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IMPROVEMENT OF *in vitro* PROPAGATION AND ACCLIMATION OF *Helichrysum arenarium* L. Moench

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Abstract. Explants of apical buds of sandy everlasting *Helichrysum arenarium* L. Moench sterilized with calcium hypochlorite – $Ca(OCl_2)$ were placed onto Murashige-Skoog (MS) growth medium enriched with 1 mg·dm^{-3} KIN (kinetin) in two consecutive passages. To optimize the procedure of *in vitro* micropropagation, the axillary shoots of *Helichrysum arenarium* L. Moench were transferred during the third passage onto 9 different combinations of MS medium without plant growth regulators (PGRs-free medium), with different concentrations of KIN (1.0, 3.0, 4.0, 5.0 mg·dm^{-3}) and KIN (1.0, 3.0, 4.0, 5.0 mg·dm^{-3}) with IAA (indole-3-acetic acid) (0.5 mg dm^{-3}). The highest mean number of shoots (24.7) was observed on MS medium with 5 mg·dm^{-3} KIN and 0.5 mg·dm^{-3} IAA. The achieved branched shoots were rooted and acclimatized. Rhizogenesis was intensified by the presence of growth hormones: 0.5 mg·dm^{-3} IAA or 0.5 mg·dm^{-3} IBA (indole-3-butyric acid). At the stage of acclimation of plantlets the application of a water solution of MS salts (25%) for watering the plants increased the efficacy of plant acclimation from 56 to 75%.

Key words: sandy everlasting, phytohormones, rooting, *in vivo* conditions, medicinal plant

INTRODUCTION

Sandy everlasting (*Helichrysum arenarium* L. Moench) is a perennial plant of the Asteraceae family. Plants of this species are used as ornamentals in flower beds, particularly in the naturalistic gardens or for fresh and dry bouquet arrangements, because

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after drying the flowers retain their natural colours for a long period of time. Due to its very specific insect-repelling smell, sandy everlasting was formerly used as a household remedy against the cloth moths [Sawilska 2015]. In pharmacy the inflorescences of *H. arenarium* are used because of their content of different flavonoids, mainly kaempferol-3-glucoside, quercetin-3-glucoside, apigenin 7-glucoside, luteolin 7-glucoside, naringenin 5-glucoside, and chalcones. Moreover, the presence of various polyphenols (especially chlorogenic and caffeic acids) and essential oils is also important for its medical uses [Czinner et al. 1999, 2000, Radusiene and Judzentiene 2008, Sawilska and Mielcarek 2009].

The composition of biologically active substances of sandy everlasting decides about its diuretic, cholagogue, hepatoprotective, bile building, antithrombotic and capillary-sealing, detoxifying, antioxidative, antifungal, antiviral, and antibacterial properties [Chinou et al. 1996, Czinner et al. 2000, Lembercovics et al. 2002, Pawlaczyk et al. 2009, Albayrak et al. 2010a, 2010b, Eroğlu et al. 2010, Stanojević et al. 2010]. Furthermore, it is used for the production of substances for the treatment of diseases of the gastrointestinal tract and of the liver or gall bladder.

In Poland, the cultivation of *H. arenarium* on a commercial scale using conventional methods is prohibited, because this species is under partial protection due to the Regulation of the Minister of Environment of 9th October 2014 on the protection of plant species. The main threat is the collection of plants from natural habitats for medicinal purposes [Piękoś-Mirkowa and Mirek 2006]. One of the allowed methods for obtaining propagation material is the application of plant tissue and cell culture techniques. However, in the literature the information about this topic is limited [Sawilska and Figas 2006, Pawełczak and Bryksa-Godzisz 2008, Bryksa-Godzisz and Pawełczak 2010]. The first micropropagation attempts were conducted in France in order to restore natural habitats [Clasquin and Henry 2002]. However, they were considered to be ineffective. As a consequence, the present study aimed at improving the method of its propagation in *in vitro* culture.

