



(REVIEW ARTICLE)



Comparative investigation of sesquiterpene components of essential oils originating from intact plants and hairy root chamomile cultures

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GSC Advanced Research and Reviews, 2021, 06(02), 028–049

Publication history: Received on 06 January 2021; revised on 04 February 2021; accepted on 06 February 2021

Article DOI: <https://doi.org/10.30574/gscarr.2021.6.2.0016>

Abstract

The importance of chamomile (*Chamomilla recutita*) inflorescence is widely known in classical and folk medicine, with the largest group of its effective constituents forming the essential oil (chamazulene, α -bisabolol, α -farnesene, trans- β -farnesene, spathulenol, cis/trans-en-in-dicycloethers). Among cultivated species, the Hungarian BK-2 contains more chamazulene in its essential oil than the German Degumil type, which is mainly cultivated for its α -bisabolol. Both components have important antiinflammatory activities.

Wild populations can be easily distinguished from cultivated ones by their high amount of bisaboloides, particularly the flower of Hungarian Szabadkígyós wild type, which contained on average 48 % of the biologically active (-)- α -bisabolol. The population of Szabadkígyós has good salt tolerance which is important owing to global warming, because the proportion of saline areas is increasing worldwide.

To keep the genome of Szabadkígyós having high (-)- α -bisabolol content, Szőke and research team used biotechnological methods.

Sterile plantlets, were infected by *Agrobacterium rhizogenes* strains #A-4, #15834, #R-1601. The hairy root clones possessing the best growing and biosynthetic potential were multiplied for phytochemical investigations. Pharmacologically important compounds of their essential oils were followed in great detail. The amount of *in vitro* cultured terpenoids and polyin compounds was compared with that of *in vivo* plants.

GC-MS studies showed that sterile chamomile cultures generated the most important terpenoid and polyin compounds characteristics of the mother plant. Berkheyaradulene, geranyl-isovalerat and cedrol as new components were identified in these sterile cultures. The main component of hairy root cultures (D/400, D/1, D/100 and Sz/400) was tr- β -farnesene and in addition one new compound: α -selinene was identified. Hairy root culture originated from chamomile collected in Szabadkígyós was intensive increased the essential oil content and pharmacological active compounds: (-) - α -bisabolol and β -eudesmol was also synthesized in large quantity. Furthermore, *in vitro* organized cultures were made from this population to obtain propagation material containing numerous active substances.

Keywords: *Chamomilla recutita*; Hairy root cultures; Essential oil: (-)- α -bisabolol; Chamazulene, β -eudesmol; Selinenes; Farnesenes; Cedrol; Geranyl-isovalerate; Berkheyaradulene

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1. Introduction

By naturally occurring gene-transformation Ri-plasmids of *Agrobacterium rhizogenes* integrate into the plant genome, thereby inducing the formation of hairy roots. It is characterized by a high growth rate and genetic stability. Hairy root cultures have been proven to be an efficient means of producing secondary metabolites that are normally biosynthesized in roots of differentiated plants [1, 2]. Clones possessing the best biosynthetically potential were multiplied for phytochemical investigations. The amount of terpenoid and polyin compounds in genetically transformed cultured was compared with that of *in vivo* plants chamomile.

The effect of chamomile is made up by several groups of active substances, among which terpenoids in the inflorescences are of greatest importance. Among cultivated species, the Hungarian BK-2 contains more chamazulene in its essential oil than the German Degumil type, which is mainly cultivated for its (-)- α -bisabolol. Both components have important anti-inflammatory activities [3, 4, 5, 6, 7].

Chamomile is used worldwide as a medicinal plant and it is one of the most important crops for pharmaceutical and cosmetic purposes [8]. Its importance is widely known in both official and folk medicines. Chamomile shows different pharmacological activities like anti-inflammatory, anti-cancer, treatment of stress and depression, anti-allergic etc. The therapeutic activity of different parts of chamomile is due to the active ingredients of various chemical structures (essential oils are the most important) which make up the complex effect of the *Matricariae flos* in numerous Pharmacopoeias [9, 10, 11, 12, 13, 14, 15].

Matricariae flowers are yet highly variable in quality depending on their source of raw material collections of diploid wild types or the cultivated di- and tetraploid chamomile varieties (induced by 1colchicine treatment), and their variable compositions of the main ingredients, respectively [16, 17, 18]. Chamomile breeding started 70 years ago and included diploid as well as tetraploid varieties [19]. The Czech and Slovak Republics, Hungary, Poland and Germany were the nations which the first experiments on breeding and cultivation took place [20, 21]. Unfortunately, information about the natural populations serving as origins is scarce. The use molecular approaches could help to resolve the genetic relationship between different chamomile varieties [22]. Several molecular approaches to maintain breeding processes were conducted in the past. [23, 24, 25]. The exploitation of the mitochondrial diversity of *M. chamomilla* L. is just at the beginning [26].

The essential oil content of plant parts under and above ground depends on different chemotypes [21]. According to the bisabololoxide content, commercial chamomile populations are classified as types of bisabolol, bisabololoxide A and B, and bisabololoxide [27]. During the ontogenesis the essential oil content changes, reaching a maximum in the flower just before flowering (0.3-1.5%), and decreasing after the process of flowering. This is certainly the case for matricin, bisabolol content and E- β -farnesene [12, 17, 28].

The root of chamomile contains only traces of essential oil (0.02-0.11%). Despite the fact that some therapeutically active compounds such as chamazulene and (-)- α -bisabolol are missing, other substances such as E- β -farnesene, α -farnesene, en-in-dicycloethers, β -caryophyllene and caryophyllene-epoxide can be detected. The other active substances in the flowers of chamomile are flavonoids, coumarins and polysaccharides [21, 29].

The positive effects derived from chamomile use are related to the presence of its numerous flavonoid constituents. Regarding their structure many of these constituents are either flavone- (apigenin, luteolin) or flavonol-derivatives (quercetin, patuletin, isorhamnetin). Some of these constituents have been directly associated with specific therapeutic effects; luteolin and the quercetin aglyca for instance have demonstrated protective and delaying effects on the development of diabetic complications [30]. Furthermore apigenin and its -7-O-glucoside derivative are among the most important therapeutic flavonoids; their spasmolytic and antiphlogistic effects are especially prominent [11], in addition to anti proliferative and apoptotic effects in various human cancer cell lines [31].

Chamomile has also been evaluated for its anticancer property evaluated the effect of a botanical supplement (TBS-101) on invasive prostate cancer in animal models. TBS-101 contains seven standardized botanical drugs including chamomile [32]. Zu et al. [33] studied the effect of chamomile essential oil on three human cancer cell lines: A-549 (human lung cancer), PC-3 (human prostate cancer), and MCF-7 (human breast cancer) The viable MCF-7 cells were reduced to 6.9% by chamomile oil treatment. These results suggested that chamomile oil could possess potential anticancer activity.

The main anti-inflammatory activity is due to chamazulene (which is formed from matricine during the steam distillation of the oil) and (-)- α -bisabolol, further bisabololoxide A and B play a role. Spasmolytic effects are attributed to apigenin and bisabololoxides [13] and wound healing properties to chamazulene, apigenin and (-)- α -bisabolol [34]. In addition, the chamomile oil has an antibacterial effect on Gram-positive germs and a fungicidal effect on *Candida albicans* when used in concentrations of more than 0.025% [35]. It should be noticed that chamomile flowers of the so called bisabololoxide B-type also contain an allergic compound: anthecotulide [36].

Ahmadi and research team [23] used the inter simple sequence repeat (ISSR) markers to assess the genetic diversity of 23 populations of chamomile. The chamomile populations collected from different geographic regions of Iran and from Italy and Hungary were used. Morphological data revealed relatively high variation among studied populations. Hungary population had the highest essential oil yield.

Both cultivated and wild chamomile types are used in Hungary for therapeutical purposes. In earlier investigations [3, 18] has found an increase of proazulenes in the flower of chamomiles, whereas [37] have found an increase in the bisaboloid content over the years. It was established that the content and composition of the essential oil of wild chamomile populations are related to the type of chamomile occurring in different areas of Hungary.

