

IN VITRO MICROPROPAGATION OF *Helichrysum arenarium*
(Asteraceae) AS A TOOL FOR INTRODUCING THE SPECIES
IN AGRICULTURE

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Abstract

Helichrysum arenarium, known as sandy everlasting, is a perennial plant species, used in traditional medicine. In Bulgaria, it is protected by the Biodiversity Act and under a special regime of use based on the Medicinal Plants Act. Seeds from five Bulgarian populations were in vitro germinated and seedlings were sub-cultured on five media supplemented with different plant growth regulators (PGRs) and control MS medium free of PGR. The type of cytokinin turned out to be of crucial importance, as the presence of kinetin stimulated formation of rhizomes and direct organogenesis, BAP caused callogenesis, indirect organogenesis and hyperhydricity, and Meta-topolin – necrosis. Best results were obtained on medium with reduced macrosalt and sucrose concentrations as rhizomes gave rise to numerous well-developed and spontaneously rooted plantlets, up to 55 originating from a single seed. Plants were potted in soil mixture, adapted to phytotron conditions, and transferred to the greenhouse for acclimation, then planted on the ex situ collection. All plants flowered during the first summer outdoor. Nuclear DNA amount was measured by flow cytometry to check possible abnormalities of the in vitro multiplied plants. The estimated DNA amount varied in the range $1C = 0.85\text{--}0.89$ pg. No deviations in the ploidy level were detected. No difference between the studied populations

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was found. In vitro micropropagation was shown to be an appropriate method for rapid plant multiplication of *H. arenarium* thus offering the opportunity for its introduction into agriculture.

Key words: sandy everlasting, immortelle, plant growth regulators, flow cytometry

Introduction. *Helichrysum arenarium* (L.) Moench, also known as sandy everlasting and as immortelle, is a herbaceous perennial plant species. It is native to Europe, Central Asia, and China, and used as a medicinal plant in traditional medicine, as the herb exhibits choleric, hepatoprotective, diuretic, antibacterial, and detoxifying activities [1–3]. The inflorescences are a rich source of flavonoids, flavanones, essential oils, phtalides, carotenoids, glycosides and coumarins [4–6]. The species is protected in a number of European countries as ‘requiring care’ and included in the Red Data Books of Serbia and Estonia. In Bulgaria, it is included in Annex 4 of the Biodiversity Act (2002) [7] and is under a special regime of protection and use as a medicinal plant, based on the Medicinal Plants Act (2000) [8]. Recently, six populations have been confirmed in Bulgaria, located in a limited area in the north-eastern part of the country, between the towns Shumen and Varna [9]. Both the high pharmaceutical value and the protected status of the species highlight the need to develop efficient technology for its cultivation. Some attempts to cultivate *H. arenarium* were reported, but with moderate success, as the inflorescences yield was only slightly higher than that of the wild plants [10]. Further research pointed out the influence of mycorrhizal fungi associations on the plant growth [11]. In vitro propagation was another approach to produce numerous homogenous plants as starting material for agriculture; however, the literature data is limited and somewhat contradictory [12–14]. The evaluation of the efficiency of *H. arenarium* cultivation from in vitro seedlings and from achenes sown directly into the ground showed that both methods had their advantages and disadvantages, but the in vitro techniques allowed the production of an unlimited number of well-shaped plants flowering at the same time [15].

The aim of the present study was to test the possibilities for rapid propagation of *H. arenarium* by means of the in vitro micropropagation method, using seeds gathered from several Bulgarian populations, to check the ploidy level of the in vitro multiplied plants, and to introduce them in a pilot culture on the ex situ collection of IBER.