MATERIALS AND METHODS

Shoot propagation. The plant material was collected from natural stands in Łosiny near Chojnice, Bory Tucholskie, Poland (N 53°37'13''; E 17°58'43'') in October 2012. Permission of sampling was granted by the Provincial Nature Conservation Office in Bydgoszcz, Poland. Primary explants of *Helichrysum arenarium* (L.) Moench were initialized apical buds infolded into two leaves. Two hundred apical buds were used in the experimental setting and the experiment was repeated three times. Isolated explants were chemically sterilized. At the first stage the plant parts were rinsed with tap water and then dipped for 1 min in a 70% ethanol water solution (v/v) for degreasing, degasing, and surface pre-sterilization. Thereafter the explants were treated with a 9% $Ca(OCl_2)$ water solution (w/v) with Tween 20 for 12 min.

Finally, the buds were washed three times in pure water. The so-sterilized explants of apical buds were transferred onto MS growth medium [Murashige and Skoog 1962] of pH 5.7 and enriched with 1 mg·dm^{-3} KIN (kinetin). The medium comprised 3% su-

crose as carbon source and was solidified by agar (0.8%). The growth medium was autoclaved at a pressure of 0.5 MPa at a temperature of 121°C for 25 min. During the first two weeks the test tubes with explants were stored in darkness at a temperature of 25 ±2°C. Later, the test tubes with explants were incubated in the phytotron with a 16-hour photoperiod at a light intensity of 40 $\mu mol \cdot m^{-2} s^{-1}$ (daylight lamps of 40 W, PILA, Poland), relative air humidity of 60%, and a temperature of 25 ±2°C.

After 8 weeks of culture under aseptic conditions the axillary shoots were isolated and transplanted twice onto the same medium in order to stabilize the culture. During the third passage the explants were inoculated onto 9 different combinations of MS media: without plant growth regulators (PGRs-free medium), with different concentrations of KIN (1.0, 3.0, 4.0, 5.0 $mg \cdot dm^{-3}$; Sigma – Aldrich, Germany) and KIN (1.0, 3.0, 4.0, 5.0 $mg \cdot dm^{-3}$) with IAA (indole-3-acetic acid) (0.5 $mg \cdot dm^{-3}$; Sigma – Aldrich, Germany).

In vitro cultures were conducted in a phytotron under the above mentioned strictly controlled environmental conditions. Within each combination 50 explants were transferred onto the specific growth medium and the experiment was repeated three times.

The percentage of explants forming axillary shoots, the mean number of newly formed shoots and the number and area of leaves were determined.

Root formation. After 6 weeks the amplified axillary shoots of sandy everlasting were transferred onto a growth medium inducing rhizogenesis. As rooting growth medium a solid MS medium without any growth regulators as well as a MS medium supplemented with 0.5 $mg \cdot dm^{-3}$ IAA or 0.5 $mg \cdot dm^{-3}$ BA (indole-3-butyric acid) (Sigma – Aldrich, Germany) were used. On each combination of growth medium 100 explants were transferred. Finally, the percentage of rooted explants was determined, the length of roots measured and their number counted.

Growth of plants under in vivo conditions. After 10 weeks the rooted plantlets were acclimated to the *ex vitro* conditions by transferring them to square plastic pots with a side length of 6 cm filled with a sterile organic-mineral substrate. They were cultivated under a 16-hour photoperiod as described above and at constant temperature of 24°C. In this part of the experiment 200 rooted micro-plantlets of 9 combinations of growth media were used. In order to improve the acclimation efficacy 100 plants were irrigated with a water solution of MS salts (25%, v/w) and the remaining 100 with tap water. The plants were covered with a foil tent to reduce transpiration. At weekly intervals over a period of 28 days the plants were observed and their survival was evaluated. The experiment was repeated three times. After 28 days of acclimation to *in vivo* conditions sandy everlasting plantlets were transferred to the pots in the greenhouse.

Statistical analyses. The obtained results were subjected to statistical analyses, viz. the analysis of variance and the significance of differences between means using the Tukey test with a significance level of $\alpha = 0.05$. Thereafter the homogenous groups were build. The statistical analyses were performed using the software program Statistica for Windows Pl.

RESULTS AND DISCUSSION

Shoot propagation. In the presented research the apical buds of sandy everlasting were used as the primary explants. This kind of explant is commonly used for the initiation of *in vitro* cultures in the *Asteraceae* family, because it guarantees a high genetic stability and a high probability to obtain sterile cultures [Kanwar and Kumar 2008]. Axillary shoot meristems have the same histologically and genetically structures as apical shoot meristems. Their use allows achieving plants that are genetically identical with their mother plant [Hu and Wang 1983, Assim et al. 2008]. Using the axillary shoot meristems of sandy everlasting enabled obtaining an effective regeneration of up to 24.7 axillary shoots per explant. The application of the growth regulators resulted in a higher number of axillary shoots (tab. 1).