Based on examinations made earlier ago chamomile populations from the best areas were studied again [7, 38, 39,40]. The aim was to study the features of the essential oil production of the chamomile types in these 4 regions of Hungary in order to select chamomile types, which are rich in therapeutically active substances that can be retained using biotechnological methods.

2. Experimental

2.1. Plant material

Chamomilla recutita (L.) Rausch. (syn.: *Matricaria recutita* L., *Matricaria chamomilla* L.) the family Asteraceae.

Wild chamomile populations were obtained from sodic areas of Vésztő, Szabadkígyós (southern area of Hungary) and the National Park in Hortobágy in June. The specified plant and seed samples were deposited in our collection. They have common morphological features and are rich in (-)- α -bisabolol.

The improved Hungarian polyploid BK-2 and German Degumil genotype chamomile were obtained from the fields of Kerepes (Hungary).

2.2. Sterile chamomile cultures

Sterile chamomile plants were obtained by sterilization of seeds of intact plants [39, 41, 42]. Young plantlets were then cultivated in a climatized growth room at $22 \pm 2^\circ\text{C}$, under at 2500 Lux (cool and warm white fluorescent lamps, 16 hours' light, 8 hours' dark photoperiod), on solid $\frac{1}{2}$ MS [43] hormone-free media (Fig. 1).



Figure 1 *In vitro* chamomile cultures (6 weeks) in the climatized growth room

2.3. Hairy root cultures

Juvenile micropropagated plants, grown from sterile seeds, were cultured in light on Murashige-Skoog medium (1/2 MS 3%). After 3 months, they were infected with *Agrobacterium rhizogenes* (syn: *Rhizobium rhizogenes*) strains #A-4Y, #15834 and #R-1601, to induce so called hairy roots [44]. Different strains showed various abilities to induce hairy roots in this plant. The promoting effect of acetosyringone on the *Agrobacterium* mediated hairy roots initiation in chamomile has been investigated (Fig. 2).



Figure 2 Hairy roots initiation in chamomile and hairy root clones

In order to obtain media free from bacteria, hairy root cultures of A4-Y and 15834 strains were cultured on MS medium supplemented with carbenicillin (800 mg/l). Hairy root cultures of R-1601 strain were cultivated on MS medium supplemented with cephotoxime + ampicillin (250 + 1000 mg/l) for several subcultures to eliminate the bacteria. The hairy roots exempted.

from bacteria were cultivated on solid and then in liquid hormone-free B5 [45] and MS medium at $24 \pm 2^\circ\text{C}$. The established hairy root clones were maintained on solid medium containing 2% saccharose (MS-2) and subcultured every 6 weeks (Fig.3). The control of gene transformation: detection of opine by electrophoresis and PCR methods.

Analysed the effect of MgSO_4 we added it to the culture medium MS-2 and B5 medium (370 and 250 mg/l in doses reduced to one - half (185 and 125 mg/l) and redoubled (740 and 500 mg/l), respectively, in addition to the 370 mg/l concentration prescribed by Murashige – Skoog [43].

2.4. Extraction of the essential oil

The essential oil of both intact and sterile roots, herbs, and flowers was extracted by steam distillation with apparatus Clavenger according to the Pharmacopoeia (Ph.Eur.).

For the purpose of chemical analysis the hairy roots were refrigerated at -40°C . The samples were homogenized with biomix (1000 r min^{-1}), and the essential oil was obtained by steam distillation with apparatus Clavenger (Ph.Eur.).

The essential oil content was measured gravimetrically [46]. The qualitative and quantitative composition of the essential oil was examined by gas chromatographic (GC) and mass spectrometric (GC-MS) methods, which procedures were carried out by validated conditions.

2.5. Chemicals

Organic solvents (n-hexane, chloroform) were of reagent-grade and supplied by Reanal Ltd. (Hungary). Carbon dioxide (95-96% w/w pure) was purchased from Messer Griesheim Hungaria Ltd. The standards originated from Fluka Chemie GmbH (Switzerland) and Carl Roth GmbH (Germany) firms (purum; 97-99%, GC). β -Eudesmol was sent and structure determined by Professor Hisayuki Kanamori (Physics and Chemistry Division, Hiroshima Prefectural Institute of Public Health and Environment). The standard and investigation samples were stored in cooling apparatus at $+5^\circ\text{C}$.

2.6. Investigation of the essential oil

Gas chromatographically, standard addition and/or GC-MS methods were used to identify the oil components. The compounds were identified by comparing their relative retentions with those of authentic standards, essential oils of known composition and peak enrichment. The confirmation of identity was done by comparison of their mass spectra

with those reported in the literature and reference compounds. The percentage evaluation of the oil components was made by area normalization, on the basis of three parallel measurements. The deviation from average was (\pm) 6-8% at each compound.

2.7. Gas chromatographic (GC) parameters

Gas chromatograph: FISIONS GC 8000; Capillary column: 30 m \times 0.32 mm I.D.; Film thickness: 0.25 μ m; Stationary phase: β -DEXm; Oven temperature: 60-230 $^{\circ}$ C, 8 $^{\circ}$ C min $^{-1}$, 230 $^{\circ}$ C, isotherm 3 min; Detector temperature: flame ionisation, 240 $^{\circ}$ C; Carrier gas: Nitrogen, pN $_2$ =0.05 MPa, flow rate 6.8 cm 3 min $^{-1}$; Injector temperature: 200 $^{\circ}$ C; Injection: splitless 10 s, split rate 1 : 10; Injected volume: 0.4 μ L of solution 5 μ L oil in 1 mL chloroform. For evaluation was used Chrom Card computer program.

2.8. Gas chromatographic–mass spectrometric parameters

The GC-MS analyses were performed on a Hewlett–Packard and Finnigan GCQ instruments.

2.8.1. GC-MS parameters (Hewlett–Packard)

- *Gas chromatographic parameters*: chromatograph type Hewlett–Packard 6890 GC; Capillary column: 30 m \times 0.25 mm, I.D.; Stationary phase: DB-WAX (polyethylene glycol ester, J & W Scientific); Temperature program: 40 $^{\circ}$ C for 3 min, 1.5 $^{\circ}$ C min $^{-1}$ to 45 $^{\circ}$ C, 3 $^{\circ}$ C min $^{-1}$ to 80 $^{\circ}$ C, 5 $^{\circ}$ C min $^{-1}$ to 180 $^{\circ}$ C, 10 $^{\circ}$ C min $^{-1}$ to 240 $^{\circ}$ C, 240 $^{\circ}$ C for 3 min; Carrier gas: He, pHe = 0.20 MPa; flow rate 1.0 cm 3 min $^{-1}$; Injector temperature: 220 $^{\circ}$ C; transfer line temperature: 250 $^{\circ}$ C;

- *MS-parameters*: Hewlett–Packard 5973 mass-selective detector; MS source 230 $^{\circ}$ C; quadrupole temperature: 150 $^{\circ}$ C; Mode of operation: to identify and quantify the products MS was run in the electron impact (EI) an electron energy of 70 eV; Scanning the 10-400 m/z range.

2.8.2. GC-MS parameters (Finnigan GCQ)

- *Gas chromatographic parameters*: chromatograph type: Finnigan GC (San José, CA, USA); Capillary column: 30 m \times 0.22 mm I.D.; Film thickness: 0.25 μ m; Stationary phase: BPX5 (non polar); Oven temperature: 60-230 $^{\circ}$ C, 8 $^{\circ}$ C min $^{-1}$, 230 $^{\circ}$ C isotherm 3 min.; Carrier gas: He, pHe = 0.20 MPa; flow rate 4.0 cm 3 min $^{-1}$; Injector temperature: 200 $^{\circ}$ C; The injector was operated in the splitless mode 6 s, split rate 1 : 10; Injected solution volume: 0.4 μ L.

- *MS-parameters*: Detector: Finnigan MS; Start: 3 min after injection; Mode of operation: Electron-impact-ionisation (EI) positive ion with an electron energy of 70 eV; Mass range: 40-650 m/z ; Scanning: 1 analyse per second; Evaluation: Finnigan GCQ 2.0 computer program.

