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ENHANCEMENT OF *ARNICA MONTANA IN-VITRO* SHOOT MULTIPLICATION AND SESQUITERPENE LACTONES PRODUCTION USING TEMPORARY IMMERSION SYSTEM

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ABSTRACT: *Arnica montana* L. (Asteraceae) is a valuable medicinal plant species, endemic to Europe, which is threatened in many countries due to its overharvesting. The increasing market demand requires development of an effective method for *A. montana* rapid propagation, offering the possibility for its field cultivation as an alternative to plant gathering from nature. Three *in -vitro* culture systems were compared to determine the best shoot multiplication of *A. montana*: agar-gelled medium, static liquid medium and Temporary Immersion System (TIS RITA®). Murashige and Skoog (MS) nutrient medium supplemented with 1 mg/l BA and 0.1 mg/l IAA was used in all experiments. The highest micropropagation rate (18.2 shoots/explant, for 5 weeks) was obtained via TIS culture. Besides, these plants showed higher sesqiuterpene lactones content than those derived from the other tested systems. The shoots were successfully *in -vitro* rooted and *ex-vitro* adapted. The elaborated method for mass multiplication of *A. montana* allows significant enhancement of the *in -vitro* process and could be applied to produce planting material, thus contributing to the conservation of the species as well.

INTRODUCTION: *Arnica montana* L. (Asteraceae) is a medicinal plant, largely applied in pharmacy and cosmetics. The species is rich in sesquiterpene lactones and possess antiseptic, anti-inflammatory, antibacterial and antioxidant effects[1]. *A. montana* is endemic to Europe, included in the European Red List of Vascular Plants (LC), and threatened in many European countries due to loss of habitats and overharvesting from natural populations[2].

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The plant deficiency stresses the need of elaboration of effective propagation methods. Several protocols have been reported on *in-vitro* multiplication of *A. montana* on agar gelled medium differing by their efficiency; however, the propagation rate needs to be improved [3-5]. The conventional *in-vitro* propagation method on agar medium requires a considerable amount of handwork: shoots separation and regular sub-culturing on fresh medium every 4 to 6 weeks[6].

The recent trend is changing the state of the nutrient medium to liquid which allows automation and commercialization of the *in-vitro* process. Applied tissue culture technique for micropropagation of several plant species in liquid medium was found to be more appropriate for mass

plant production[7-9]. The advantages of liquid medium over agar solidified medium include lower cost, less manual labor, simple transfer of fresh media, uniform culture conditions[10, 11]. However, the asphyxia and hyperhydricity often occur in liquid medium culture[12]. These problems could be overcome using temporary immersion system (TIS) which provides partial or short immersion of explants and guarantees aeration[13, 14]. TIS has been used to improve the micropropagation in many economically important plants[15-18].

The multiplication rate was significantly increased; moreover, this system ensured obtaining of healthy plants of high quality. TIS integrates advantages of both solid and liquid media, namely maximum gas exchanges and increased nutrient uptake, reduction of hyperhydricity and decrease of labor cost, thus ensuring better plant growth[19, 20]. Plant tissues and organs derived from TIS can be used for secondary metabolite production[21-23]. The aim of the present study was to enhance *A. montana* shoot multiplication using TIS, taking into account the sesquiterpene lactones biosynthesis as well.

MATERIALS AND METHODS:

Initial plant material: *Arnica montana* seeds (German origin) were used to initiate *in-vitro* culture. Sterilization of the mature seeds was performed according to our previous trials[24]. Shoots (1.0 cm) were isolated from three-month old *in-vitro* micropropagated *A. montana* plants and used as explants for all experiments.

Nutrient medium and culture conditions: Murashige and Skoog[25] (MS) nutrient medium supplemented with 1 mg/l N^6-benzyladenine (BA) and 0.1mg/l indole-3-acetic acid (IAA) was used. The experiments were performed with liquid and agar solidified (0.6% agar) medium adjusted to pH 5.8 before being autoclaved. The cultures were maintained in a growth chamber at 25±2 ºC in 16 h photoperiod provided by cool-white light (40µmol $m^{-2}s^{-1}$). Three *in-vitro* techniques for shoot multiplication were applied: agar-gelled medium culture, static paper-bridge support liquid culture and periodical liquid-immersion culture in a temporary immersion system (RITA®, France). In the first two cases glass tubes (150 x 25 mm) with 8ml medium were used, one explant per tube. Explants were vertically cultivated, slightly inserted into the gelled medium or supported over the liquid medium by wet paper-bridges made of filter paper with ends reaching the tube bottom. In the case of temporary immersion system, 10 shoots obtained on agar solidified medium and 200 ml liquid medium were transferred in each vessel connected to an air supply of 0.25 vvm, with 5 min immersion four times a day.

