Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 76, No 8, 2023

BIOLOGY

Plant physiology

CLONAL IN VITRO PROPAGATION AS A TOOL FOR PRODUCTION OF HIGH QUALITY PLANTING MATERIAL OF *HELICHRYSUM ITALICUM* (ASTERACEAE)

Asya Kozhuharova[*,#], Marina Stanilova[*], Vladimir Vladimirov[*,], Milena Nikolova[*], Boryanka Traykova[*], Strahil Berkov[*]**

Received on January 19, 2022
Presented by D. Ivanov, Corresponding Member of BAS, on June 21, 2022

Abstract

Helichrysum italicum (immortelle), Asteraceae, is a perennial plant species with increasing market demand for its essential oil possessing anti-inflammatory, antibacterial, and antioxidant properties. The present study deals with clonal in vitro propagation of high-productive immortelle individuals. In vitro culture was initiated from stem tips on basal MS medium and sub-cultured several times on the same medium or on media supplemented with activated charcoal or with different plant growth regulators. Plants differed by their height, ramification, in vitro rooting, and callus formation. The highest propagation rate of 13.5 ± 4.2 new shoots per explant, along with spontaneous rooting, was noticed in spring on the control MS medium. About 180 plants were successfully ex vitro adapted first in a phytotron, then in a greenhouse, and 14 were transferred outdoor in the ex situ collection where they acclimatized and bloomed in the first season. The nuclear DNA amount in 65 individuals was measured by flow cytometry, and no deviations in the ploidy level were detected. The results suggest the genome of the in vitro obtained plants is stable. Essential oil was extracted by water distillation from two pooled samples, one for the mother plants and another one for the ex vitro adapted plants. The main components identified by GC/MS in both samples were α-pinene, nerol acetate,

[#]Corresponding author.
DOI:10.7546/CRABS.2023.08.06

and γ-curcumene. The results proved that the method of the in vitro clonal propagation is reliable for obtaining of numerous true-to-type plants in a short time, by direct organogenesis of high-productive individuals of *H. italicum*.

Key words: immortelle, in vitro micropropagation, essential oil

Introduction. *Helichrysum italicum* (Roth) G. Don (immortelle), Asteraceae, is a perennial plant species, valuable for its essential oil. The latter is present in the whole aerial part of the plants and possesses anti-inflammatory, antibacterial, antiallergic, and antioxidant properties [1,2]. Plant extracts were used in traditional medicine in the Mediterranean region [1]. Nowadays, the market demand of the immortelle essential oil is increasing, especially for the perfume industry [3]. The composition of the essential oil of *H. italicum* was found to depend on many factors including the population, the altitude and the climate conditions [4,5]. In the last decades *H. italicum* cultivars have been introduced into agriculture. However, seed quality and crops' growth are variable. The method of clonal in vitro propagation is known as a suitable tool for rapid vegetative multiplication of selected individuals with desired qualities. The effect of different cytokinins on the multiplication rate of *H. italicum* was studied along with different medium composition [6]. In order to check for possible multiplication (polyploidy) of the monoploid genome (x) of a large number of in vitro cultivated and adapted plants, flow cytometry was used. The latter technology proved to be very informative for estimating the variation in genome copy numbers [7]. It happens that the biosynthesis of secondary metabolites decreases under the conditions of in vitro cultivation [8] or some changes in their composition occur including appearance of non-typical compounds [9]. Usually, the concentration of the bioactive compounds recovers quickly after the ex vitro adaptation of the plants and their transfer to normal field conditions [10].

The aim of the present study was to verify the reliability of the clonal in vitro propagation method as a tool for rapid multiplication of true-to-type plants of *Helichrysum italicum* issued from high-productive 'mother' individuals.