In the present experiment, from 200 sterilized apical buds 158 were able to regenerate, resulting in a regenerative efficacy of 79%. The in this way from regenerated axillary shoots were characterized by a normal growth habit, green colour, and did not respond negatively to the active substances applied during the sterilization process. After 8 weeks single axillary shoots developing from the primary explants were used as secondary explants for further propagation.

Table 1. The effect of plant growth regulators on micropropagation of axillary shoots of *Helichrysum arenarium* L. Moench in *in vitro* culture after 6 weeks of experiment

Plant growth regulator ($mg \cdot dm^{-3}$)		Mean number of explants forming shoots (%)	Mean number of shoots/explant	Mean axillary shoot length (cm)	Mean number of leaves per shoot	Mean leaf area (mm^2)
KIN	IAA					
0.0	0.0	10.67 ±3.05(21)c	0.63 ±0.15g	2.94 ±0.11a	3.72 ±0.86b	31.23 ± 5.03b
1.0	–	38.00 ±9.64(76)a	2.20 ±1.61f	3.05 ±0.24a	3.60 ±0.26b	28.90 ± 5.71b
1.0	0.5	34.00 ±6.50(67)b	3.98 ±1.30e	3.37 ±0.98a	4.30 ±0.65b	29.42 ± 5.78b
3.0	–	35.00 ±7.21(70)a	12.90 ±2.91d	4.20 ±0.92a	5.60 ±0.50ab	34.20 ± 8.70ab
3.0	0.5	41.00 ±4.58(82)a	14.80 ± 2.43c	4.15 ±0.59a	7.60 ±0.41a	37.58 ± 8.72a
4.0	–	50.00 ±0.00(100)a	15.30 ±4.28bc	3.73 ±0.60a	7.80 ±0.87a	32.69 ±11.55ab
4.0	0.5	50.00 ±0.00(100)a	16.45 ±3.52b	4.07 ±0.52a	7.90 ±0.82a	38.52 ± 6.63a
5.0	–	50.00 ±0.00(100)a	16.98 ±3.50b	4.49 ±0.86a	7.50 ±0.33a	40.14 ± 7.00a
5.0	0.5	50.00 ±0.00(100)a	24.67 ±1.52a	4.60 ±0.73a	8.10 ±0.58a	40.78 ± 9.43a

Results are mean ±SD (standard deviation); within a column means followed by the same letter do not differ significantly at $\alpha = 0.05$ (Tukey test), KIN – cytokinin, IAA – indole-3-acetic acid (auxin)

In contrast to the patent claim [Sawilska and Włodarczyk 2014, PL 216755 B3] at the last stage of micropropagation the MS medium contained either only the cytokinin (KIN) or a combination of KIN and the auxin (IAA). The significantly highest amount of axillary shoots per explant (24.7) was achieved on MS medium enriched with 5 $mg \cdot dm^{-3}$ KIN and 0.5 $mg \cdot dm^{-3}$ IAA (3-indoleacetic acid) (tab. 1, fig. 1). This combination of phytohormones also influenced other parameters such as the length of individual shoots and the number as well as the area of leaves in dwarf everlasting. A high content of cytokinin and low amount of auxin favoured the micropropagation of plants in

in vitro cultures. Cytokinins derived from adenine (BAP, KIN) overrule the dominance of the apical bud and stimulate at the same time the growth of axillary shoots from axillary buds [Beyl 2011]. In the presented experiment a mean shoots number obtained on MS medium enriched only with KIN at a concentration of 4 and 5 mg·dm^{-3} resulted in 15.3 and 17.0 units, respectively, which is 93 and 69% of the number of shoots observed on the media with 4 and 5 mg·dm^{-3} of KIN and IAA (tab. 1).

