos.

In the case of characterisation of the plantation we used the followed principles and methods of the evaluation of native populations.

Sample collection in the native and the progeny populations

In 2010 and 2011 we collected mass samples all of the wild population in flowering phenophase. The relative frequency of different chemotypes in a population was represented by shoots cutting to a minimum of 20 individuals.

In 2011, the individuals of progeny populations reached the full bloom phenophase to Autumn , because they were in their first year, so the cutting of samples was made at the end of September. In 2012, we could cut the samples in the usually expected summer season, in July.

Chemical analysis of the samples of populations (nutritional assessments)

In case of assessment of the native populations and the plantations were followed same principles and methods. The main goal of the chemical analyses of the different native populations was to get an overall picture of the chemical profile of every population.
Considering that all populations of their habitat represented a gene poll during developed natural selection, the balance of the chemical profile determined genes of the individuals in the population resulting the balance of chemotypes at any given time without gene flow. (PECSENYE, 2006, PEDRYC, 2001)

Investigation of essential oil

Measuring of the essential oil content

The essential oil content was determined according to the PH. HG. VII. (1986) with hydrodistillation by Clevenger-type apparatus and its amount was given in ml/100 g dry material.

Identification of the essential oil composition

Identification of components was carried out according to the methods of capillary-column gas chromatography and mass spectrometry. The data of the composition assaying : GC-MS 6890 N gaschromatograph equipped with 5975 inert mass selective detector (Agilent Technologies); colonna - HP-5MS (5% phenyl methyl siloxane); temperature programme: 60-240 °C by 3 °C/min; ionization energy: 70 eV. Detection of the compounds was done by comparing their mass spectra to librarian references (NIST) and by calculating their linear retention indexes (LRI).

Examination of non-volatile constituents

Flvaonoid content

The total flavonoid content

The total flavonoid content was determined by Ph. Hg. VIII. (PH. HG. VIII., 2004). Due to the literature of *Origanum* genus are mentioned aglycones and glycosides also, two basic - and in the Pharmacopoeia as methodological description available - were tested two suitable flavonoids measuring methods. In preliminary experiments to the comparison of the populations in the general screening were selected and used the method, which can find under the paragraph of *Crataegi folium cum flore* (PH. HG. VIII., 2004). The absorbance was measured spectrophotometrically at 410 nm, against the compensation solution. Percent of the total flavonoid content of each samples was calculated to hiperoside, using the following formula: 1.235 * A / m, where *A* is the measured absorbance, *m* is the exact weight of the tested substance in grams, 4 decimal places.

Identification of flavonoid components

The sample collected year of 2012 was identification with the aim to obtain an overall view regarding to Hungarian wild marjoram populations.

The qualitative and quantitative determination of the flavonoid components were used the thin-layer chromatography method developed by JANICSÁK and MÁTHÉ (1997). The assay was performed with the available standards for us, which were in according to running order as follows:rutin, hiperoside, iso-quercetin, cynarine, quercetrin, *rosmarinic acid*, *caffeic acid*, luteoline, apigenine, kempferol, naringenine. Because they are visible at same wavelength, identification of two additional in Lamiaceae family typical flavonoid components were inserted in this measure, these were the rosmarinic acid and caffeic acid. The measure was performed by Desaga TLC system, with horizontal chmaber system, on

TLC-silicagel 60 (Merck) stationary phase. The identification was made under UV-lamp at 254 nm, which range the spots of flavonoids are perceptible to the eye.

Determination of the total phenol content

The determination of TPC was used the modified method of Singleton and Rossi (1965), with Folin-Ciocalteau reagent, spectrophotometrically, in water and ethyl alcoholic extracts of the samples. The absrobance is measurable at 760 nm. Gallic acid was applied as standard for calibration (dissolved in 80% MeOH, 0,3M). The concentration of the sample extracts was determined as mg of gallic acid equivalent per ml of sample (mg GAE/ml) with Analysis ToolPak Add-in of MS Excel and regression calculation, then the values were expressed relation to dry weight content of each solution as mg of gallic acid equivalent per g dry weight (mg GAE/ g d.w.).

This modified method of SINGLETON and ROSSI (1965) in our country is a pharmaceutical, applied chemical, horticultural research and other researches at our department became more common practice, so we chosen to this research work are also this.