Material and methods. *In vitro multiplication and ex vitro acclimation.* In vitro culture was initiated from stem tips of four *H. italicum* plant individuals selected for their essential oil composition, kindly offered by the firm "Bio Cultures". Stem segments were surface sterilized by soaking consecutively in 70% ethanol for 1 min, 50% commercial bleach (chlorine $< 5\%$) for 6 min, then rinsed trice with sterile distilled water. Stem tips with 1 cm length were excised and put on MS nutrient medium [11], then sub-cultivated every three months. Four media were tested, two repetitions about 20 explants each, on each medium: basic MS (control medium), MS supplemented with 1 g/l activated charcoal (medium MS-AC), or MS containing the plant growth regulators (PGRs) 6-benzylaminopurine (BAP), α-Naphthaleneacetic acid (NAA), kinetin (Kin), and indole-3-butyric acid (IBA) in the following combinations: 2 mg/l

BAP + 0.2 mg/l NAA (medium $B2N_2$) or 0.2 mg/l Kin + 0.1 mg/l IBA (medium K_2I_1). All media contained 3 g/l sucrose and were solidified with 6.5 g/l Plant agar (Duchefa, NL). The propagation coefficient (PC) was calculated as a number of the obtained new shoots per initial explant. Cultures were maintained under 16/8 h light regime, at temperature $23 \pm 2\,^{\circ}C$. Rooted shoots were potted in substrate mixture (soil lightmix, sand and coconut fibres in proportion 2:1:1), three per pot, and consecutively ex vitro adapted in a growth chamber (POLEKO Aparatura) for 5 weeks, under strictly controlled round-the-clock temperature and light regime (8 h dark at 18 °C; 10 h 3000 lx at 23 °C; and two 3h-intermediate periods of 2000 lx at 20 °C) thus simulating the natural dynamics of the ambient conditions, and gradually decreasing the air humidity from 90% to 60%. Survived plants were moved in a room phytotron with wider temperature and light amplitudes for at least 2 months, then transferred into larger pots, one per pot, and acclimated first in a greenhouse and then outdoor in the ex situ collection of the Institute of Biodiversity and Ecosystem Research (IBER).

Flow cytometry. Nuclear DNA content was measured by CyFlow SL Green flow cytometer (PARTEC, Germany), equipped with a green (532 nm) solid-state laser. Fresh leaves from in vitro multiplied and then cultivated plants of *H. italicum* were used for the study. *Pisum sativum* 'Kleine Rheinländerin' (1C = 4.38 pg, [12]) was applied as an internal standard. The plant material was treated with the extraction and staining kit 'CyStain® PI Absolute P' (SYSMEX) following the protocol prescribed by the manufacturer. To reduce the negative impact of secondary metabolites in immortelle, respectively, to minimise the coefficient of variation (CV) of measurements, the following additives (1% added) in each preparation were used: PVP-40, β-mercaptoethanol, and Triton X-100. Each preparation comprised five plants of *H. italicum* + the internal standard. Altogether 5000 PI-stained particles were measured per run. Additionally, three immortelle individuals were measured separately, each with the internal standard, in order to obtain precise value of the nuclear DNA content of the species. In the latter case, five runs per preparation with 5000 PI-stained particles per run were conducted. Runs with a CV above 5% were discarded.

Isolation of essential oil and determination of its composition. Essential oil was extracted by hydrodistillation using Clevenger apparatus, from two pooled samples, one for the mother plants and another one for the ex vitro adapted plants, consisting of the aboveground part of the plants (samples of 50 g DW in a flask with 500 ml water, for 2 h). The obtained essential oil was dried with anhydrous sodium sulphate and stored in a glass vial at 4 °C until the analysis. Essential oil composition was analysed by GC/MS as previously described [13].