The appearance of shoots from the base of the explant was taken into consideration for calculating the multiplication efficiency. The average number of shoots induced per explant, and the mean height of shoots were recorded after 5 weeks of cultivation. All shoots derived from the three culture systems were collected and their dry weight was measured.

Rooting and *ex-vitro* adaptation: The obtained shoots from the three culture systems at the end of the 5-week shoot multiplication period were separated from one another and transferred to agar solidified half strength MS medium supplemented with 0.5mg/l indole-3-butyric acid (IBA). The rooting was carried out in glass tubes containing 8 ml of medium for 4 weeks. The plants with newly formed roots were planted into small plastic pots (6 cm diameter) with substrate mixture consisting of peat, perlite, and coconut fiber (2:1:1 v/v/v). The pots were placed in crate with transparent plastic cover which was removed after two weeks, thus allowing gradual adaptation to the lower air humidity in room conditions. The survival percentage of plants was assessed after six weeks.

Gas chromatographic (GC) analysis of sesquiterpene lactones:

For phytochemical analysis dried *in-vitro* micropropagated shoots from the three culture systems were collected. The samples for GC analysis were prepared according to Malarz et al.[26]. A quantitative analysis of sesquiterpene lactones in *A. montana in-vitro* plants was carried out on a Trace GC Ultra gas chromatograph fitted with a Tri Plus auto sampler.

Thermo Scientific, Bremen, Germany and DB 225 capillary column (J&W Scientific, California, USA) 60m x 0.25 mm ID, 0.25 mm film thickness were used. Helium was the carrier gas at a constant flow rate of 1ml/min. Split injection was used at

split ratio 1:10, injection volume 1µl and inlet temperature of 260 °C. The oven was programmed from initial temperature 60 °C to 220 °C at 15 °C/min and to 240 °C at 1 °C/min, and held at final temperature for 10 min. Flame-ionization detection was used with detector temperature of 260 °C. The absolute calibration method was used for the quantification, using santonin as a standard.

Statistical analysis: Twenty explants were used for each treatment and the experiment was repeated twice. Data were subjected to one-way ANOVA analysis of variance for comparison of means using a statistical software package (Statigraphics Plus, version 5.1 for Windows). Data were reported as means ± standard error.

RESULTS AND DISCUSSION:

Comparison of agar-gelled medium, static liquid medium and TIS culture on the *A. montana* shoot formation: Our preliminary results showed that MS agar-gelled medium supplemented with 1 mg/l BA and 0.1mg/l IAA gave the best shoot multiplication, compared to media containing kinetin, zeatin or 2-iP combined with the same level of IAA[27]. Therefore this medium was applied in the present study. The frequency of shoot formation for the three tested systems was 100%; however, the number of shoots per explant was significantly influenced by the used *in-vitro* technique **(Table 1)**.

TABLE 1: MICROPROPAGATION OF *A. MONTANA* USING DIFFERENT CULTURE SYSTEMS.

Culture system	Frequency of shoot formation %	No. of shoots/explant	Shoot height (cm)	Dry weight of shoots (g)
Agar-gelled medium culture	100	7.60 ± 0.63^{b}	1.81 ± 0.10^{a}	1.12 ± 0.12^{a}
Liquid culture with static paper-bridge support	100	3.90 ± 0.39^{a}	2.34 ± 0.17^{b}	1.09 ± 0.10^{a}
TIS culture RITA®	100	18.20 ± 0.93^{c}	2.62 ± 0.11^{b}	2.47 ± 0.12^{b}

Data are presented as means of 20 individuals per treatment ± standard error (SE). Different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

The best system was TIS, remarkable for the good quality of the shoots and their high number: an average of 18.2 shoots per explant. Some of the explants formed large shoot-clumps with up to 25 shoots **(Fig. 1)**. Moreover, shoots grown in TIS were higher than those obtained in the other two systems, with an average height of 2.62 cm. Few of them (5%) formed roots.