Determination of antioxidant capacity

The measurement of reducing ability were applied modified FRAP (ferric reducing antioxidant power) method of Benzie and Strain (1996), in the case of water and ethyl alcoholic extracts of the samples. The absorbance is measurable spectrophotometrically at 593 nm. The standard curve was determined by known concentrations of ascorbic acid (0,001 M). With the measured absorbance values from related to ascorbic acid concentration of sample extracts (mg AA/(sample) ml), with Analysis ToolPak Add-in of MS Excel and regression calculation, the antioxidant effect was expressed relation to dry weight content of each solution as mg of ascorbic acid equivalent per g dry weight (mg AA/ g d.w.).

Statistical analysis

The assessment of the results were completed by using univariate and multivariate analysis of variance with SPSS PASW Statistics 18 and 20 softwares, furthermore with Analaysis ToolPak Add-in of MS Excel 2010.

The add-ins were also used as follows: function and detection devices, correlation analysis.

NEW SCIENTIFIC RESULTS AND DISCUSSIONS

We have confirmed that the subsp. *barcense* described by SOÓ and BORHIDI (1968) is still present in Hungary in addition to subsp. *vulgare* typical in Hungary according to IETSWAART (1980). We verified the correctness of *lusus* and *forma* categories (only phenotypic occurrence) for all the categories described by SOÓ and BORHIDI (1968), namely forma *thymiflorum*, forma *procumbens*, lusus *albiflorum*, lusus *roseum* and lusus *carneum,* within subsp. *vulgare*. During the study of the *ex situ* progeny populations we revealed the disappearance or change of these morphological properties among different environmental conditions. It has been proved, that the different colours of flowers (*lusus*) are not individual phenotypical properties, sometimes 3 different colours of flowers can occur on one individual.

In the case of the 9 investigated Hungarian wild marjoram populations we have proved the low essential oil content (0.005-0.402 ml/100 g), the presence of variable data for the same gene-pool (within this scale)depending on the different vegetation periods. Furthermore, the growing conditions provided by us caused decrease in the essential oil content of 4 populations. Moreover it has been confirmed, that the dispersion between the essential oil content of populations decreased in the same environmental conditions.

Our new scientific results in connection with the essential oil composition are as follows. Among the 9 surveyed populations 8 ones are sesquiterpene chemotypes and 1 population is a monoterpene-sesquiterpene chemotype. However, we have proved that the changes of environmental factors influence the essential oil composition: the appearance and percentage of the mono- and sesquiterpenes, the dominance of non-oxidized or oxidized sesquiterpenes, which facts all together additionally can generate a shift of a chemotype.

The available data regarding the specific groups of non-volatile constituents (as flavonoids and phenols) are few and contradictory; additionally, there are no data on the accumulation level of these compounds in the case of Hungarian populations.

It has been demonstrated that the TFC of the examined *Origanum vulgare* L. populations changes on a wide range (0.87-2.892%), however a tendency cannot be detected, neither in gene-pools (populations), nor according to vintages (equal in the nature and under the conditions of growing).

We have proved with the measures of TPC that the alcoholic extracts of the analysed Hungarian wild marjoram populations have uniform lower total phenol content (47.95-212.01 mg GAE/g dry weight), than the aqueous extracts (120.89-380.87 mg GAE/g dry weight).

In case of TPC in water extracts we confirmed appearing of less relative dispersion between the samples of the surveyed populations (4 samplings), so the quantity is more stable and the genes have stronger influence on synthesis of alcohol-soluble phenolic compounds. Assessing all the measured results an additional statement can be described. The vintages have a standard, but stronger effect on the alcoholic soluble TPC of the native and the progeny populations, furthermore the same vintage resulted approximately similar changes both in the wild and growing populations. In connection with this, it has been confirmed that the more stable water soluble TPC in the populations means highly variable quality of the drug, so the selection for higher TPC is justified. We have confirmed that the ethyl-alcoholic soluble TPC is genetically less determined, but the vintages have stronger effect, so it has lower influence on the breeding goals.

The measurement of antioxidant activity of wild marjoram populations examined by us has proved that water extracts have stronger effect (124.45-348.26 mg AAE/g dry weight) than in alcoholic extracts (35.73-241.81 mg AAE/g dry weight), with similarly lower relative deviation between the 4 samplings.