Materials and methods. In vitro micropropagation. Seeds of *H. arenarium* were gathered in July 2019 from five Bulgarian populations: Kanarata locality, Pobitite Kamani locality, Shumen Plateau, near the village Dobrina, and south of Beloslav. One hundred seeds from each population were disinfected by a standard procedure: consecutive soaking into 70% ethanol for a minute, commercial bleach (chlorine < 0.5%) for 6 min, and thrice rinsing for 10 min with sterile distilled water; then germinated on basic MS medium [16] supplemented

with 30 g/l sucrose, in plastic containers. Seedlings were sub-cultured on MS medium free of plant growth regulators (PGRs) as a control, and five media supplemented with different PGRs: medium B1 (1 mg/l BAP), medium B2N₁ (2 mg/l BAP + 0.1 mg/l NAA), medium K2N₁ (2 mg/l Kin + 0.1 mg/l NAA), medium K1I₅ (1 mg/l Kin + 0.5 mg/l IBA), and medium MT1I₅ (1 mg/l Meta-topolin + 0.5 mg/l IBA). All in vitro obtained plants were transferred on medium 3/4 MS-20 with reduced concentration of the macrosalts and 20 g/l sucrose. The conditions in the culture room were constant: temperature of 23 ± 2 °C and 16 h light daily.

Ex vitro adaptation. Rooted plants were potted in sterilized soil substrate: light mix, sand, and coco fibres in proportion 2:1:1, and ex vitro adapted first in a growth chamber (POLEKO Aparatura) for 6 weeks under strictly controlled conditions simulating the natural dynamics of the round-the-clock temperature, light regime, and relative air humidity (8 h dark at 18 °C; 10 h 3000 lx at 23 °C; and two 3h-intermediate periods of 2000 lx at 20 °C), with gradual decrease of the air humidity from 90% to 60%. Plants were then moved for a period of 3–4 months in a room phytotron with ambient conditions varying over a wider range. In October 2020 plants were transferred to the greenhouse for acclimatization, and finally planted outdoor in the ex situ collection of IBER.

Flow cytometry. Nuclear DNA amount was measured by a flow cytometer CyFlow SL Green (PARTEC, Germany), equipped with a green (532 nm) solid-state laser, to check possible abnormalities of the in vitro multiplied plants. Fresh leaves from the outdoor cultivated plants and from an internal standard, *Pisum sativum* ‘Kleine Rheinländerin’ (1C = 4.38 pg) [17], were used for this purpose. The plant material was treated with CyStain PI Absolute P extraction and staining kit (SYSMEX), following the protocol provided with the kit. The samples were then measured at a rate of 10–20 nuclei per second, with 5000 nuclei for each measurement; measurements with CV > 6% were discarded. Altogether 11 plants from the outdoor ex situ collection were checked for their ploidy level.

Results and discussion. In vitro micropropagation. Seed germination occurred between the first and the fifth week of the in vitro inoculation, and reached up to 44% for Shumen population; however, heavy microbial contamination was observed, and 22% of the seedlings free of contamination necrotized. Well-developed one month old seedlings with several leaves were sub-cultured on media containing different plant growth regulators. The type of cytokinin was of crucial importance, as the presence of Kin stimulated formation of direct organogenesis and rooting of shoots, BAP caused callogenesis, indirect organogenesis and hyperhydricity, and Meta-topolin – necrosis (Fig. 1A-D). Other authors also reported kinetin as the most effective PGR for induction and growth of immortal axillary shoots, supplemented in the medium alone or in combination with IAA, but in higher concentration of 4–5 mg/l [12,14]. Although BAP is the most commonly used cytokinin, its application is not suitable for some species. Thus, a