In another experiments on micropropagation of sandy everlasting (*Helichrysum arenarium* L. Moench) Sawilska i Figas [2006] obtained 16.1 axillary shoots per explant by application of MS growth medium enriched with 4 mg·dm^{-3} KIN without addition of auxin, whereas Pawełczak and Bryksa-Godzisz [2008] achieved 17.59 shoots per explant using a BAP-enriched medium (1.0 mg·dm^{-3}). In the presented study, an increase of kinetin from 4 to 5 mg·dm^{-3} in the medium and enriching it additionally with 0.5 mg·dm^{-3} IAA resulted in 53% higher amounts of axillary shoots (8.6 axillary shoots per explant).

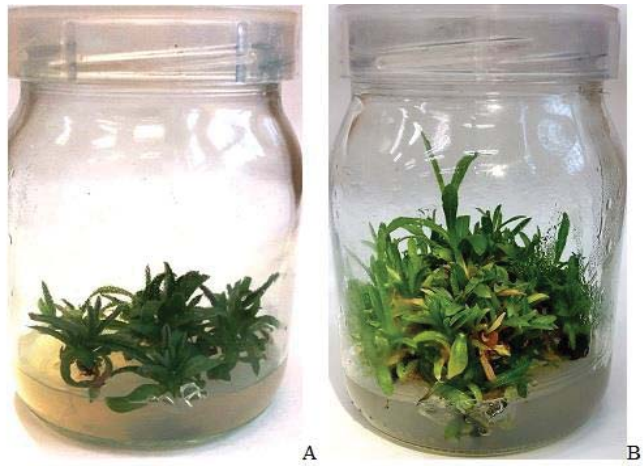

Fig. 1. Shoot propagation of *Helichrysum arenarium* L. Moench on MS medium with 1 mg·dm^{-3} KIN (A) or 5 mg·dm^{-3} KIN + 0.5 mg·dm^{-3} IAA (B) after 6 weeks of culture

Rooting of shoots. The induction of rhizogenesis in *in vitro* cultures was obtained on MS medium without any growth regulator and also on growth medium containing the auxins IAA or IBA (tab. 2). In their preliminary research on micropropagation of sandy everlasting Sawilska and Figas [2006] recognized, that propagated shoots rooted sufficiently well on a medium without any growth regulator. In addition, Tomaszewska-Sowa and Figas [2011] in their investigation on micropropagation of cup plant (*Silphium perfoliatum* L.), another member of the *Asteraceae* family, achieved 100% of rooted plants using a medium without any phytohormone. However, in the case of sandy everlasting the addition of auxins intensified the process of root formation (tab. 2). The application of IAA or IBA for the induction of rhizogenesis significantly increased the percentage of explants that developed roots from 88 to 93–96%, respectively (tab. 2). Similarly, Bryksa-Godzisz and Pawełczak [2010] reported an increased

root formation from 94% on a medium without any phytohormone to 98 and 100% by addition of IAA (0.5 mg·dm^{-3} and 1.0 mg·dm^{-3}) or NAA (0.1 mg·dm^{-3} and 0.2 mg·dm^{-3}), respectively. In the presence of applied phytohormones the number of roots per explant as well as their length were higher, and the best results were achieved at the presence of 0.5 mg·dm^{-3} IBA (tab. 2, fig. 2).

Table 2. The effect of plant growth regulators on rhizogenesis of *Helichrysum arenarium* L. Moench in *in vitro* cultures after 10 weeks of culture

Plant growth regulator mg·dm^{-3})	Mean of explants forming roots (%)	Mean number of roots per explant	Mean root length (cm)
0.0	88.0 ±2.45 b	8.40 ±1.73c	2.95 ±0.48b
0.5 IAA	93.0 ±4.58 a	10.30 ±2.51b	3.45 ±0.86b
0.5 IBA	96.0 ±2.00 a	12.67 ±1.90a	3.85 ±0.73a

Results are mean ±SD (standard deviation); within a column means followed by the same letter do not differ significantly at α = 0.05 (Tukey's test), IAA – indole-3-acetic acid (auxin), IBA – indole-3-butyric acid (auxin)

Fig. 2. Rooted plants of *Helichrysum arenarium* L. Moench on MS medium with 0.5 mg·dm^{-3} IBA after 10 weeks of culture time

The presented results are well in line with earlier reports [Ahmed et al. 2007, Anbazhagan et al. 2010, Tomaszewska-Sowa and Figas 2011], which stated, that rhizogenesis occurs on media without any growth regulator, while the supply of IAA, IBA, or NAA can accelerate this process. An effective influence of IBA at a concentration of 0.5 mg·dm^{-3} is also confirmed by the reports of Jitendra et al. [2012], who conducted research on the micropropagation of *Stevia rebaudiana*, a further member of the *Aster-*

aceae family. They found, that in the presence of this auxin the roots were longer and their score higher.

