Studies on the thiophene metabolism in genetically transformed hairy root cultures of *Tagetes patula* L.

Ph.D. thesis

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1. Introduction

Plants biosynthesize many valuable biologically active products that can be used by the flavour, fragrance, pharmaceutical industries, and agriculture. The demand for natural active ingredients is rising day by day. Extensive development of the analytical methods facilitates the discovery of more and more pharmaceutically active compounds.

In vitro plant cell cultures are an attractive alternative source to whole plant for the production of high-value special metabolites. Significant achievements of the past decades resulted in the successful application of the genetically transformed hairy root cultures. Hairy roots have a real advantage over the traditional callus or cell suspension cultures. They produce growth hormones, so they will grow fast in hormone-free media. A remarkable proportion of the cells are specialized and differentiated, which is essential for a high level special metabolite synthesis. The metabolite production of hairy root cultures usually exceeds that of callus and cell suspension cultures; moreover, the metabolite profile is comparable to the parent plant. Their biochemical potential is stable and predictable. Because of cellular differentiation, the cell proliferation is better controlled, thus these cultures are unique in their genetic stability as well. Moreover, the genetically transformed hairy roots are able to regenerate whole viable transgenic plants.

A significant proportion of the biologically active special metabolites is undoubtedly involved in the defense mechanisms of plants. Thiophenes are characteristic special metabolites occurring in the Asteraceae plant family. Due to their remarkable phototoxic activity, and beneficial mechanisms of action, they have potential application in pharmaceutical and agrochemical fields. The roots of *Tagetes patula* L. (French marigold) is a significant source of the biologically active bi- and terthiophenes. Therefore this plant seems to be promising for the induction of an efficient *in vitro* plant tissue culture system characterized by high-level thiophene production.

2. Aims

The subject of my doctoral dissertation is to study the thiophene metabolites in intact plants and genetically transformed *in vitro* hairy root cultures of *Tagetes patula*.

For the qualitative analysis and quantitative determination of thiophenes in complex biological matrix we aimed to develop a gas chromatographic method coupled with mass spectrometric detection (GC-MS), which is one of the most efficient analytical and structure elucidation techniques nowadays.

We aimed to produce genetically transformed hairy root cultures of *T. patula* that can be characterized by high-level stable thiophene production. The quantitative determination of the characteristic thiophene metabolites is essential for the selection of the hairy root clone possessing the optimal performance.

The studies on the characteristic growth and thiophene production parameters are important to work out the optimal culture conditions. Afterwards, we wished to compare the thiophene metabolism of the transformed hairy root cultures with intact field-grown parent plants.

In order to increase the special metabolite production of hairy root cultures, we aimed to optimize the cultivation conditions. Manipulation of the culture environment must be effective in increasing the metabolite accumulation. The expression of many special metabolite pathways is easily altered by external factors such as nutrient levels. For the highest possible biomass and special metabolite production the selection of the optimal basal medium is of great importance. For the further enhancement of the thiophene production, the influence of sulphur sources (e.g. $MgSO_4$ and sulphur-containing amino acids) was aimed to study.

Moreover, the thesis was aimed to summarize the research results of the past three decades. We hope that our results will provide useful information for the better understanding of the biosynthetic properties of *T. patula* hairy root cultures.

3. Materials and methods

3.1. Plant material

Tagetes patula L. seeds were collected in Murau (Austria). The harvested plants were raised in a garden in Magyaratád (Hungary). The seeds were sown in March and collected in August, during the full flowering period.

3.2. Genetically transformed hairy root cultures

The hairy root cultures were obtained by transformation of *T. patula* seedlings with *Agrobacterium rhizogenes* strains (R-1601, A4Y) using the microinjection technique. In order to eliminate bacterial growth, the isolated hairy root clones were transferred into solid MS medium containing antibiotics. The bacteria-free cultures were maintained in liquid MS cultivation medium with half rate nitrate content (1/2 NMS) supplemented with 2 % sucrose, in a programmable incubation shaking cabinet (100 rpm, dark, 22±1 ºC). Thereafter the cultures were transferred every three or four weeks.