2.8.3. Solid phase microextraction (SPME) and GC-MS conditions

The fresh plant samples (0.3 - 0.5 g), or the powdered plant material and the acetone extracts were put into 20 mL headspace vials sealed with a silicone/PTFE septum prior analyses. The extracts were evaporated to dryness at room temperature in the vials. The static headspace solid phase microextraction (sHS-SPME) sample preparation was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 μ m StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min, the extractions were performed by exposing the fiber for 10, 20, or 40 min at 60, 80, or 100 $^{\circ}$ C to the headspace of a 20 mL vial. Then the fiber was transferred immediately into the heated GC-MS injector, and desorbed for 1 min at 250 $^{\circ}$ C. The SPME fiber was cleaned and conditioned in pure nitrogen atmosphere at 250 $^{\circ}$ C for 15 min after desorption.

The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA). Separations were performed using an SLB-5ms capillary column (30 m \times 250 μ m \times 0.25 μ m). The GC oven temperature was programmed from 60 $^{\circ}$ C (3 min isothermal) to 200 $^{\circ}$ C at 8 $^{\circ}$ C/min (2 min isothermal), 200-230 $^{\circ}$ C at 10 $^{\circ}$ C/min (5 min isothermal) and finally 230-250 $^{\circ}$ C at 10 $^{\circ}$ C/min (1 min isothermal). Helium was the carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode. The injector temperature was set to 250 $^{\circ}$ C and the split ratio was 1:15.

The mass selective detector was equipped with a quadrupole mass analyzer and was operated in electron ionization mode at 70 eV. The MS was operated in full scan mode (40-500 amu at 3.2 scan/s), and data were evaluated by MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing

retention times, retention indices and recorded spectra with data known from the literature and the NIST 05 library was also consulted.

Percentage data of the total ion current chromatograms were calculated by the area normalization method without applying response factor correction.

2.8.4. Cultivated and wild chamomile populations

The total essential oil content and the percentile distribution of its components were evaluated in selected wild chamomile populations chosen according to the previous investigations. In addition, comparison with cultivated *BK-2* type chamomile concerning the essential oil was done. Table 1 shows that the highest amount of the total essential oil is found in the flowers of wild *Hortobágy* population. A similar tendency was observed in the same chamomile type concerning the herbs [39].

Concerning the herbs, its essential oil content is equally low both in cultivated and wild chamomile populations (Table 1).

Table 1 Total essential oil content (%) of flowers, herbs and roots in cultivated and wild chamomile populations

Chamomile	Total essential oil content (%)		
	Flowers	Herb	Root
BK-2	0,33	0,08	0,13
Degumil	0,78	0,07	0,04
Robumil	0,52	0,06	0,05
Hortobágy	0,70	0,12	0,02
Vésztő	0,31	-	-
Szeghalom	0,55	0,07	0,05
Szabadkígyós	0,43	0,07	0,12

The GC analysis (Table 2, 3, 4 and Fig.3) it was clear that the oils from the **flowers** of the *Szabadkígyós* population were the richest in (-)- α -bisabolol (increasing 42→54%) and that from the flowers of *BK-2* in chamazulene (decreasing 24→18%). The ratio of (-)- α -bisabolol relative to that chamazulene was always higher in wild samples. The highest bisabolol-oxide A content was found in the oil of *BK-2* (36 %), whereas the *Hortobágy* population had the highest bisabolol-oxide-B concentration. Cycloethers occurred in about the same proportion in the studied samples; the content of *cis-en-in-dicycloethers* showed the higher amount of the *trans*-isomers, except for the *BK-2* type where the amount of *trans-en-in-dicycloether* exceeded that of *cis*-isomer.

It was interesting that the (-)- α -bisabolol content in the oil of *Szabadkígyós* population was 48 % (average on three consecutive years, Fig. 3); therefore we planned to keep the genome of this type using biotechnological methods in order to produce chamomiles with high content of active substances.

Concerning the **herb**, its essential oil content was equally low both in cultivated and wild chamomile populations [39]. Both in the herbs of the cultivated *BK-2* and the wild populations from *Szabadkígyós*, *trans*- β -farnesene was the main component. *Cis*-spiroethers exceeded the content of *trans*-isomers in all cases. Further in the herbs four sesquiterpene compounds (M⁺204): germacrene-D, α -selinene, berkheyaradulene and 4-(2', 4', 4'-trimethyl-bicyclo [4.1.0] hept-2'-en-3'-yl)-3-buten-2-one were identified firstly. We could detected germacrene-D, a monocyclic sesquiterpene in the flower too. A bicyclic sesquiterpene hydrocarbon: α -selinene and berkheyaradulene a tricyclic sesquiterpene hydrocarbon were earlier described in other species of Asteraceae family. Further the herbs contained one oxygenated sesquiterpene: 4-(2',4',4'-trimethyl-bicyclo [4.1.0] hept-2'-en-3'-yl)-3-buten-2-one bicyclic sesquiterpene as minor oil constituent.

The characteristic main component of the **root** oil was again *trans*- β -farnesene, but its amount was lower than in herbs (Table 3, 4). In the essential oil of *BK-2* root it reached nearly 40 %. Wild populations were also rich in percentile

distribution of components similarly to the cultivated *BK-2* type. In the oil of the roots β -eudesmol was determined and identified by GC/MS, this component was characteristic for wild populations [39]. The highest value of β -eudesmol was found in the oil of roots of *Szeghalom*. The four compounds above mentioned were present also in the root oil.

Table 2 Percentile distribution of oil components in the total essential oil of flowers in cultivated and wild chamomile populations

FLOWERS/components	Degumil	BK - 2	Szeghalom	Vésztő	Hortobágy	Szabadkígyós
β -caryophyllene	0,15	0,07	0,20	0,65	0,68	0,90
tr- β -farnesene	12,80	8,22	9,53	7,20	11,07	15,28
germacrene-D	0,55	0,16	0,83	0,53	0,62	5,78
α -muurolene	2,86	1,00	0,37	0,24	0,33	1,43
α -farnesene	0,30	0,27	1,62	1,34	2,43	0,15
α -cadinene	0,90	0,85	2,61	1,93	2,99	3,75
spathulenol	0,46	0,90	1,01	0,92	0,81	0,77
bisabolol-oxide B	7,32	8,25	18,37	15,10	20,42	3,61
(-)- α -bisabolol	30,00	1,59	20,72	34,91	24,00	41,45
bisabolon-oxide	0,55	4,13	3,55	2,60	2,51	1,07
chamazulene	24,50	23,41	10,84	5,23	9,31	8,71
bisabolol-oxide A	6,00	36,27	16,67	13,07	11,24	0,42
cis-spiroether	7,48	3,43	5,64	4,12	4,28	6,05
trans-spiroether	0,90	6,01	2,66	1,08	1,87	3,70

Table 3 Percentile distribution of oil components in the total essential oil of herbs (stem plus leaves) in cultivated and wild chamomile populations

HERBS/components	Degumil	BK - 2	Szeghalom	Hortobágy	Szabadkígyós
M+ 204	-	0,29	0,16	0,32	0,36
berkheyaradulene	0,20	1,21	0,72	0,98	1,65
α -selinene	+	0,21	0,21	0,21	0,28
β - caryophyllene	+	0,22	0,15	0,22	0,16
tr- β - farnesene	56,74	59,03	52,47	59,01	58,51
germacrene-D	0,43	0,74	0,52	0,70	0,28
α -muurolene	2,17	1,68	1,27	1,68	0,62
α -farnesene	3,60	6,61	4,40	6,02	2,06
α -cadinene	0,50	1,77	2,65	2,32	1,16
spathulenol	0,86	2,85	5,96	3,00	2,81
bisabolol-oxide B	0,45	0,67	1,37	+	3,10
(-)- α -bisabolol	3,93	0,47	3,28	0,47	1,01
bisabolon-oxide	0,20	0,29	0,39	+	0,33
bisabolol-oxide A	0,26	0,28	0,51	0,20	+
cis-spiroether	6,70	9,95	7,23	9,95	4,92
chamomilla ester	0,14	0,11	0,57	0,11	0,39
trans-spiroether	3,70	1,82	3,21	1,82	1,82

Table 4 Percentile distribution of oil components in the total essential oil of roots in cultivated and wild chamomile populations