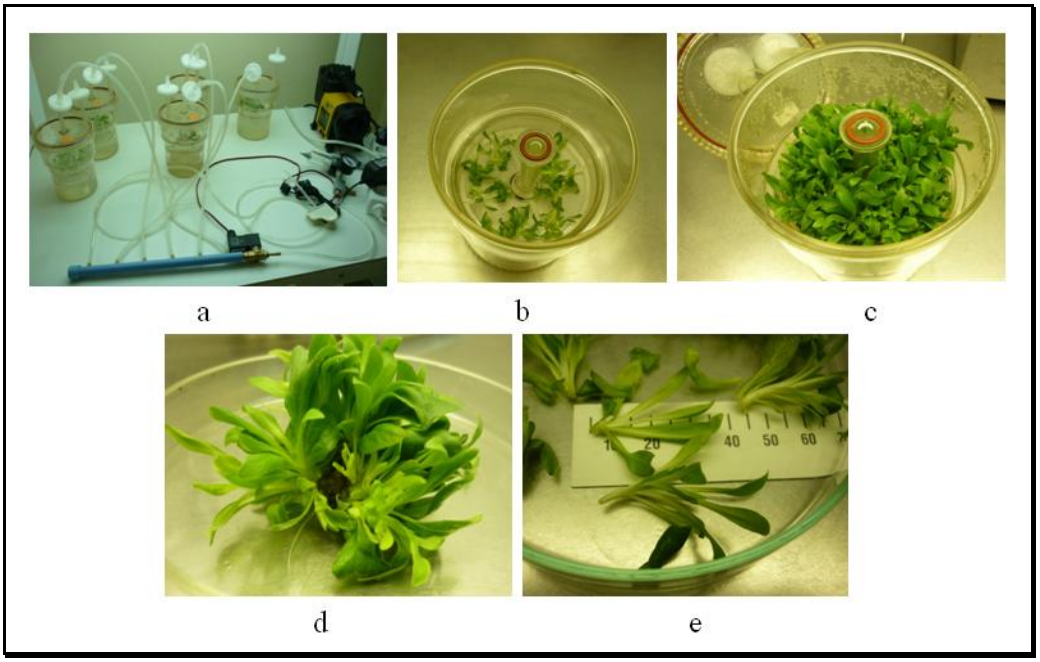

FIG.1: MICROPROPAGATION OF *A. MONTANA* USING TIS RITA® CULTURE: A) VIEW OF THE WHOLE RITA® SYSTEM; B) INITIAL EXPLANTS; C) SHOOTS OBTAINED AFTER 5 WEEKS OF CULTIVATION; D) MULTIPLE SHOOTS (SHOOT-CLUMP) DERIVED FROM A SINGLE EXPLANT; E) SHOOT HEIGHT MEASUREMENT.

The bridge-support liquid culture was the less effective one for shoot propagation – only 3.9 shoots per explant were obtained **(Fig. 2a)** and some cases of hyperhydricity were observed as well. The propagation rate of the shoots cultivated on agar-gelled medium was much higher; however, they formed smaller leaf blade and remained lower **(Fig. 2b)**.

In the present study, the number of *A. montana* multiple shoots obtained under the three different culture systems showed significant difference in the shoot numbers and the quantity of dry biomass. The shoot growth was the best in temporary immersion culture. This was expected because of the better availability of nutrients as well as the faster and efficient uptake of nutrients from the liquid medium in the TIS[28]. Some other medicinal plant species were also successfully micropropagated via TIS[29-31].

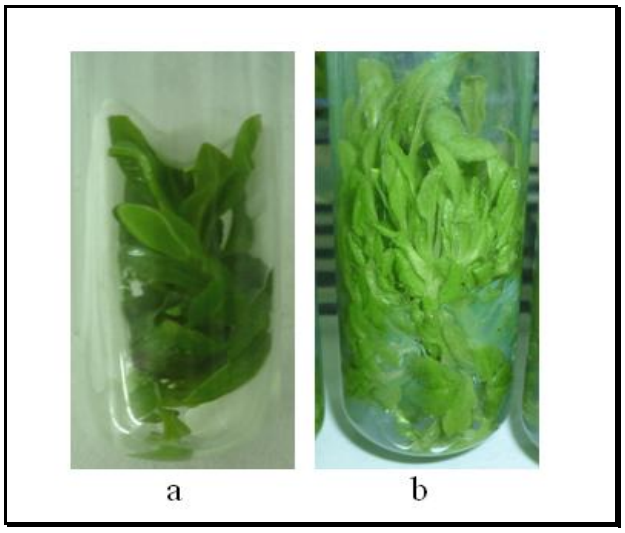

FIG. 2: SHOOT MICROPROPAGATION OF *A. MONTANA* USING: A) STATIC LIQUID MEDIUM WITH PAPER-BRIDGE SUPPORT; B) AGAR GELLED MEDIUM.