3.3. PCR analysis for the confirmation of the genetic transformation in hairy roots

Fresh plant tissue was quickly frozen in liquid nitrogen and grounded to fine powder in a mortar. Genomic DNA was extracted according to the Promega Wizard® Genomic DNA Purification Kit.

The bacterial plasmid DNA was isolated according to the QIAGEN Plasmid Mini Kit.

The polymerase chain reaction analyses were performed with a Bio-Rad iCycler Thermal Cycler 3.021 using primers designed for the amplification of *rol* B and *man* 2 genes. The PCR products (*rol* B: 862 bp and *man* 2: 513 bp) were separated by gel electrophoresis and detected under UV light (λ=312 nm).

3.4. Sample preparation and extraction methods

Steam distillation

The volatile compounds were extracted by steam distillation for 3 h in a modified Clevenger apparatus, and trapped in organic solvent. The essential oil content was determined by gravimetry.

Static headspace analysis (sHS)

The analyses were performed by a CTC Combi Pal multipurpose autosampler. After a 15 min equilibration at 25 or 60 ºC, 250 µL sample was extracted from the headspace of the plant samples by a 2.5 mL gas-tight syringe. The syringe temperature was set to 70 ºC.

Solid phase microextraction (SPME)

The analyses were performed by a CTC Combi Pal multipurpose autosampler. SPME was performed with a Stableflex fiber coated with 65 µm PDMS/DVB stationary phase. After an incubation period of 5 min, the extractions were performed by exposing the fiber for 10 min at 25 or 60 ºC to the headspace of the samples. Then the fiber was transferred immediately into the heated GC-MS injector.

Supercritical fluid CO_2 extraction (SFE)

SFE was performed with an ISCO SFX 2-10 supercritical fluid extractor at different pressures (10, 20, 30 and 40 MPa) and 40 ºC temperature using pure CO_2 or supplemented with 20 % methanol co-solvent. SFE method consisted of a 30 min static mode, followed by a 60 min dynamic mode, and the extracts were collected in 5 mL methanol.

Solvent extraction for GC-MS analyses

The extraction was performed with 70 % methanol with sonication. The combined crude extract was purified by partitioning three times using a 1:1 (*v*/*v*) mixture of hexane/*tert*-butyl methyl ether. The upper phases were combined and evaporated to dryness under vacuum.

3.5. Chromatographic analyses

Fractionation of the solvent extracts by flash chromatography

Concentration and clean-up was achieved using a FlashMaster II system equipped with an automatic fraction collector.

Normal-phase separation was performed on a packed silica column with a hexane-dioxane solvent system using a step-gradient method. The flow rate was 5 mL/min, and thiophenes were detected at 340 nm.

Reversed-phase chromatography was performed on a modified, octadecyl (C18) silica column. For the separation, 0.1 % formic acid-methanol gradient elution was used at a flow rate of 2 mL/min.

GC-MS analysis of the essential oil

The analyses were carried out by a Finnigan MAT GC-MS. Separations were performed using an MDN-5S capillary column. The oven temperature was maintained at 60 ºC for 3 min after injection, then programmed at 8 ºC/min to 200 ºC (2 min isothermal), then 200-230 ºC at 10 ºC/min (5 min isothermal) and finally 230-250 ºC at 10 ºC/min (10 min isothermal). Helium was the carrier gas at 1 mL/min flow rate. Injector temperature was 220 °C, and the split ratio was 1:62. The 0.5-1.0 µL samples were injected manually.

The instrument was operated in electron impact (EI) mode with an electron energy of 70 eV, and the analyses were performed in full-scan mode.

SPME-GC-MS and sHS-GC-MS analysis of the volatile compounds

The analyses of volatile compounds were carried out with an Agilent 6890N/5973 GC-MSD coupled with an automatic multipurpose CTC Combi Pal sampler. Separations were performed using an Agilent HP-5MS capillary column. The GC oven temperature was programmed from 60 ºC (3 min isothermal) to 200 ºC at 8 ºC/min (2 min isothermal), 200-230 ºC at 10 ºC/min (5 min isothermal) and finally 230-250 ºC at 10 ºC/min (1 min isothermal). Helium was the carrier gas at 1.0 mL/min in constant flow mode. The injector temperature was 250 ºC, and the split ratio was 1:50.