Results. ***In vitro multiplication and outdoor acclimation.*** Most of the primary explants dropped out due to fungal contamination, and the survived ones were sub-cultured several times on MS medium to obtain enough shoots for the experiments. In January 2020 shoots were distributed randomly between the four

medium variants. At the end of the third month of cultivation, plants grown on the tested media differed by their height, ramification, in vitro rooting, and callus formation. Explants grown on medium $B2N_2$ formed numerous short adventitious shoots (PC 8.45 ± 4.44) and callus instead of roots. Those cultivated on medium K_2I_1 rooted and had fewer but higher new shoots that were additionally cut to segments during the sub-cultivation, which resulted in a similar propagation rate (PC 8.38 ± 3.67). The multiplication was less effective on the media without PGRs: 4.85 ± 1.82 and 6.18 ± 1.87 shoots per explant for the control MS medium and medium MS-AC, respectively. However, significant growth acceleration was observed in the next sub-cultivation in spring (April-June) and an average of 13.48 ± 4.15 new shoots per explant were obtained for only 2 months on MS control medium. All shoots rooted spontaneously on media free of PGRs, and some of them bloomed in vitro. The survival rate of the plants during the ex vitro adaptation was very high: 96% of the plants strengthened in the growth chamber, and 82% of them survived in the room phytotron. All 185 adapted plants were transferred to the greenhouse (September-November, related to their size), where their development successfully continued. In April 2021, 14 plants were planted outdoor in the ex situ collection, where they easily acclimatized and bloomed in June (Fig. 1).

Nuclear DNA content. The nuclear DNA amount was estimated in 65 individuals that represent 35% of all adapted plants. The estimated DNA amount measured for the three individual specimens varied in the range 1C = 1.37–1.41 pg (Table 1). The DNA amount in the preparations with five specimens of *Helichrysum* in each tube varied in the range 1C = 1.28–1.34 pg, with a CV of ca. 5% for the measured *Helichrysum* nuclei and ca. 3.6% for the measured standard nuclei in each run.

Essential oil composition. Thirty-eight components were identified in the essential oil and the profiles of both samples were almost similar. The main components were α-pinene, γ-curcumene, and nerol acetate (Table 2). Several compounds were detected only in the essential oil of the mother plants or only in that of the in vitro multiplied plants acclimated outdoor, although in relatively small quantities except for linalool (7.55%) (Table 2).

T a b l e 1

DNA content in three individuals of in vitro multiplied plants of *H. italicum*, with *P. sativum* 'Kleine Rheinländerin' (1C = 4.38 pg) used as an internal standard

Sample	1C [pg] I run	1C [pg] II run	1C [pg] III run	1C [pg] IV run	1C [pg] V run	Mean 1C [pg]	CV [%]
H. italicum 1	1.415	1.405	1.409	1.412	1.414	**1.41**	0.29
H. italicum 2	1.368	1.369	1.374	1.371	1.376	**1.37**	0.23
H. italicum 3	1.376	1.380	1.380	1.379	1.379	**1.38**	0.11