Comparison of agar-gelled medium, liquid medium and TIS concerning *in-vitro* rooting and *ex-vitro* adaptation of *A. montana* plants:

The multiplied shoots of *A. montana* from each culture system were successfully rooted on half strength MS medium supplemented with 0.5 mg/l IBA. The root primordia were observed first in plants derived from TIS culture after only two days of cultivation. The roots emerged directly from shoot base without callus formation. After 4 weeks of cultivation they were counted and the plantlets were transferred to substrate mixture **(Table 2)**.

TABLE 2: *IN-VITRO* ROOTING AND *EX-VITRO* ADAPTATION AFTER APPLICATION OF DIFFERENT PROPAGATION SYSTEMS

Culture system	*In -vitro* rooting %	Root number/plant	*Ex -vitro* adapted plants, %
Agar-gelled medium culture	90	4.73 ± 0.18^{a}	80
Liquid culture with static paper-bridge-support	75	3.80 ± 0.24^{a}	85
TIS culture RITA®	100	6.42 ± 0.33^{b}	95

Data are presented as means of 20 individuals per treatment ± standard error (SE). Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

The used culture system influenced subsequently the *ex-vitro* adaptation of the plants. TIS was noticed as the most appropriate one for successful application of the method: all plantlets were rooted and the number of the roots was the highest (6.42 roots per *in-vitro* plant).

Besides, the plants obtained via TIS distinguished with best survival rate during the period of *ex-vitro* adaptation (95%). All plants reached similar height range of 4.0-5.5 cm after 6 weeks of adaptation, but those obtained via TIS had more branches and produced bigger leaves compared to the other ones grown on agar-gelled medium **(Fig. 3)**.

The better developed roots of the plants cultivated in TIS ensured their fast growth in the substrate mixture which allowed their transfer to soil after only 2 months of *ex-vitro* adaptation, while the plants from the other two tested culture types required longer time (4 months).

According to Berthouly and Etienne[19] temporary immersion generally improves plant material quality resulting in increased shoot vigour and higher frequency of morphologically normal plants. Plants of *Calathea orbifolia* produced via TIS had much higher photosynthetic rates and subsequently higher leaf area, fresh and dry weight during *ex-vitro* adaptation than those from semi-solid media[32].

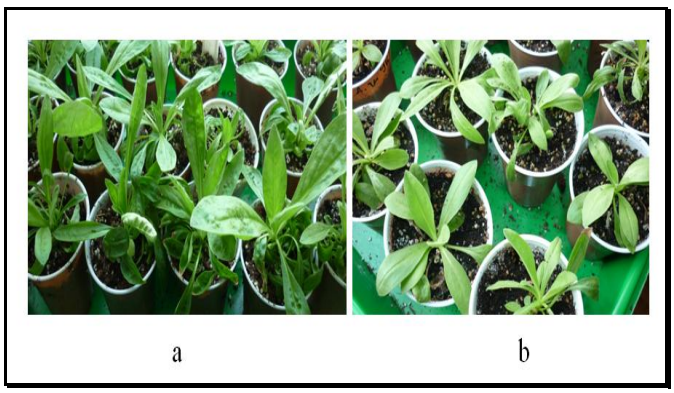

FIG. 3: PLANTS AFTER 6 WEEKS OF *EX-VITRO* ADAPTATION ORIGINATED FROM A) TIS CULTURE, B) AGAR GELLED MEDIUM

Sesquiterpene lactones content: The content of sesquiterpene lactones in plants derived from TIS culture (15.34 mg/g DW) was higher compared to plants collected from paper-bridge support liquid culture (13.81 mg/g DW) and agar-gelled medium (7.62 mg/g DW) **(Table 3)**. Two types of lactones – helenalin (H) and dihydrohelenalin (DH) were detected **(Fig. 4)**. Dihydrohelenanolides were twice more in TIS culture plants (14.74 mg/g DW) than in agar derived plants (7.00 mg/g DW). The content of dihydrohelenanolides reached 11.08% mg/g DW in static liquid medium with paper-bridge support.