ROOTS/components	Degumil	BK - 2	Szeghalom	Hortobágy	Szabadkígyós
M ⁺ 204	0,18	2,02	0,60	0,79	0,60
berkheyaradulene	0,83	7,20	2,27	2,94	1,75
α -selinene	0,21	1,32	0,58	0,65	0,59
β -caryophyllene	0,13	0,91	0,33	0,49	0,40
tr- β -farnesene	25,80	39,8	30,7	35,80	30,2
germacrene-D	0,11	0,42	0,80	0,22	0,73
α -muurolene	0,74	1,97	1,15	2,36	2,98
α -farnesene	1,20	3,06	1,72	2,85	1,17
α -cadinene	0,07	+	0,12	0,17	0,15
geranyl-isovalerate	3,37	2,50	1,53	1,30	1,48
spathulenol	0,56	0,60	0,93	0,48	0,73
cedrol (M ⁺ 222)	24,9	6,42	24,2	6,95	17,54
bisabolol-oxide B	0,70	1,50	1,12	1,51	2,63
β -eudesmol	8,23	1,10	9,25	3,16	4,87
bisabolon-oxide	0,26	0,18	0,26	0,16	0,22
cis-spiroether	13,85	15,00	11,25	24,50	16,10
chamomilla ester	0,55	1,10	0,14	0,39	0,25
trans-spiroether	4,03	1,12	1,42	2,00	3,10

+ in traces

We can conclude that, although a change was observed in the essential oil content and also in the proportion of pharmacologically active compounds in comparison with the results of the earlier survey, the fundamental characteristics of the oil of cultivated and wild chamomile populations remained the same.

We planned to keep the genome of wild type Szabadkígyós using biotechnological methods in order to produce chamomiles with high content of active substances (Fig. 3) [40].

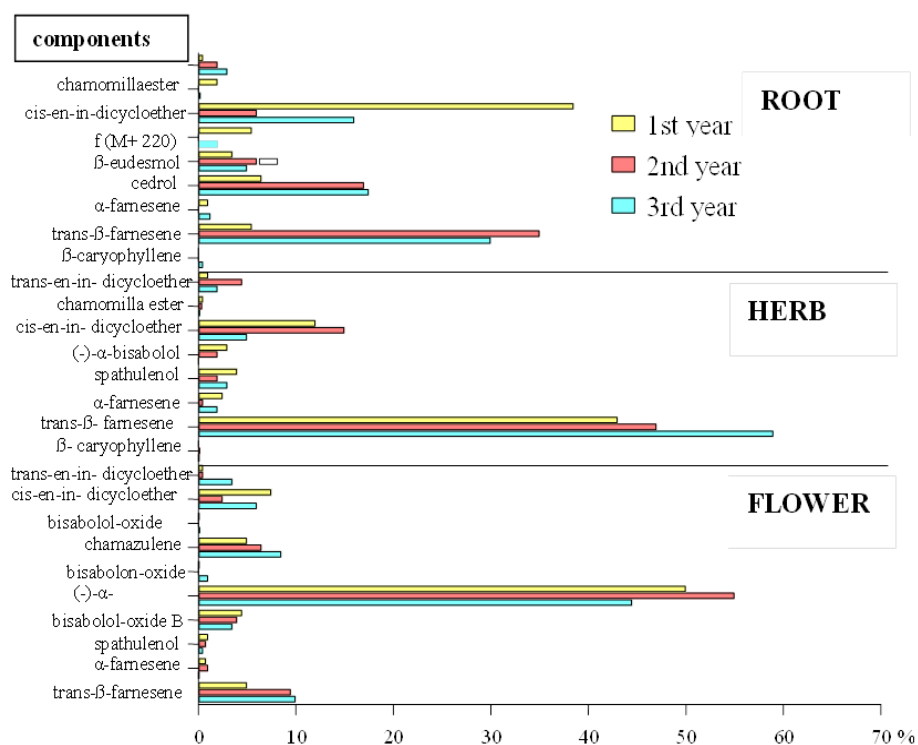


Figure 3 Percentile distribution of some components in essential oil of roots, herbs and flowers of wild chamomile from Szabadkígyós (during 3 years) [40]

Hairy root cultures were mostly used in research on the biogenesis of root specific metabolites and dependency of biosynthetic activity on the degree of physiological differentiation of the plant cell. The full identity of compounds produced by transformed roots with those of intact plants was shown. Some differences between qualitative and quantitative composition of biologically active metabolites synthesized by hairy roots and the intact plants were determined by GC and GC-MS.

3. Sterile *in vitro* chamomile cultures

Among wild chamomile populations in Hungary, a population was found in the area of Szabadkígyós containing significant amounts - on average 48 % - of α -bisabolol in its inflorescences. We planned to keep the genome of this type using biotechnological methods [38, 47, 48,] in order to produce chamomiles with high content of active substances.

Sterile organized chamomile cultures were then cultivated on solid $\frac{1}{2}$ Murashige-Skoog hormone-free media, in a climatized growth room (Fig.1).

The essential oil content (%) of herbs and roots in cultivated (Degumil) and wild chamomile (Szabadkígyós) populations *in vivo* and *in vitro*, shows Fig 4. [40].

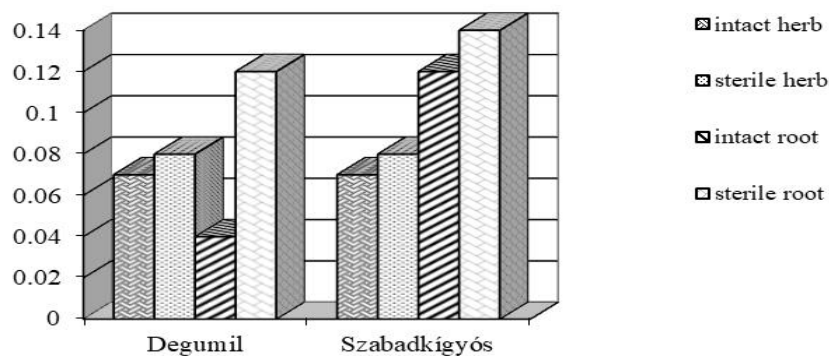


Figure 4 Total essential oil content (%) of herbs and roots in cultivated (Degumil) and wild chamomile (Szabadkígyós) populations *in vivo* and *in vitro*

Gas chromatographically and mass-spectroscopically studies showed that sterile chamomile cultures generated the most important terpenoid and polyin compounds characteristics of the mother plant (Table 5, Fig. 5). Berkheyaradulene, α -selinene, geranyl-isovalerate and cedrol as new components were identified in these sterile cultures. Furthermore, *in vitro* cultures were made from this population to propagation material containing a high number of other active substances too.

The sterile roots contained also no α -bisabolol but a new sesquiterpene alcohol β -eudesmol was firstly identified from roots of the sterile plants [48]. Although β -eudesmol seemed a characteristic component in organized sterile roots of Degumil and Szabadkígyós types was also present (Table 5) [40].

Table 5 Comparing of percentile distribution of oil components in the total essential oil of sterile organised culture (1/2 MS medium) from cultivated (Degumil) and wild (Szabadkígyós) chamomile populations

Component	Degumil		Szabadkígyós	
	sterile herb	sterile root	sterile herb	sterile root
M+ 204	0,96	3,20	0,35	1,84
berkheyaradulene	2,89	11,80	1,14	4,17
α -selinene	0,53	2,41	0,25	1,25
β -caryophyllene	0,76	1,80	0,68	1,20
tr- β -farnesene	14,24	26,19	8,42	33,57
germacrene-D	2,57	0,52	2,51	0,50
α -muurolene	-	-	-	-
α -farnesene	35,74	3,93	27,52	0,82
α -cadinene	0,42	-	0,17	-
geranyl-isovalerate (M+238)	1,26	6,50	0,72	9,63
spathulenol	0,68	-	0,55	-
cedrol (M+ 222)	3,62	8,71	0,51	1,40
bisabolol-oxide B	-	+	+	0,24
(-)- α -bisabolol	2,46	-	1,26	-
β -eudesmol	-	2,81		1,23
bisabolon-oxide	+	+	0,4	1,00
cis-spiroether	2,60	0,67	0,64	0,25
trans- spiroether	1,34	+	1,55	+

+ in traces

Regarding the numerous pharmacological tests proving the biological activity of β -eudesmol the aim was to research plants occurring in Hungary which contain β -eudesmol as oil constituent [40]. It was established that the chamomile root oils contain this compound between 1-10 %, at the same time in flower oils it was not detectable, but the flower oils are in general rich in (-)- α -bisabolol [48].