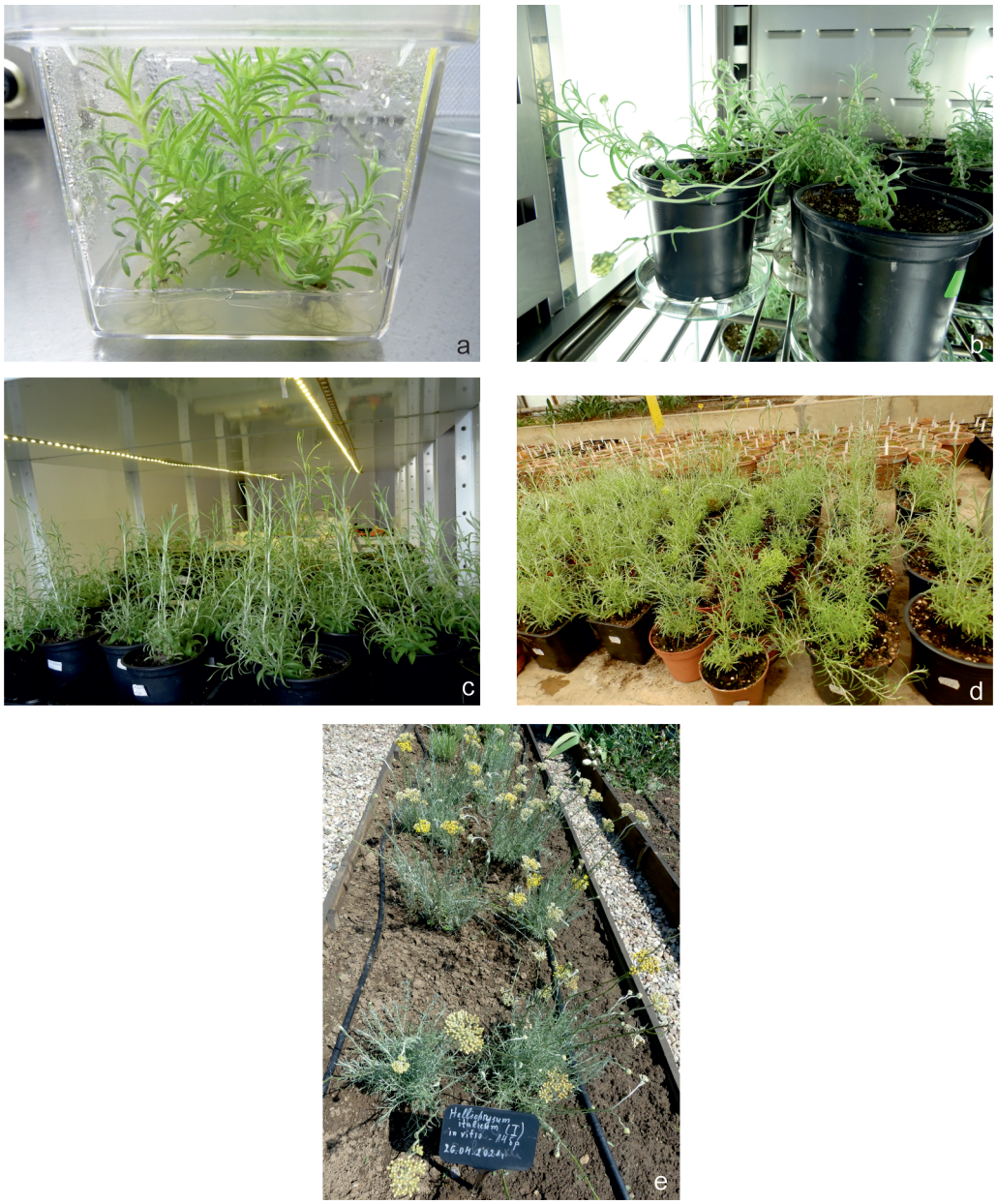


Fig. 1. Clonal propagation of selected plant individuals: a) In vitro plant multiplication and rooting; b) Ex vitro adaptation in a growth chamber; c) Adaptation in a room phytotron; d) Acclimatization in the greenhouse; e) Acclimatization and blooming outdoor in the ex situ collection of IBER

Discussion. In vitro multiplication was successful on media supplemented with a combination of Kin and IBA, as well as on media free of PGRs. Kinetin