The instrument was operated in EI mode with an electron energy of 70 eV, and the analyses were performed in full-scan mode.

GC-MS analysis of thiophenes in solvent extracts and SFE

The analyses were performed with an Agilent 6890N/5973 GC-MSD coupled with an automatic multipurpose CTC Combi Pal sampler. Separations were performed using an Agilent HP-5MS capillary column. The GC oven temperature was programmed from 60 ºC (1 min isothermal) to 280 ºC at 10 ºC/min (7 min isothermal). Helium was the carrier gas at 1.0 mL/min in constant flow mode. Injection volume was 1.0 µL. Injector parameters using different sample introduction techniques were the following:

- *split* injection was performed with a split ratio of 1:20 at 280 ºC;
- *hot splitless* injection was performed at 280 ºC injector temperature while splitless time was varied;
- *hot pulsed splitless* injection was performed at 280 ºC using 40 psi pressure pulse while splitless time was varied;
- *temperature programmed pulsed splitless* injection was performed with an injector temperature programmed from 120 to 300 ºC at 720 ºC/min using a 40 psi pressure pulse while splitless time was varied.

For further GC-MS analyses the *temperature programmed pulsed splitless* sample introduction technique was applied with 0.8 min splitless time.

The instrument was operated in EI mode with an electron energy of 70 eV, and the qualitative analyses were performed in full-scan mode. For quantitative determination purposes the selected ion monitoring mode was used.

4. Results and discussion

4.1. Chemical analysis, method development

A new, GC-MS method was developed for the qualitative and quantitative analysis of thiophenes in the genetically modified *Tagetes patula* hairy root cultures and intact field-grown plants. Numerous sample preparation techniques (steam distillation, static headspace, SPME, SFE, and solvent extraction) were studied in order to increase the efficiency of the thiophene determination.

In addition to thiophenes, terpenoids were also identified by the different sample preparation techniques (except for static headspace). The highest intensity and selectivity was observed when SPME was used. Three new sesquiterpenes, α-gurjunene, β-caryophyllene, and (E)-β-farnesene were identified for the first time in the intact roots and the genetically transformed hairy root cultures of *T. patula*.

Thiophenes could be easily identified by their characteristic mass spectra. The sulphur-containing compounds have typical isotope abundance pattern, and the intensity ratio can be used for the estimation of the number of sulphur atoms in the molecule. In the organic solvent extracts of intact roots and genetically transformed hairy root cultures of *T. patula*, the following 8 thiophene compounds were identified: 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT), 5'-methyl-5-(3-buten-1-ynyl)-2,2'-bithienyl (MeBBT), 5-(3-penten-1-ynyl)-2,2'-bithienyl (PBT), 5-(4-hidroxy-1-butynyl)-2,2'-bithyenyl (BBTOH), 2,2':5',2"-terthiophene (α-T), 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (BBTOAc), 5-methylaceto-5'-(3-buten-1-ynyl)-2,2'-bithienyl ($AcOCH_2BBT$) and 5-(3,4-diacetoxy-1-butynyl)-2,2'-bithienyl [$BBT(OAc)_2$].

The time consumed for the solvent extraction was decreased by 33 % without the loss of efficiency and reliability.

Thiophene-rich extracts were obtained by SFE. In order to maximize extraction efficiency, the process was optimized by the variation of pressure and the addition of modifier co-solvent. Finally, the analytical parameters were characterized. The SFE performed at 30 MPa without modifier co-solvent allowed the selective extraction of thiophenes for the subsequent GC-MS analysis.

Due to the lack of available thiophene standard materials, the main thiophenes of *T. patula* roots were purified by normal and subsequent reversed-phase flash chromatography. Three main compounds were isolated from the

crude methanolic extracts in high purity [BBT (97,0 %), α-T (98,4 %), and BBTOAc (95,1 %)], and the α-T was produced by crystallization. The identification and purity was confirmed by GC-MS.

Sample introduction is one of the most crucial processes of gas chromatography. Thus, several injection techniques were studied for the best performance. The pulsed splitless injection combined with 0.8 min splitless time and temperature programming provided the highest sensitivity and reliability for thiophene analysis.