The new sesquiterpenes, β -eudesmol and geranyl-isovalerate seemed also characteristic essential oil components of the intact and organized root of chamomile which were identified by GC and GC/MS methods (Fig.5) [40]. The confirmation of identity was done by comparison of mass spectra with those reported in the literature and reference compound. The percentage evaluation of the oil component was made by area normalization, on the basis of three parallel measurements. Among the cultivated and wild chamomile species examined in the wild species from the areas of Szeghalom occurred in the biggest quantity of β -eudesmol (9.25% in the total essential oil).

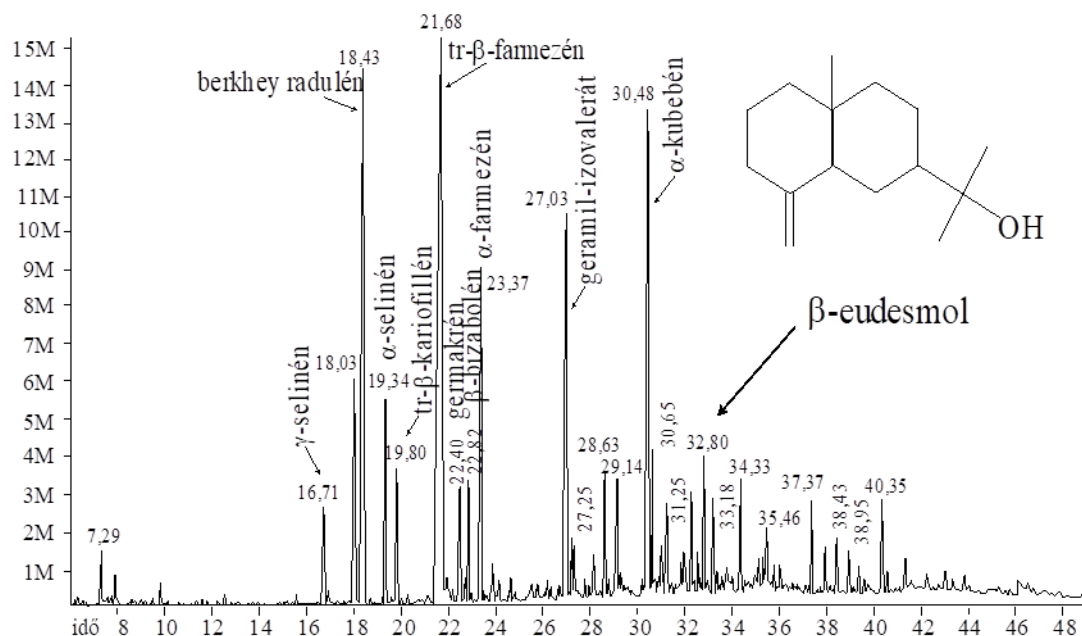


Figure 5 GC-MS identification of β -eudesmol and geranyl-isovalerate in essential oil of root [40]

4. Salt Stress

Szöke and her team checked the salt tolerance of the cultures of Szabadkígyós, given that the proportion of saline areas is increasing all over the world, including in Europe, due to global warming.

Salt stress is one of the most common abiotic stresses. High salt concentrations cause water deficit, ion nutrient imbalance, and oxidative stress. These can cause modification in root morphology and even death of the plant. A study was conducted to determine the effect of salt stress and growth of the plant and the quality and quantity of the essential oil in chamomile. It was found that an increase in salinity caused a reduction in the growth of the plant. High salinity level decreased plant height, number of branches, number of flowers, and essential oil content. The effect of salinity was highest on the dry flower weight. The effect of salinity on the growth of the chamomile plant was also studied by Ghanavati and Sengul [49].

Tai et al. [50] reported that with the increasing concentrations of NaCl in the soil, the morphological, anatomical, and biochemical changes occurred in the chamomile plant. Biochemically, the level of chlorophyll increased, and then decreases. The soluble sugars, proline, and peroxidase activity increased [21]. Laxa et al. [51] studied the role of the plant antioxidant system in drought tolerance.

Cellárová et al. [52] the salt tolerance were examined according to both fresh and dry matter increases of callus cultivated on the media supplemented with various sodium chloride concentrations as well as a high amount of K^+ , Na^+ , Ca^{2+} , and Mg^{2+} ions as found in the East Slovakia salt soil lowland. The cells tolerate salinity at least up to 85 mM.

Máday [53] studied the influence of different concentrations of Na_2SO_4 on the organized chamomile cultures (herb and root) originating from Szabadkígyós (Fig.6). The concentrations 0, 10, 25 and 50 mM Na_2SO_4 both the herb and root dry weight (g) increased. 100 mM Na_2SO_4 inhibited the growth of cultures. Influence of different concentrations of Na_2SO_4 to the essential oil (Fig. 7) amount obtained by steam distillation from organized chamomile cultures (herb and root).

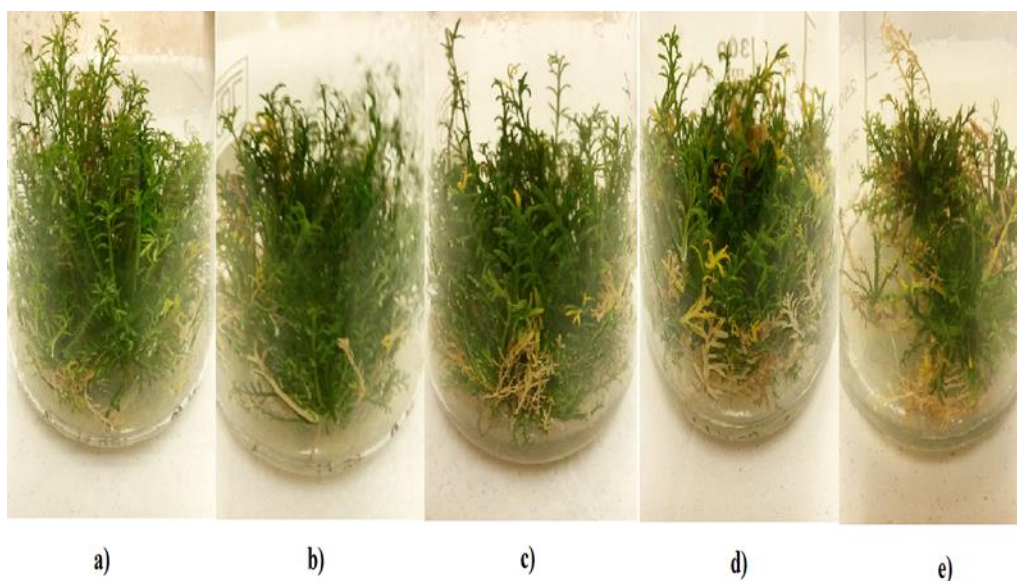


Figure 6 Chamomile sterile cultures cultivated on a variety of salt concentrations (mM Na_2SO_4) MS medium Symbols: a) Control 0 mM, b) 10 mM, c) 25 mM d) 50 mM, e) 100 mM Na_2SO_4

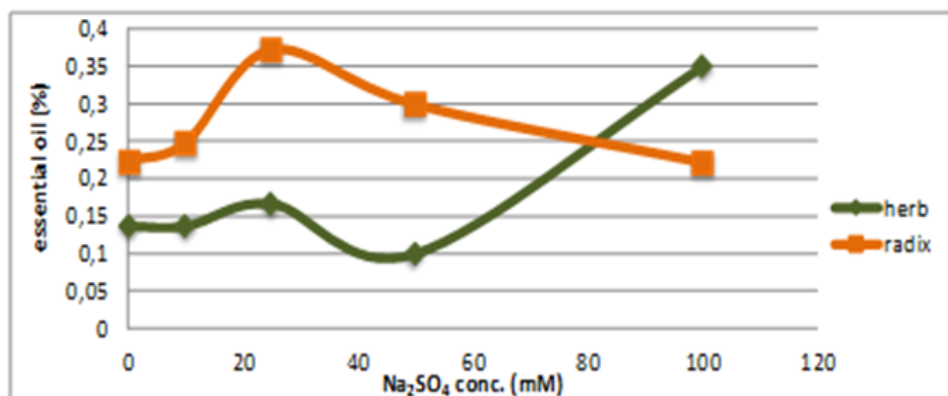


Figure 7 Change of essential oil content of organized cultures depending on concentrations of Na_2SO_4

The percentage distribution of 18 essential oil components detected in herb from *in vitro* cultures grown on various media containing different concentrations of Na_2SO_4 was changed during the treatments. The effect of treatment with 100mM Na_2SO_4 the pharmacologically active α -farnesene was synthesized in higher amount (19,2%) but the quantity of guaiadiene decreased drastically (5%). At the same time, the α -farnesene content of the essential oil increased (8%) similarly to the spiroether content (23,6%) which increased drastically [53]. The spiroethers increased significant the antiphlogistic and spasmolytic effects of the chamomile extracts [10, 21].