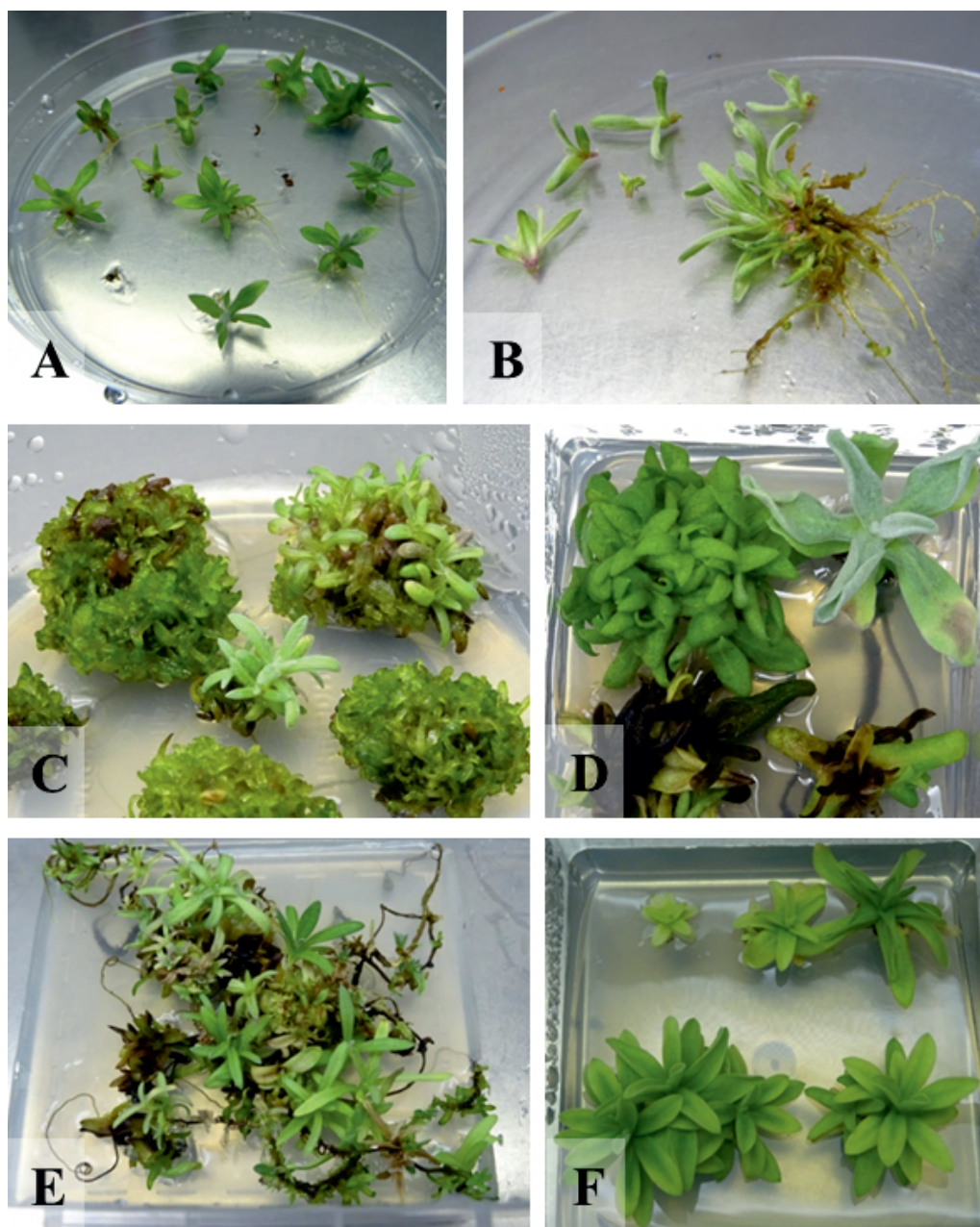


Fig. 1. In vitro cultivation of *H. arenarium*: A) Seedlings obtained on MS basal medium; B) Plantlets multiplied on medium K2N₁; C) Callogenesis, indirect organogenesis and hyperhydricity on medium B2N₁; D) Necrosis and hyperhydricity on medium MT1I₅; E) Plants formation on the rhizomes on medium $\frac{3}{4}$ MS-20; F) Plants growing on medium $\frac{3}{4}$ MS-20

negative influence of BAP was observed in *P. degenii* resulting in hyperhydricity and plant loss [18]. In nature, both species are growing on soil poor in nutrients:



Fig. 2. Consecutive ex vitro adaptation of in vitro multiplied *H. arenarium* plants: A) In the growth chamber; B) In the room phytotron; C) In the greenhouse; D) Formation of a cluster of plants; E) In the ex situ collection