The GC-MS method was validated according to ICH Q2(R1) (2005) guidelines, FDA Guidance (2001) and guidelines proposed by Balla (2006). The repeatability of retention data ($RSD < 0.005$ %) and peak areas ($RSD < 10.7$ %) met the requirements. Quantitative determination of α-T was performed by internal standard calibration, the correlation coefficients were always higher than 0.99. The limit of detection (LOD) was 3.1 ng/mL, and the lower limit of quantification (LLOQ) was 1.2 μg/mL. The accuracy was 11.1 % in LLOQ, 2.0 % in mid-range, and 0.4 % in upper-range. The extraction efficiency of α-T was 96 ± 0.16 % ($RSD = 0.2$ %), and the recovery data obtained at three concentration levels were 94.6 ± 0.72 % (75 μg), 89 ± 4.9 % (150 μg), and 95 ± 8.2 % (300 μg). The results of the post-preparative stability test confirmed that no significant qualitative and quantitative changes occurred during the 72 h time period at room temperature.

4.2. Thiophenes in the intact Tagetes patula

The BBT, α-T, BBTOAc main compounds, and PBT, BBTOH, $AcOCH_2BBT$ and $BBT(OAc)_2$ minor constituents were identified in the solvent extracts of intact plant roots. The α-T content was 575 ± 23.1 μg/g on dry weight basis.

The flowers were characterized by two dominant thiophene compounds, PBT and α-T (312 ± 4.2 μg/g). Other thiophenes were not detected, only BBT occurred in traces. The extracts were rich in long-chain organic acids.

The leaves did not contain considerable amounts of thiophenes, but the high level of organic acids was also detected. The α-T content was below the LLOQ.

4.3. Genetically transformed hairy root cultures of Tagetes patula

Several hairy root clones (#TpR, #TpA1, #TpA5, #TpA6, and #TpA7) formed after the microinjection of sterile *T. patula* seedlings with *Agrobacterium rhizogenes* R-1601 and A4Y strains. A clone selection study was performed in order to select the clone that has high-level biomass production and thiophene synthesis. The intensively growing #TpA1 and #TpA6 clones had remarkable α-T production (118 ± 38.4 μg/culture and 121 ± 5.2 μg/culture, respectively).

The amounts of the other thiophenes was the highest in the #TpA6 hairy root culture, therefore this clone was selected for further experiments.

The confirmation of the genetic transformation was achieved by PCR analysis and subsequent gel electrophoresis. The integrated bacterial T-DNA (*rol* B and *man* 2 genes) was identified in the genomic DNA of #TpA6 hairy root cultures.

Afterwards, the biomass and thiophene production characteristics of the selected #TpA6 hairy root clone were studied in detail. Thiophene synthesis showed no direct correlation to culture's growing. The highest α-T content (212 ± 58.3 μg/g) was observed at the end of the third week, during the intensive growing period. The amount of BBT was also the highest at the end of the third week, while the contents of two end products of the thiophene biosynthesis, BBTOAc and $BBT(OAc)_2$, significantly increased at the end of the studied period (7th week). The biomass yield, thiophene content and production were rather stable for a long time period (1.5 years). The average growth value was 10 ± 2.1 (*RSD* = 20.8 %), the long-term α-T content (143 ± 34.6 μg/g; *RSD* = 24.1 %), α-T production (95 ± 25.3 μg/culture; *RSD* = 26.6 %), and the relative amount of thiophenes were also fairly balanced.

The thiophene metabolisms of the transformed hairy root cultures and the intact field-grown plants were then compared. In hairy root tissues the content of thiophenes containing two heterocycles [BBT, BBTOAc, and $BBT(OAc)_2$] exceeded significantly the contents in intact roots. However, the highest tricyclic α-T content was detected in intact plant roots (590 ± 11.1 μg/g), in hairy root cultures it was reduced by two-thirds (169 ± 9.6 μg/g).

4.4. Enhancement of the biomass formation and the thiophene production of T. patula hairy root cultures (#TpA6)

Selection of the optimal cultivation medium

The three commonly applied cultivation media, Gamborg's B5, Murashige and Skoog (MS) and half rate nitrate content MS (1/2 NMS) media did not have any influence on the biomass formation. Thiophene content, however, changed significantly. The highest BBT accumulation was observed in B5 medium, while the MS medium considerably increased the BBTOAc amount. In the modified MS medium (1/2 NMS) both the α-T content (122 ± 31.1 μg/g) and production (98 ± 25.1 μg/culture) was about 2-fold over the values measured in cultures cultivated in MS or B5 media.