Summarizing: on the effect of medium containing Na_2SO_4 not only the amount of the total essential oil content increased but also the rate of spiroethers and farnesene isomers.

By increasing the NaCl concentration of the medium, we can say that the sterile culture obtained from the population of Szabadkígyós which originally grows on saline soils, grew better on the medium with high NaCl content than the Degumil variety, i.e. it is more salt tolerant. This is probably due to the fact that this population is already genetically adapted to high salt concentrations on saline soils. But it is necessary to note that according to previous studies the NaCl concentration (Fig. 8) did not influence the biosynthesis of the therapeutical important chamomile compounds [50].



Figure 8 Camomile sterile cultures cultivated on a variety of salt concentrations (NaCl 0-11.6 g/l) in MS medium (30 day)

Finally, it is important to note that the proportion of saline areas which is increasing due to global warming gives a special feature of this salt tolerant experimental results.

Nowadays it is especially important to research, examine, and reserve the genotype of salt- and drought-tolerant plant species.

5. Genetically transformed hairy root cultures

It is noteworthy that the population of Szabadkígyós, whose essential oil content and the quantity of (-) - α -bisabolol are extremely high (on average 48%), also has good salt and drought tolerance. Szőke and Máday [54] used biotechnological methods to preserve the genome in order to produce chamomile with a high pharmacologically active ingredient content.

Sterile organized cultures were infected by microinjection of *Agrobacterium rhizogenes*. By naturally occurring gene-transformation the transfer T-DNS of Ri-plasmids from *Agrobacterium* integrate into the plant genome. The Ri-plasmid is inducing the formation of hairy roots (Fig. 2). The clones of hairy root possessing the best growing and biosynthetically potential were multiplied for phytochemical investigations. The amount of terpenoid and polyin compounds in genetically transformed cultured was compared with that of in vivo plants.

The hairy root cultures growing on liquid MS-2 medium (Fig. 9), the best biomass formation of clone Sz/400 (Table 6).

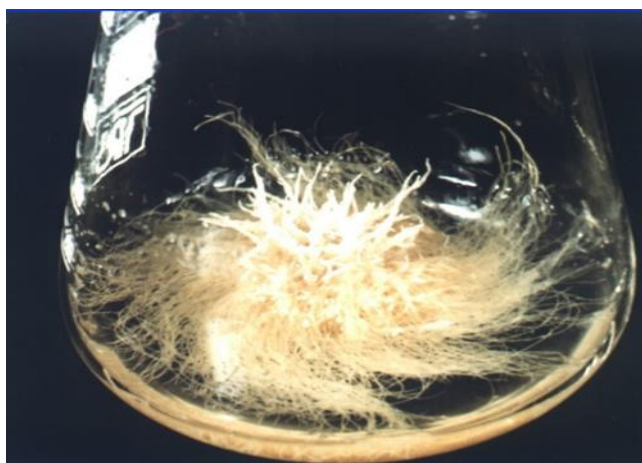


Figure 9 Hairy root culture of chamomile, cultivated on liquid medium MS-2

Table 6 Biomass formation in hairy root clones of chamomile Degumil (D/1, D/100, D/400) and Szabadkígyós Sz/400)

Medium	Hairy root clones fresh weight (g/culture)			
	D/400	D/1	D/100	Sz/400
solid MS-2	0,46 ± 0,19	0,35 ± 0,08	0,40 ± 0,03	0,47 ± 0,02
liquid MS-2	0,93 ± 0,05	2,02 ± 0,10	0,61 ± 0,03	9.60 ± 1.5
solid B5	0,20 ± 0,01	0,30 ± 0,02	0,30 ± 0,02	-
liquid B5	0,71 ± 0,03	1,57 ± 0,04	0,11 ± 0,04	-

The hairy root cultures growing also on liquid MS media where the essential oil formation also increased. The total essential oil content and the percentage composition of the oils were determined in hairy root clones, from cultivated and wild chamomile populations (Fig.10).

Comparing the total essential oil content of the hairy root cultures cultivated on different media, the liquid cultures presented higher value than the solid cultures. Their essential oil content was ten times higher than that of the intact root, and many times higher than that of the sterile plant root. The Sz/400 hairy root clone obtained from population of Szabadkígyós having high bisabolol content has the highest total essential oil content (1.17 %).

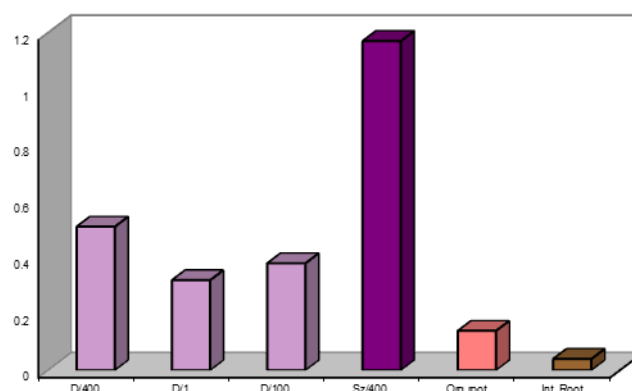


Figure 10 Essential oil content (%) of Degumil (D/400, D/1, D/100), Szabadkígyós (Sz/400) hairy root clones (MS-2 liquid medium) further organized and intact roots

Gas chromatographical and mass-spectroscopical studies showed that genetically transformed chamomile cultures generated the most important terpenoid and polyin compounds characteristics of the parent plant (*in vivo* and *in vitro*). Sterile cultures earlier 7 new components were identified [40, 48]; 6 compounds of them were synthesized in the hairy root oil as characteristic components. The main components of hairy root cultures were *tr*- β -farnesene, α -farnesene, geranyl-isovalerate and cedrol (Table 7). The Szabadkígyós (Sz/400) hairy root clones synthesized (-)- α -bisabolol in larger quantity, too.

Identified α -, and β -selinene, of them the latter one was new component in the genetically transformed cultures. The characteristic fragments of mass spectra, *m/z* (rel. int.) were following:

α -Selinene, 2-isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a-octahydro-naphthalene (Mw 204, C₁₅H₂₄): 204 [M]⁺ (25), 189(77), 175(10), 161(38), 147(50), 133(38), 121(42), 108(100), 93(40), 79(38), 67(17), 55(18).

β -Selinene, 7-isopropenyl-4a-methyl-1-methylene-decahydro-naphthalene (Mw 204, C₁₅H₂₄): 204 [M]⁺ (20), 189(18), 175(6), 161(20), 147(18), 134(20), 121(20), 109(100), 105(33), 94(68), 79(78), 69(22), 55(18).