TABLE 3: SESQUITERPENE LACTONES (mg/g dw) IN *A. MONTANA IN-VITRO* SHOOTS ORIGINATED FROM THREE DIFFERENT CULTURE SYSTEMS

Sesquiterpene lactones*	Agar-gelled medium culture	Liquid culture with static paper-bridge-support	TIS RITA®
H-Ac	0.34 ±0.01	0.33 ±0.09	0.08 ±0.01
dH-Ac	1.58 ±0.18	2.50 ±0.32	3.70 ±0.38
H-iBu	0.00	0.06 ±0.01	0.02 ±0.01
dH-iBu	0.76 ±0.08	0.61 ±0.21	1.32 ±0.29
H-Met	0.12 ±0.15	1.92 ±0.28	0.32 ±0.44
dH-Met, traces H-2MeBu	3.26 ±0.33	3.91 ±0.28	6.78 ±0.42
H-iVal	0.00	0.17 ±0.02	0.02 ±0.01
dH-2MeBu	0.14 ±0.06	0.13 ±0.03	0.30 ±0.10
dH-iVal	0.82 ±0.12	2.06 ±0.43	1.60 ±0.24
H-Tig	0.16 ±0.12	0.25 ±0.15	0.16 ±0.01
dH-Tig	0.44 ±0.09	1.87 ±0.24	1.04 ±0.32
Total H	0.62 ±0.02[a]	2.73 ±0.34[b]	0.60 ±0.10[a]
Total DH	7.00 ±0.28[a]	11.08 ±0.32[b]	14.74 ±0.44[c]
TOTAL	7.62 ±0.32[a]	13.81 ±0.38[b]	15.34 ±0.52[c]

*H-Ac (helenalin acetate); dH-Ac – (11α,13-dihydrohelenalin acetate); H-iBu (helenalin isobutirate); dH-iBu (11α,13-dihydrohelenalin isobutirate); H-Met (helenalin methacrylate); dH-Met (11α,13-dihydrohelenalin methacrylate), traces H-2MeBu (helenalin 2-methylbutirate); H-iVal (helenalin isovalerate); dH-2MeBu (11α,13-dihydrohelenalin 2-methylbutirate); dH-iVal (11α,13-dihydrohelenalin isovalerate); H-Tig (helenalin tiglate); dH-Tig (11α,13-dihydrohelenalin tiglate); total H – helenalin type lactones; total DH – dihydrohelenalin type lactones.

The concentrations of helenanolides were almost similar in TIS and in agar-gelled medium samples (0.62 and 0.60 mg/g DW) and four-fold more in static liquid medium (2.73 mg/g DW). The proportion of DH: H type esters was higher in plants cultured in TIS compared to that in plants from agar-gelled medium and static liquid culture.

The high content of dihydrohelenanolides in plants cultured in TIS can be due to their intensive growth and increased nutrient uptake. The main sesquiterpene lactones of the studied leaf extracts were methacryloyl and acetyl esters of dihydrohelenanolides. The results showed that TIS could be promising for future studies on *in-vitro* biosynthesis of sesquiterpene lactones because of the significantly higher production of both, biomass and dihydrohelenanolides, compared to the other two tested culture types.

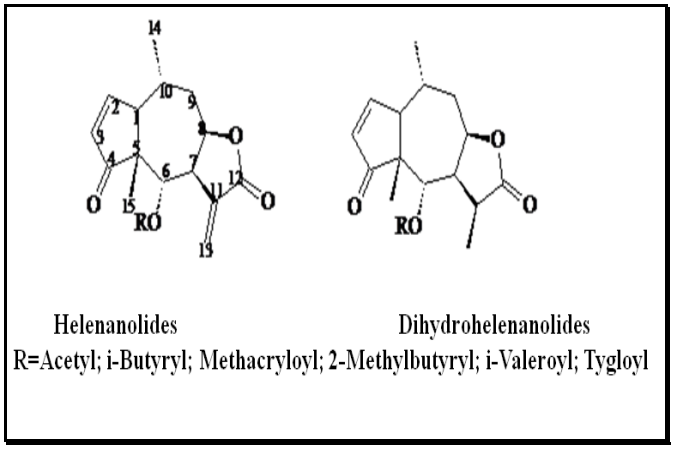

FIG. 4: STRUCTURES OF LACTONES IN *A. MONTANA*

CONCLUSIONS: Temporary immersion system has been successfully applied to enhance the *in -vitro* micropropagation of *Arnica montana*. Among three tested culture systems (agar-gelled medium culture, static liquid medium culture with paper-bridge support, and TIS RITA®) TIS was the most appropriate one for *in -vitro* propagation of this valuable medicinal plant species, endemic to Europe and threatened in many countries.

This system ensured production of numerous *A. montana* plants of good quality in a short time, as well as biosynthesis of high concentration of sesquiterpene lactones. TIS could be used for rapid production of *A. montana* plants designed for planting material needed for establishment of field cultivation. Further studies could be aimed to increase the *in-vitro* biosynthesis of sesquiterpene lactones under controlled conditions.

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