T a b l e 2

Identified compounds (GC/MS) in the essential oil of *H. italicum* samples

Compounds	RI	Mother plants	Ex vitro acclimated plants
α-Pinene	939	**21.69**	**20.54**
Camphene	954	1.34	0.69
β-Pinene	979	0.36	0.25
Pseudocumene	983	0.16	
β-Myrcene	991		0.15
Decane	1000	0.25	0.07
α-Terpinene	1017	0.10	0.13
o-Cymene	1026		0.04
D-Limonene	1029	3.10	7.19
Eucalyptol	1031	0.98	
γ-Terpinene	1060	0.19	0.19
p-Mentha-1,4(8)-diene	1088	0.65	
2-Nonanone	1091		0.12
Linalool	1097		**7.55**
Nonanal	1101		0.35
β-Fenchol	1117		0.10
2-Methylbutyl angelate	1134	1.14	0.05
Cyclohexene-1-methanol, α, α, 4-	1143		0.52
Isoborneol	1162	0.40	0.16
Terpinen-4-ol	1177	0.60	0.15
α-Terpineol	1189	0.70	
Cis Geraniol	1253	1.03	0.11
Amyl angelate, 4-methyl-	1286	1.18	
2-Undecanone	1290		1.42
Nerol acetate	1362	**16.76**	**12.91**
Unidentified	1376	0.49	0.28
Italicene	1406	1.31	
Caryophyllene	1419	1.37	1.82
8-Decene-3,5-dione, 4,6,9-trimethyl	1449	5.96	6.09
Neryl propionate	1455	2.78	
γ-Curcumene	1483	**14.91**	**29.64**
8-Decene-3,5-dione, 2,4,6,9-tetramethyl-	1486	7.49	1.20
β-Eudesmene	1490	1.40	
γ-Gurjunene	1744	0.53	
Azulene	1516	0.37	0.26
9-Undecene-4,6-dione, 3,5,7,10-tetramethyl-	1583	2.84	
β-Bisabolol	1672	0.74	0.53
α-Bisabolol	1685	0.74	0.55

was reported as the best cytokinin for obtaining of *H. italicum* plants with good quality even if the multiplication rate was similar on media supplemented with BAP or zeatin [6]. The method of in vitro clonal propagation proved to be reliable for production of true-to-type plants as no deviations in the ploidy level

were detected. The results suggest the genome of the in vitro cultivated plants is stable. Similar pattern for the nuclear DNA content was reported for seedlings and in vitro shoots of *Teucrium scorodonia* as well [14]. The quantities of the main components α-pinene and nerol acetate was similar in the samples of the mother plants and the in vitro derived plants of *H. italicum* while γ-curcumene doubled in the ex vitro acclimated plants (Table 2). Variation in the concentrations of some components was also noticed for the essential oil of the seedlings and the in vitro shoots of *T. scorodonia* although their chemical profiles did not differ [14]. Significant differences were noticed for a few components such as linalool, eucalyptol, and neryl propionate. However, all these discrepancies in the profiles of the essential oils of both samples could be due to the short time between the ex vitro adaptation and the analysis. Thus, in *Mentha piperita* great differences were noticed in the first vegetation season concerning the main components of the essential oils of the control plants and those acclimated on the field after in vitro multiplication. However, in the second year the chemical profiles of all plants were approximately the same [8]. Based on the stable level of ploidy of the in vitro multiplied plants, it is assumed that the differences observed in the essential oils profiles of the two samples of *H. italicum* will decrease in the next growing season. The cluster analysis revealed ten chemotypes of *H. italicum* according to the main compounds of the essential oil [3]. The essential oil profile of the studied individuals is consistent with the data reported for *H. italicum* plants from Bosnia and Herzegovina and from the Adriatic region [5].

Conclusions. The results proved that the method of the in vitro clonal propagation is reliable for obtaining of numerous true-to-type plants in a short time, by direct organogenesis of high-productive individuals of *Helichrysum italicum*. The whole procedure from the beginning of the in vitro experiment in January 2020 to the obtaining of flowering plants in the ex situ collection in June 2021 lasted 18 months.

REFERENCES

[1] Mastelic J., O. Politeo, I. Jerkovic, N. Radosevic (2005) Composition and antimicrobial activity of *Helichrysum italicum* essential oil and its terpene and terpenoid fractions, Chem. Nat. Compd., **41**, 35–40.

[2] Rosa A., M. Deiana, A. Atzeri, G. Corona, A. Incani et al. (2007) Evaluation of the antioxidant and cytotoxic activity of arzanol, a prenylated a-pyrone-phloroglucinol etherodimer from *Helichrysum italicum* subsp. *microphyllum*, Chem. Biol. Interact., **165**(2), 117–126.