Table 7 Percentile distribution of essential oil components (%) of hairy root clone (#D/400) in various media (comparing with oil composition of the sterile and intact chamomile roots)

Sign.	Components	Hairy root / medium				Sterile root	Intact root
		solid MS-2	liquid MS-2	solid B5	liquid B5		
a	4-(2', 4', 4'-trimethyl-bicyclo [4.1.0] hept-2'-en-3'-yl)-3-buten-2-one	1.7	3.6	1.5	1.8	3.2	0.18
b	Berkheyaradulene	2.6	6.5	2.0	3.2	11.8	0.83
c	α -Selinene	0.7	2.6	0.9	0.9	2.41	0.21
d	β -Caryophyllene	0.8	2.3	1.0	0.7	1.80	0.13
e	<i>trans</i> - β -Farnesene	20.4	24.9	16.1	10.1	11.8	25.80
f	β -Selinene	0.3	2.8	1.1	0.6	-	-
g	α -Farnesene	7.1	6.6	9.0	7.9	26.19	1.20
I	Unknown	6.0	1.4	4.3	5.9	-	-
h	Geranyl-isovalerate	19.1	7.0	27.6	11.5	6.50	3.37
i	Spathulenol	0.4	1.2	0.15	0.3	0.55	0.56
j	Cedrol	9.5	2.5	14.5	21.4	8.71	24.9
II	Unknown	5.8	2.2	3.3	5.6	-	-
k	Bisabolol-oxide B	2.0	1.7	2.0	0.67	-	0.70
l	(-)- α -Bisabolol	4.7	2.0	2.4	4.1	-	-
m	<i>cis</i> -Spiroether	in traces ^a	in traces	in traces	in traces	0.67	13.8
n	<i>trans</i> -Spiroether	in traces	in traces	in traces	in traces	in traces	4.03

The percentage evaluation was carried out on the basis of three parallel measurements.

The deviation from average was (\pm) 6-8% at each compounds.

^a in traces: below 0.10%

It was visible that *tr*- β -farnesene was present in significant amount in the essential oils of hairy roots cultivated on the various media, but it was contained in the highest amount in the hairy roots cultivated on solid and liquid media MS-2. Its percentile value reached the 24.9 % in essential oil of clone #D/400 cultivated on liquid medium MS-2 (Fig. 11, Table 7). In all cases the percentage occurrence of α -farnesene was lower. On medium B5 the volatile compound newly identified by us synthesized in significant amount: on liquid B5 the cedrol, on solid B5 the geranyl-isovalerate was the main component (Fig. 11) [44]. The geranyl-isovalerate was earlier identified in intact plants and in organized cultures (Fig. 5) [40, 48]. This compound was also present in high percent in the essential oil of the hairy root clone cultivated on solid medium B5.

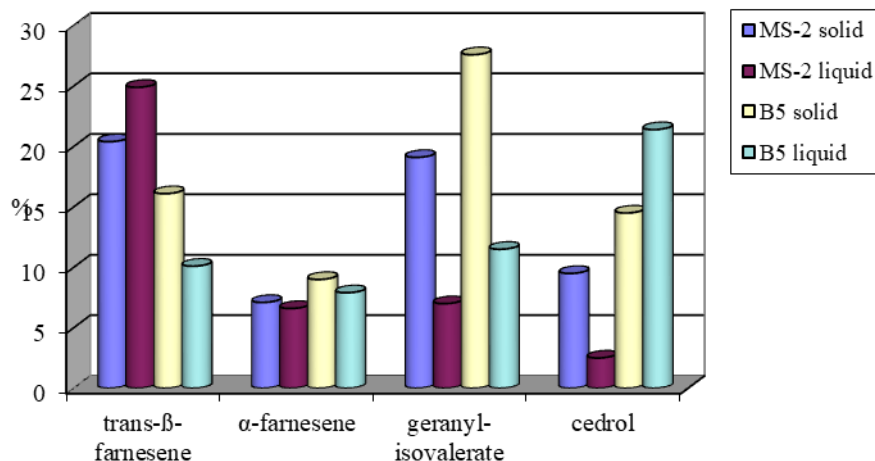


Figure 11 Percentile distribution of the characteristic oil components (%) of hairy root clone (#D/400) cultivated in various media

5.1. Influence of magnesium on the essential oil production in chamomile cultures

Magnesium ions play a significant role in all living cells and biochemical processes. As $MgSO_4$ has an exceptionally positive effect on rooting of chamomile organized cultures, it is interesting to demonstrate its effect on the growth of isolated roots and genetically transformed hairy root cultures. Observed the biomass production and essential oil formation in the cultures with respect to the therapeutically important compounds (Fig.12.) [55].

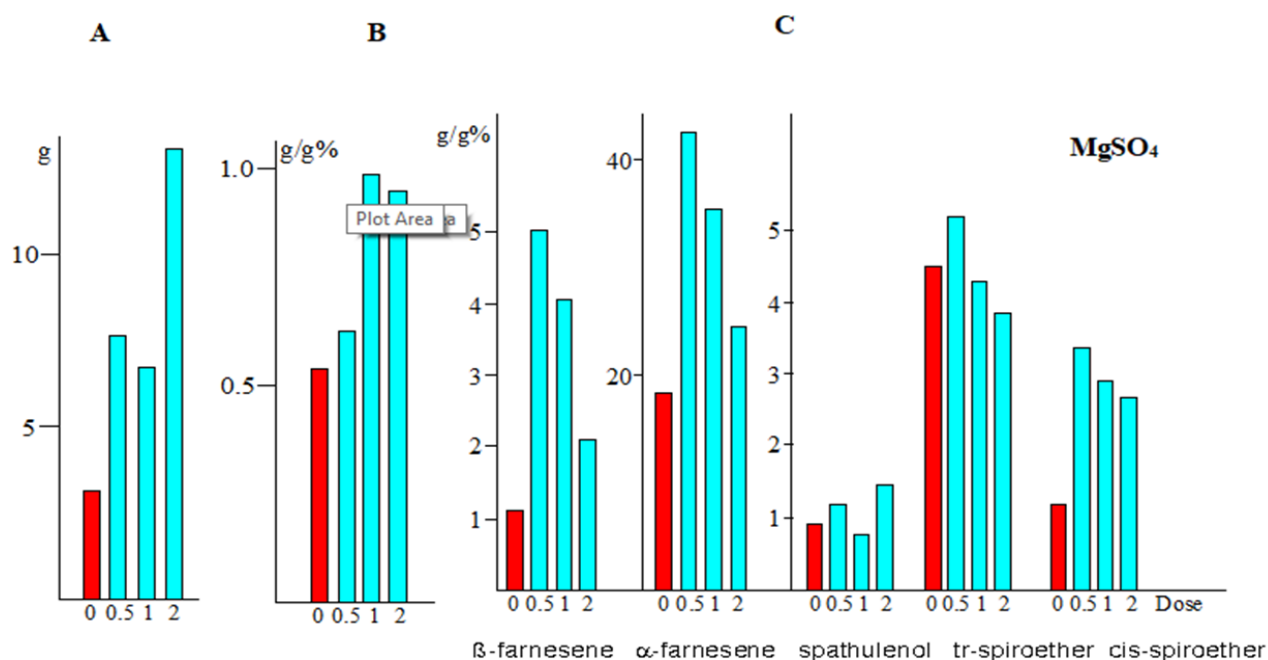


Figure 12 Effect of $MgSO_4$ on fresh weights (A) and essential oil content (B) and composition (C) of sterile organized cultures from cultivated (BK-2) chamomile herb

The hairy root cultures growing also on liquid MS media where the biomass formation increased with the Mg concentrate on which is correlated with the results of linear growth of cultures on solid MS medium (Fig. 13, Table 8) [56].