Growth of plants under ex vitro conditions. The results of plant irrigation experiments revealed that watering the plants at the stage of acclimation to *ex vitro* conditions with tap water resulted in a survival rate of 56%, while in case of plants irrigated with a MS water solution (25%, w/v) survival rate accounted for 75% (tab. 3). A positive influence of irrigation with a 25% MS-water solution was also observed by Jitendra et al. [2012]. The presence of exogenously applied ions in the aqueous nutrient solution reduced the negative effects of stress, which accompanies the transfer of plants to *ex vitro* conditions. This enabled the plantlets to acclimatize to greenhouse environment and later to the field conditions more rapidly and to achieve a normal growth rate as well as plants habit (fig. 3).

Table 3. The effect of using a water solution of MS salts (25%, v/w) for watering the plants of *Helichrysum arenarium* L. Moench during acclimatization to *ex vitro* conditions on the acclimatization rate of plants in percentage during a period of 28 days

	Aqueous solution of MS salts used for watering	Application time after the start of acclimatization				
		1 day	7 days	14 days	21 days	28 days
Mean value of acclimatized plants (%)	tap water (0%)	100 ±0.00a	79.00 ±2.64cd	64.00 ±2.65e	60.00 ±5.57e	56.00 ±4.00e
	MS salts solution (25%)	100 ±0.00a	88.00 ±2.00b	81.00 ±3.00c	77.33 ±2.52d	74.67 ±4.04d

Results are mean ±SD (standard deviation); means followed by the same letter do not differ significantly at α = 0.05 (Tukey's test), MS – Murashige-Skoog

Fig. 3. Plants of *Helichrysum arenarium* L. Moench acclimatized to greenhouse conditions and then planted onto the field

CONCLUSIONS

Due to the high rate of regenerated shoots of sandy everlasting (*Helichrysum arenarium* L. Moench) obtained from the apical buds of plants, their high rate of root formation, and the uncomplicated acclimation of plantlets to *ex vitro* conditions the presented method may be used for the production of plants on a commercial scale.

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OPTYMALIZACJA PROCEDURY MIKROROZMNAŻANIA *in vitro* I AKLIMATYZACJA *Helichrysum arenarium* (L.) Moench

Streszczenie. Wysterylizowane za pomocą podchlorynu wapnia – $Ca(OCl_2)$ pąki szczytowe kocanek piaskowych *Helichrysum arenarium* (L.) Moench wykładano w dwóch kolejnych pasażach na pożywkę MS [Murashige i Skoog 1962] wzbogaconą w 1 $mg \cdot dm^{-3}$ KIN (kinetyna). W celu optymalizacji procedury mikrorozmnażania w kulturach *in vitro* w trzecim pasażu pędy boczne inokulowano na 9 wariantów pożywek MS bez regulatorów wzrostu, z dodatkiem KIN (1,0; 3,0; 4,0; 5,0 $mg \cdot dm^{-3}$) oraz KIN (1,0; 3,0; 4,0; 5,0 $mg \cdot dm^{-3}$) i kwasu indolilo-3-octowego – IAA (0,5 $mg \cdot dm^{-3}$). Największą średnią liczbę pędów (24,7) odnotowano na pożywce z dodatkiem 5 $mg \cdot dm^{-3}$ KIN (kinetyna) i 0,5 $mg \cdot dm^{-3}$ IAA (kwas indolilo-3 octowy). Uzyskane pędy boczne poddano ukorzenianiu i aklimatyzacji. Proces ryzogenezy intensyfikowała zawartość regulatorów wzrostu 0,5 $mg \cdot dm^{-3}$ IAA i 0,5 $mg \cdot dm^{-3}$ IBA (kwas indolilo 3-masłowy). Na etapie aklimatyzacji mikrosadzonek zastosowanie do nawadniania 25% roztworu soli MS spowodowało poprawę efektywności aklimatyzacji roślin z 56 do 75%.

Słowa kluczowe: kocanki piaskowe, fitohormony, ukorzenianie, warunki *in vivo*, rośliny lecznicze

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