Effect of $MgSO_4$

The study was performed in liquid 1/2 NMS cultivation medium containing different amounts of $MgSO_4$: 0; 370 (control, corresponding to the $MgSO_4$ content of the basic 1/2 NMS medium); 600 and 1600 mg/L. Both the biomass yield and the total thiophene amounts decreased significantly on $MgSO_4$-free medium (α-T content: 42 ± 1.2 μg/g). The α-T content was 149 ± 18.2 μg/g in the cultures cultivated on the control 1/2 NMS medium. The increase of $MgSO_4$ caused an increase of the α-T and BBTOAc contents, while the amounts of other thiophenes and the biomass production reached a steady-state condition at the control level. The maximal α-T content (242 ± 29.5 μg/g) was observed on the medium supplemented with 1600 mg/L $MgSO_4$ and it was a 1.5-fold increase over the control. The experimental results performed in our Department show that high $MgSO_4$ concentrations (approx. 1000 mg/L) considerably reduced both the biomass yield and special metabolite production of other plant species. It is interesting to note that this trend was not observed, high concentrations of $MgSO_4$ (e.g. 1600 mg/L) was well tolerated; moreover, further increase in the contents of α-T and BBTOAc can be expected.

Effect of sulphur-containing precursor amino acids

L-cysteine and L-methionine are important precursor amino acids involved in the thiophene biosynthesis pathways. The effect on biomass and thiophene production of hairy root cultures was studied by varying amino acid content in the cultivation medium. Cysteine increased the thiophene amounts significantly, however, considerably inhibited the biomass formation in a dose-dependent manner, therefore thiophene production decreased. On the medium supplemented with 1.0 mM cysteine the α-T production decreased by 50 % compared to control (cysteine-free 1/2 NMS medium). The biomass yield did not increase by methionine, but it should be emphasized that 1.0 mM methionine caused a 2.4-fold increase in the BBT content, and a 1.3-fold increase in the $AcOCH_2$BBT content.

5. Conclusions

The genetic transformation by *Agrobacterium rhizogenes* is suitable for the production of *Tagetes patula* hairy root cultures possessing high-level thiophene synthesis.

The steam distillation and the SPME were found to be optimal and efficient methods for the chemical analysis of the volatile terpenoid essential oil constituents. Although thiophenes were also detected by these two techniques, excessive discrimination was observed, as the extracts were dominated by the

non-polar, more volatile compounds, while some polar thiophenes having higher molecular mass [BBTOH and $BBT(OAc)_2$] were not detected at all. Among the different sample preparation techniques (steam distillation, static headspace, SPME, SFE, and solvent extraction), SFE and solvent extraction proved to be suitable for the analysis of thiophenes.

The GC-MS analysis of the solvent extracts proved to be very efficient for the identification, quantitative determination, and monitoring of thiophenes in hairy root tissues. The analytical parameters and validation data suggest, that the method is selective, sensitive, provides precise and accurate data of the five thiophene metabolites [BBT, α-T, BBTOAc, $AcOCH_2BBT$ and $BBT(OAc)_2$], thus it should provide the means to be useful for the routine analytical task of the proposed biotechnological experiments.

The thiophene distribution was different in the intact *T. patula* plant parts. The GC-MS analysis confirmed that thiophenes were remarkably enriched in the roots; therefore this organ can be regarded as the primary thiophene source. The flowers accumulated considerable amounts of α-T and PBT, while other thiophenes could only be detected in traces. The thiophene content of leaves was negligible. Compared with data reported in literature, considerably high α-T content was observed in the roots studied; therefore it seemed to be promising for the induction of root-like tissue cultures, the hairy root cultures, characterized by high-level thiophene production.