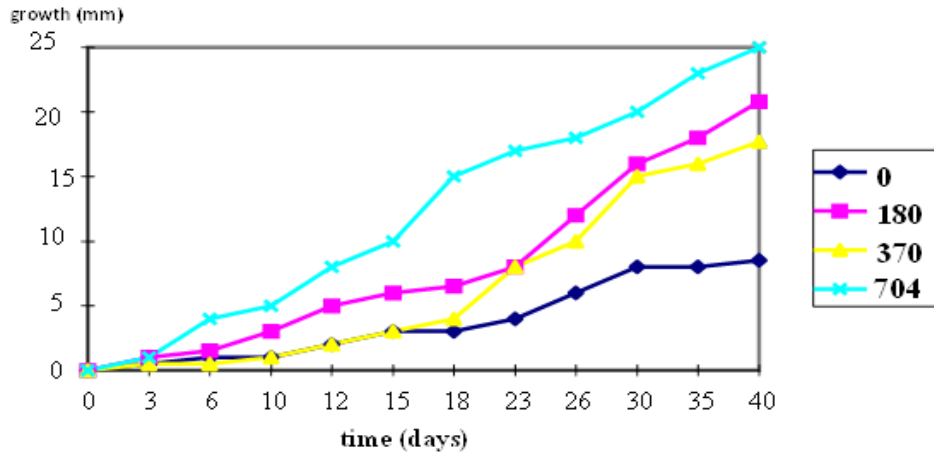


Figure 13 The effect of magnesium in different concentrations on the linear growth of chamomile hairy root cultures

Table 8 Magnesium effect on biomass formation of hairy root cultures (D/400 clone) on MS-2 and B-5 media

Medium	Fresh weight (g/culture)			
	MgSO ₄ mg/l			
	0	185	370	740
Solid MS-2	0.18 ± 0.03	0.66 ± 0.03	0.53 ± 0.04	0.94 ± 0.02
Liquid MS-2	0.22 ± 0.03	1.21 ± 0.03	1.10 ± 0.09	1.36 ± 0.03
	0	125	250	500
Solid B5	0.15 ± 0.03	0.33 ± 0.03	0.32 ± 0.03	0.57 ± 0.03
Liquid B5	0.28 ± 0.05	0.69 ± 0.08	0.76 ± 0.04	0.92 ± 0.05

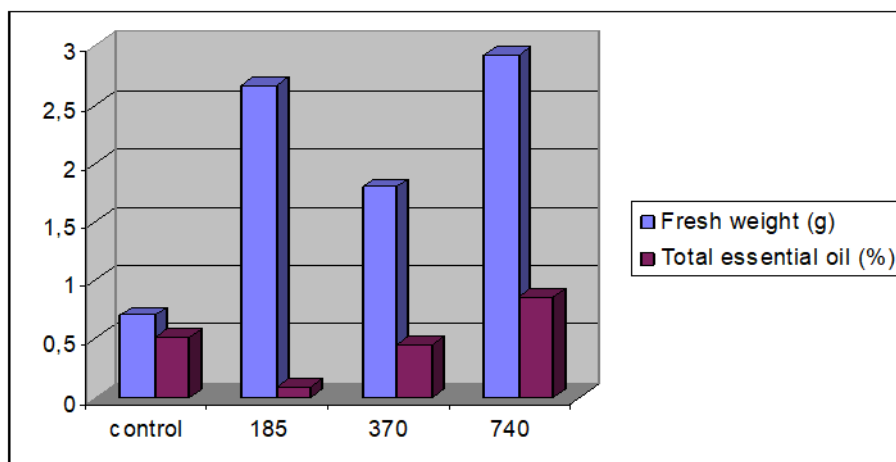


Figure 14 The effect of MgSO₄ in different concentrations on the fresh weight and essential oil content of chamomile hairy root cultures

The total essential oil content of hairy roots increased with the magnesium concentration, but in contrary, the relative percentile distribution of the main essential oil components (e.g. tr-β-farnesene, α-farnesene) decreased while that of

sesquiterpenes increased. These results correspond to those made previously in the organized chamomile cultures (Fig. 12 and 14).

According to the gas chromatogram the composition of the essential oil of the hairy root cultures growing on liquid MS media of different magnesium concentration is similar to each other but they differ in proportion: cultures at 185 mg/l mg-conc. are rich in tr- β -farnesene while at 740 mg/l Mg-concentration the main component is berkheyaradulene (sesquiterpene component of 204 molecule weight) which occurs in the intact plant as a minor component (Table 9, Fig. 15).

Table 9 Effect of MgSO₄ on essential oil components of hairy root cultures (chamomile Degumil) cultivated on MS medium

Components (%)	hairy root cultures (D-400 clone)			sterile roots	intact roots
	185 mg/l MgSO ₄	370 mg/l MgSO ₄	740 mg/l MgSO ₄		
M+ 204	1,9	1,2	8,6	3,2	0,18
berkheyaradulene	3,6	2,1	21,9	11,8	0,83
α -selinene	1,6	1,1	2,9	2,41	0,21
β -caryophyllene	1,6	1,3	1,3	1,80	0,13
trans- β -farnesene	23,4	9,2	10,6	26,19	25,80
α -farnesene	11,9	3,4	0,67	3,93	1,20
geranyl-isovalerate	18,5	19,1	9,8	6,50	3,37
spathulenol	0,7	1,4	2,4	-	0,56
cedrol	12,8	18,6	9,6	8,71	24,9
bisabolol-oxide B	1,2	1,0	0,45	+	0,70
β -eudesmol	-	-	-	2.81	8.23
(-)- α -bisabolol	1,3	0,85	1,1	-	-
cis-en-in-dicycloether	1,6	1,1	2,9	0,67	13,8
trans-en-in-dicycloether	1,6	1,3	1,3	+	4,03

+ in traces

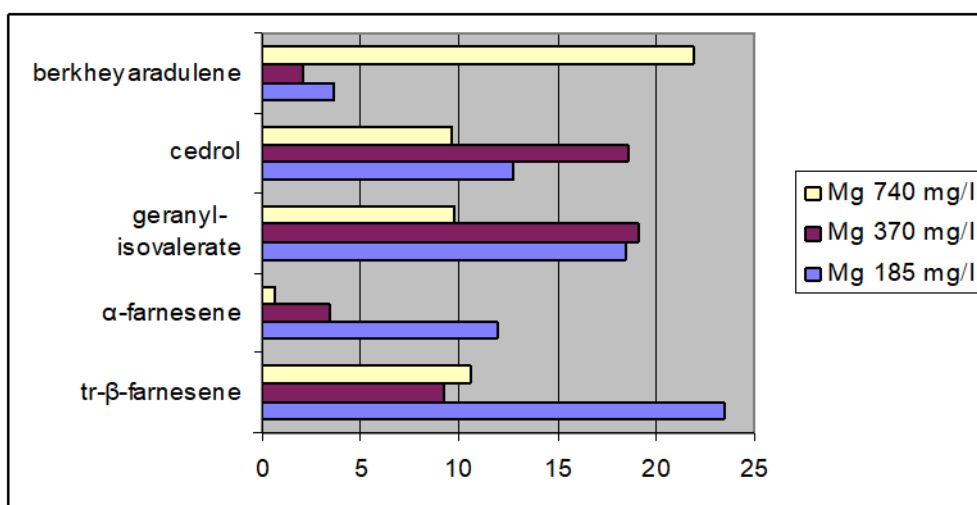


Figure 15 Percentage occurrence of some essential oil components in hairy roots

MS media favourably effects to the biomass and essential oil production of chamomile sterile cultures and hairy roots. The biggest effect was observed at 740 mg/l magnesium concentration, the essential oil production 5 times higher than

that of the control. In the contrary, the percentile distribution of the main components seems optimal at low Mg-concentration.

The Hungarian wild chamomiles first of all the population of Szabadkígyós chamomile has a high essential oil content and in it extreme high (-)- α -bisabolol content, further it synthesizes numerous other pharmacological active substances too. It has good salt and drought tolerance so it is convenient to preserve its genome and suitable for cultivation. In vitro organized cultures were made from this population to obtain propagation material containing numerous active substances. In hairy root cultures the content of essential oil and biologically active compounds increased, (-)- α -bisabolol and β -eudesmol were also synthesized in larger quantity.

6. Conclusion

The Hungarian wild chamomiles primarily the population originating from Szabadkígyós has good salt tolerance which is important due to global warming because the proportion of saline areas is increasing worldwide. Therefore, it is convenient to preserve its genome and suitable for cultivation. Analytical studies showed that sterile chamomile cultures generated the most important terpenoid and polyin compounds of the mother plant. Berkheyaradulen, α -selinene, geranyl-isovalerat and cedrol as new volatile components were identified in sterile cultures. In hairy root cultures the content of essential oil and other biologically active compounds increased in larger quantity, too.

Compliance with ethical standards

Acknowledgments

Many grateful thanks concern our co-workers - Máday E., Marczal G., Kuzovkina IN., Mészáros AM., Böszörményi A., Gershenzon J. - who were working in gathering of wild and cultivated chamomile samples, experiments of tissue cultures, and have taken part in analytical labors and chemical structure researches.

Disclosure of conflict of interest

The authors - who have contributed in the manuscript - declare that there are no conflicts of interest.

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