In case of extracts made with ethyl-alcohol we proved similar tendency as in connection with TPC and vintages with the addition that the different vintages induced bigger differences within the populations. In this case the relevance of breeding to the populations with higher AA has also been confirmed, because the biological activity is more stable but more variable too, between the gene-pools.

Analyzing the connection strength between the measured non volatile compounds (TFC, TPC) and the AA, it can be proved that the environmental factors have stronger influence than the genetic background. However, it is important to know, the constituents of total quantities in terms of their chemical structures are forming big sets, so for a more accurate evaluation it is necessary to identify compounds and the establishment of *ex situ* progeny populations on more, different localities. It is supported by the described different parameters of the localities of the 9 examined *Origanum vulgare* L. populations.

Studies to assess non-volatile constituents in case of (some) Hungarian wild marjoram (*Origanum vulgare* L.) populations were carried out by us at first. It has been confirmed that values of a steady state population are scattering over a wide range. There are a lot of publications with data from one vegetation period, so our results show the necessity of studies covering several vegetation periods. These latter ones can provide reliable results and these can be the base for further researches.

Our present comparative studies on the stability of both morphological and phytochemical properties are the first in Hungary. However our results underline the relevance of complex experiments with more vegetation periods, more locations and more *ex situ* plantation sites. This is especially significant in case of well-known morphological and chemical diversity of *Origanum* genus and *Origanum vulgare*.

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PUBLICATIONS IN RELATION TO THE PHD THESIS

(AS DEFINED CLASSIFICATION BY THE PHD SCHOOL)

Journal article with IF:

1) CSERHÁTI, B., JUHOS, K., BEGYIK, A., RADÁCSI, P., NÉMETH, É., és SZABÓ, K. [2012]: In situ morphological variability of wild marjoram (*Origanum vulgare* L.) populations in Hungary. Acta Alimentaria. 41. 12-23. IF 0,444

Peer-reviewed journal (HAS list) published Notice

1) CSERHÁTI, B., LADÁNYI, M., KOVÁCS, L., RAJHÁRT, P., NÉMETH, É., és SZABÓ, K. [2015]: Magyarországi vadon termő közönséges szurokfű populációk (*Origanum vulgare* L.) összes polifenol-tartalmának és antioxidáns kapacitásának alakulása termesztésben. Kertgazdaság (in press)

Conference full paper (Hungarian):

1) CSERHÁTI, B., JUHOS, K., BEGYIK, A., RADÁCSI, P., és SZABÓ, K. [2011]: Hazai vadon termő szurokfű (*Origanum vulgare* L.) populációk in situ morfológiai variabilitása. Erdei Ferenc VI. Tudományos Konferencia, Kecskemét, 2011. augusztus 25-26. 248-252.

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1) CSERHÁTI, B., JUHOS, K., BEGYIK, A., BERNHARDT, B., RADÁCSI, P., SÁROSI, SZ., és SZABÓ, K. [2011]: Hazai vadon termő közönséges szurokfű (*Origanum vulgare* L.) populációk morfológiai jellemzése és kémiai analízise – Első eredmények. XII. magyar Gyógynövény Konferencia és XLVI. Rozsnyay Mátyás Emlékverseny, Szeged, 2011. május 5-7. Absztrakt. E-12

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1) CSERHÁTI, B., JUHOS, K., BEGYIK, A., BERNHARDT, B., RADÁCSI, P., és SZABÓ, K. [2011]: Morphological characterisation and chemical analysis of wild *Origanum vulgare* L. populations in Hungary – First results. 1. Transilvanian Horticulture and Landscape Studies Conference, Tîrgu-Mures/ Marosvásárhely, 8-9 April 2011. Abstracts. 18.

2) CSERHÁTI, B., JUHOS, K., BEGYIK, A., RADÁCSI, P., ZSIDÁKOVITS, Á., NÉMETH, É., és SZABÓ, K. [2012]: Non-volatile compounds of wild marjoram (*Origanum vulgare* L.) in Hungary. 7th Conference on Aromatic and Medicinal Plants of Southeast European

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3) CSERHÁTI, B., SÁROSI, SZ., NÉMETH, É., és SZABÓ, K. [2012]: Essential oil content and composition of Hungarian wild marjoram (*Origanum vulgare* L.) populations. 7th Conference on Aromatic and Medicinal Plants of Southeast European Countries, Subotica/ Szabadka, Republic of Serbia 27-31 May, 2012. Book of abstracts. 40.

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