Several hairy root clones formed after the genetic transformation by *A. rhizogenes*. It became obvious that the efficient clone selection could only be carried out by the characterization of the complete thiophene profile. The #TpA6 hairy root clone was selected for further experiments, because it had remarkable biomass formation and outstanding thiophene production. Thiophene synthesis showed no direct correlation to culture's growing, considerable thiophene amounts were observed during the intensive growing phase. Since thiophenes were detected only in traces in the cultivation medium, the excretion into the medium is negligible. This suggests that the changes in tissue thiophene levels are affected by the rate of assimilation and degradation processes.

Results of the long-term retrospective study suggest that the genetically transformed *T. patula* #TpA6 hairy root clone has rather stable biomass yield and fairly balanced thiophene biosynthetic potential. Compared with published data, the biosynthetic capacity of *T. patula* hairy root cultures was much superior and more stable to that of callus and cell suspension cultures. The *T. patula* #TpA6 hairy root tissue cultures met the requirements available in literature; therefore it seems to be a promising *in vitro* plant tissue culture

system that can be used for the large-scale biotechnological production of thiophenes.

The content of three bicyclic thiophenes [BBT, BBTOAc, and $BBT(OAc)_2$] in hairy root tissues exceeded 3-fold of the contents in the roots of intact field-grown plants, however, the tricyclic α-T content was reduced by two-thirds in the hairy root cultures. This suggests that in hairy root cultures the biosynthetic flux of thiophenes is channeled from the tricyclic pathway to the bicyclic type thiophenes. The biosynthetic potential of the #TpA6 hairy root culture was comparable to that of the parent plant; therefore it was found to be suitable for the proposed biotechnological experiments aimed to increase thiophene production.

By the media selection study it was observed that the half rate nitrate content MS (1/2 NMS) medium caused a selective 2-fold increase in the α-T content and production, which can be attributed to the quantitative differences in the nitrogen source.

The study on the effects of $MgSO_4$ showed that this is an essential constituent of the cultivation media. In the absence of $MgSO_4$ both the biomass production and thiophene content decreased significantly. Cultivated on sulphur-free medium, due to the limited availability of sulphur-sources, the synthesis of the bicyclic thiophenes is more favourable, while the content of α-T, a tricyclic thiophene containing three sulphur atoms, decreased dramatically. Increasing of $MgSO_4$ caused an increase of the α-T and BBTOAc contents, while the amounts of other thiophenes and the biomass production reached a steady-state condition at the control level.

A sulphur-containing precursor amino acid, L-methionine, selectively increased the BBT content of the hairy root cultures. Although, L-cysteine significantly increased the total thiophene content, considerably inhibited the biomass formation, therefore its practical significance is negligible.

In summary, it can be concluded that manipulation of the culture environment is effective in increasing the thiophene accumulation of *T. patula* hairy root cultures; moreover, selective overproduction of an individual thiophene compound can be achieved. Concerning the results of the studies on thiophene production, the following suggestions are proposed:

- to increase α-T production the cultivation in low nitrogen content 1/2 NMS medium supplemented with high concentration of $MgSO_4$ (e.g. 1600 mg/L) is suggested;
- BBT synthesis can be increased in B5 medium supplemented with 1.0 mM methionine;

- high BBTOAc content can be achieved by longer cultivation (e.g. 5 or 6 weeks) in MS medium supplemented with 1600 mg/L $MgSO_4$;
- $AcOCH_2BBT$ amount can be increased by cultivation in MS medium supplemented with 1.0 mM methionine;
- the $BBT(OAc)_2$ content could only be increased by a longer cultivation period.

List of publications related to the thesis

Articles published in international scientific journals

1. **Szarka Sz,** Gyurján I, László M, Héthelyi É, Kuzovkina IN, Lemberkovics É, Szőke É. (2010) GC-MS studies of thiophenes in the supercritical fluid CO_2 and solvent extracts of *Tagetes patula* L. Chromatographia, 71: 1039-1047 (IF: 1,098).
2. **Szarka Sz,** Héthelyi É, Kuzovkina IN, Lemberkovics É, Szőke É. (2008) GC-MS method development for the analyses of thiophenes from solvent extracts of *Tagetes patula* L. Chromatographia, 68: S63-S69 (IF: 1,312).
3. **Szarka Sz,** Héthelyi É, Lemberkovics É, Kuzovkina IN, Farkas E, Bálványos I, Szőke É. (2007) Essential oil constituents of intact plants and *in vitro* cultures of *Tagetes patula* L. J Essent Oil Res, 19: 85-88 (IF: 0,368).
4. **Szarka Sz,** Héthelyi É, Lemberkovics É, Kuzovkina IN, Bányai P, Szőke É. (2006) GC and GC-MS studies on the essential oil and thiophenes from *Tagetes patula* L. Chromatographia, 63: S67-S73 (IF: 1,171).