H. arenarium on calcareous sandy dry terrains [9], and *P. degenii* exclusively on marble screes or rock crevices in the high mountain [18]. This is probably the reason they developed better on MS medium free of PGRs. Sandy clay was proved to be appropriate for proper development of immortelle seedlings and high inflorescence yield [11]. When in vitro *H. arenarium* plants were sub-cultured on modified MS medium containing reduced macrosalts and sucrose (3/4 MS-20) shoots not only rooted, but formed long rhizomes which ramified and gave rise to numerous well-shaped shoots on them (Fig. 1E). Shoots rooted spontaneously,

and the multiplication rate increased about ten times, as up to 55 plantlets were obtained from a single explant. Moreover, subsequent development of the plants was better on this medium (Fig. 1F). Adventitious buds formation on root explants of immortelle seedlings was also reported on MS medium supplemented with 1 mg/l BAP [13]. Obviously, the effect of the Meta-topolins depends on the species, as their presence in the nutrient medium was recommended for solving some problems such as hyperhydricity, shoot-tip necrosis, early senescence, and poor rooting [19].

The number of the new shoots formed on the survival explants varied a lot on all tested media, from 2 up to 14 per explant. All shoots rooted spontaneously on the control MS medium free of PGRs and on the media containing Kin and either NAA or IBA. According to other authors the addition of the auxins IAA, IBA or NAA in the basal MS medium improved the in vitro rooting of the shoots of sandy everlasting, increasing the percentage of rhizogenesis from 88 to 93–100% [13,14].

The soil substrate used was found to be suitable for ex vitro adaptation of the in vitro obtained plants, as 90% of a total of 73 potted plants survived in the growth chamber (Fig. 2A).

Plants originated from four populations: 33 from Shumen Plateau, 26 from Kanarata locality, 7 from Pobitite Kamani locality, and 7 from the population near the village Dobrina. Well-developed rosettes and root system were prerequisite for further successful adaptation (Fig. 2B-D). Other authors reported the beneficial effect of watering the ex vitro plants with solution of 25% MS salt, which increased the effectiveness of acclimation from 56 to 75% [14]. In the room phytotron plants were invested with mites, and treated with acaricide. All 34 survived plants were successfully acclimated in the greenhouse, and many of them developed several branches as a cluster of plants. Plants continued their growth and development

T a b l e 1

DNA content in in vitro multiplied and consequently outdoor adapted and cultivated plants of *H. arenarium*, with *P. sativum* 'Kleine Rheinländerin' (1C = 4.38 pg) used as an internal standard

Sample	1C [pg]
<i>Helichrysum arenarium</i> (Kanarata locality) – 1st	0.89
<i>Helichrysum arenarium</i> (Kanarata locality) – 2nd	0.89
<i>Helichrysum arenarium</i> (Kanarata locality) – 3rd	0.89
<i>Helichrysum arenarium</i> (Kanarata locality) – 4th	0.85
<i>Helichrysum arenarium</i> (Pobiti Kamani locality) – 1st	0.88
<i>Helichrysum arenarium</i> (Pobiti Kamani locality) – 2nd	0.85
<i>Helichrysum arenarium</i> (Pobiti Kamani locality) – 3rd	0.85
<i>Helichrysum arenarium</i> (Shumen town) – 1st	0.87
<i>Helichrysum arenarium</i> (Shumen town) – 2nd	0.85
<i>Helichrysum arenarium</i> (Dobrina) – 1st	0.85
<i>Helichrysum arenarium</i> (Dobrina) – 2nd	0.85

on the ex situ collection. All plants flowered during the first summer outdoor (Fig. 2E).

The estimated DNA amount varied in the range $1C = 0.85\text{--}0.89$ pg. It seems that this is the first report of the DNA amount for the species (Table 1). No deviations in the ploidy level were detected. No difference between the studied populations was found.

Conclusions. Medium containing combination of Kin and IBA or NAA induced direct organogenesis. However, medium $3/4$ MS-20 was chosen as the best one for further sub-cultivations, as it stimulated both formation of rhizomes and direct organogenesis on them, thus increasing the multiplication rate up to 55 plants from a single in vitro plant, for 5 months. In vitro micropropagation was proved to be a suitable method for rapid plant multiplication as the obtained plants kept their normal ploidy level. Although the conditions in the room phytotron need to be improved, the outdoor acclimation was easy, and an ex situ collection has been established, with all plants blooming in the first summer. The first trials to introduce *H. arenarium* in agriculture were promising.

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