[3] Acimovic M., J. Ljujic, J. Vulic, V. D. Zheljazkov, L. Pezo et al. (2021) *Helichrysum italicum* (Roth) G. Don essential oil from Serbia: chemical composition, classification and biological activity – may it be a suitable new crop for Serbia?, Agronomy, **11**(7), 1282.

[4] Melito S., G. Petretto, J. Podani, M. Foddai, M. Maldini et al. (2016) Altitude and climate influence *Helichrysum italicum* subsp. *microphyllum* essential oils composition, Ind. Crops Prod., **80**, 242–250.
[5] Talic S., I. Odak, T. Lukic, M. Brkljaca, A. M. Bevanda et al. (2021) Chemodiversity of *Helichrysum italicum* (Roth) G. Don subsp. *italicum* essential oils from Bosnia and Herzegovina, Fresenius Environ. Bull., **30**(3), 2492–2502.
[6] Dimitrova N., L. Nacheva (2018) Micropropagation of *Helichrysum italicum* (Roth) G. Don – a medicinal plant with ornamental value, J. BioSci. Biotechnol., **7**(2), 97–101.
[7] Suda J., P. Kron, B. C. Husband, P. Trávníček (2007) Flow cytometry and ploidy: Applications in plant systematics, ecology and evolutionary biology. In: Flow Cytometry with Plant Cells. Analysis of Genes, Chromosomes and Genomes (Eds J. Doležel, J. Greilhuber, J. Suda), Weinheim, Wiley-VCH, 103–130.
[8] Shelepova O. V., T. A. Dilovarova, A. A. Gulevich, L. S. Olekhnovich, A. V. Shirokova et al. (2021) Chemical components and biological activities of essential oils of *Mentha* × *piperita* L. from field-grown and field-acclimated after in vitro propagation plants, Agronomy, **11**(11), 2314.
[9] Luczkiewicz M., A. Jesionek, A. Kokotkiewicz, P. Migas, M. Mardarowicz et al. (2015) Production of essential oils from in vitro cultures of *Caryopteris* species and comparison of their concentrations with in vivo plants, Acta Physiol. Plant., **37**(3), Art. 58, https://doi.org/10.1007/s11738-015-1804-0.
[10] Trendafilova A., M. Jadranin, R. Gorgorov, M. Stanilova (2015) Bioactive compounds in wild, in vitro obtained, ex vitro adapted, and acclimated plants of *Centaurea davidovii* (Asteraceae), Nat. Prod. Commun., **10**(6), 839–841.
[11] Murashige T., F. Skoog (1962) Revised medium for rapid growth and bioassay with tobacco tissue culture, Physiol. Plant., **15**, 473–497.
[12] Greilhuber J., E. Temsch, J. Loureiro (2007) Nuclear DNA content measurement. In: Flow Cytometry with Plant Cells. Analysis of Genes, Chromosomes and Genomes (Eds J. Doležel, J. Greilhuber, J. Suda), Weinheim, Wiley-VCH, 67–101.
[13] Traykova B., M. Stanilova, M. Nikolova, S. Berkov (2019) Growth and essential oils of *Salvia officinalis* plants derived from conventional or aeroponic produced seedlings, Agric. Conspec. Sci., **84**(1), 77–81.
[14] Makowczynska J., E. Sliwinska, D. Kalemba, E. Piatczak, H. Wysokinska (2016) In vitro propagation, DNA content and essential oil composition of *Teucrium scorodonia* L. ssp. *scorodonia*, Plant Cell, Tissue Organ Cult., **127**, 1–13.

*Department of Plant and Fungal Diversity and Resources
Institute of Biodiversity and Ecosystem Research
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 23
1113 Sofia, Bulgaria
e-mails: asya.kozhuharova@mail.bg
marina.stanilova@gmail.com
vladimir_dv@abv.bg
mtihomirova@gmail.com
borianka_traikova@abv.bg
berkov_str@yahoo.com

**Botanical Garden
Bulgarian Academy of Sciences
P.O. Box 664
1000 Sofia, Bulgaria