Abstracts published in journals

1. **Szarka Sz,** Héthelyi É, Kuzovkina IN, Lemberkovics É, Szőke É. (2009) GC-MS studies of thiophenes for the hairy root clone selection of *Tagetes patula* L. *Gyógyszerészet*, Supplementum: 111.
2. **Szarka Sz,** Héthelyi É, Lemberkovics É, Kuzovkina IN, Bálványos I, Szőke É. (2005) Investigation of volatile compounds in *Tagetes* species. Sci Pharm, 73: 260.

Chapter in book

1. **Szarka Sz,** Héthelyi É, Kuzovkina IN, Szőke É. Effects of different media on *Tagetes patula* hairy root cultures. In: Szilágyi M, Szentmihályi K (eds.), Trace Elements in the Food Chain Vol. 3. Institute of Material and Environmental Chemistry of the HAS, Budapest, 2009: 322-326.

Selected conference presentations

1. **Szarka Sz,** Héthelyi É, Kuzovkina IN, Lemberkovics É, Szőke É. (2010) GC-MS monitoring of thiophenes in French Marigold hairy root cultures. 28th Informal Meeting on Mass Spectrometry, Kőszeg.
2. **Szarka Sz,** Héthelyi É, Kuzovkina IN, Lemberkovics É, Szőke É. (2009) *Tagetes patula* L. hairy root klónok szelekciója GC-MS vizsgálati módszerekkel. PhD Tudományos Napok 2009, Budapest.
3. **Szarka Sz,** Héthelyi É, Kuzovkina IN, Lemberkovics É, Szőke É. (2009) *Tagetes patula* L. hairy root clone selection using gas chromatography-mass spectrometry. 27th Informal Meeting on Mass Spectrometry, Retz, Austria.
4. **Szarka Sz,** Héthelyi É, Gyurján I, Kuzovkina IN, Szőke É. (2008) Biocid hatású tiofének GC-MS vizsgálata. Elválasztástudományi Vándorgyűlés, Sárvár.
5. **Szarka Sz,** Héthelyi É, Kuzovkina IN, Szőke É. (2007) GC-MS method development for the analyses of thiophenes from solvent extracts of *Tagetes patula* L. hairy root cultures. 7th Balaton Symposium on High-Performance Separation Methods, Siófok.
6. **Szarka Sz,** Héthelyi É, Lemberkovics É, Kuzovkina IN, Bálványos I, Szőke É. (2005) Investigation of volatile compounds in *Tagetes* species. Joint Meeting on Medicinal Chemistry, Vienna, Austria.
7. **Szarka Sz,** Héthelyi É, Lemberkovics É, Kuzovkina IN, Bálványos I, Szőke É. (2005) GC and GC-MS studies on the essential oil of *Tagetes patula* L. 6th Balaton Symposium on High-Performance Separation Methods, Siófok.
8. **Szarka Sz,** Héthelyi É, Lemberkovics É, Kuzovkina IN, Bálványos I, Szőke É. (2005) Essential oil constituents of two *Tagetes* species. 36th International Symposium on Essential Oils, Budapest.
9. **Szarka Sz,** Héthelyi É, Lemberkovics É, Gerencsér G, Farkas E, Kuzovkina IN, Bálványos I, Szőke É. (2004) Essential oil constituents of *Tagetes patula* L. hairy root cultures. Symposium of Pharmaceutical Biotechnology, Trieste, Italy.
10. **Szarka Sz,** Gerencsér G, Kuzovkina IN, Bálványos I, Szőke É. (2003) The secondary metabolism of *Tagetes patula* L. hairy root cultures. VIII. International Conference, The biology of plant cells *in vitro* and biotechnology, Saratov, Russia.

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