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In Vitro Propagation of *Rhododendron tomentosum* – an Endangered Essential Oil Bearing Plant from Peatland

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Rhododendron tomentosum Harmaja (formerly *Ledum palustre* L.) is a medicinal peat bog plant native to northern Europe, Asia and North America. This plant has a distinctive aroma thanks to the presence of essential oil, to which it also owes its anti-inflammatory, analgesic, antimicrobial and insecticidal properties. However, in Europe *R. tomentosum* is classified as an endangered species, mainly due to degradation of peatlands. In the present work, the micropropagation protocol for *R. tomentosum* was established for the first time, providing both an *ex situ* conservation tool and a means of continuous production of *in vivo* and *in vitro* plant material for further studies. *R. tomentosum* microshoots were initiated from leaf explants and further multiplied using Schenk-Hildebrandt (SH) medium supplemented with 9.84 μM 2iP and 1.00 μM TDZ. The shoots were elongated on the SH medium supplemented with 24.6 μM 2iP and subsequently rooted using the perlite substrate saturated with half-strength Woody Plant medium supplemented with 1.0% sucrose and 4.92 μM IBA. The regenerated plants were hardened on the phytohormone-free SH medium and acclimatized using 3:1:1 deacidified peat:perlite:gravel substrate. The identity of the mother plant was confirmed at morphological and molecular levels and Random Amplified Polymorphic DNA (RAPD) method was implemented to assess the genetic fidelity of the regenerants. The essential oil content of the maternal plant, *in vitro* shoots and the regenerants was determined by steam-distillation, and the obtained volatile fractions were analyzed by GC/MS.

Keywords: endangered species, essential oil, GC analysis, *in vitro* cultures, *Ledum palustre*, micropropagation, *Rhododendron tomentosum*

Abbreviations: AR – Anderson's Rhododendron (medium), DMRT – Duncan's multiple range test, FW – fresh weight, GC/MS – gas chromatography-mass spectrometry, Gi – growth index, IAA – indole-3-acetic acid, IBA – indole-3-butyric acid, 2iP – 2-isopentenyladenine, NAA – 1-naphthaleneacetic acid, PGR – plant growth regulator, RAPD – Random Amplified Polymorphic DNA, SH – Schenk and Hildebrandt (medium), TDZ – thidiazuron, WP – Woody Plant (medium), ½WP – half-strength Woody Plant (medium)

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INTRODUCTION

Unique medicinal plants are important components of the peatland high biodiversity (Chapman et al., 2003). One of the most recognizable bog shrubs in Europe, Asia and North America is *Rhododendron tomentosum* Harmaja (previously *Ledum palustre* L.), the representative of *Ericaceae* commonly known as the marsh tea (Harmaja, 1991). Its white flowers and ever-green, narrow leaves with rusty hairs are characterized by the presence of essential oil with a distinctive strong aroma. *R. tomentosum* has been used for centuries as a repellent and a folk remedy for rheumatic pain and respiratory diseases such as cough, bronchitis and asthma. Nowadays, the anti-inflammatory, analgesic, antimicrobial and insecticidal properties of *R. tomentosum*, associated predominantly with its terpene-rich volatile fraction, have been scientifically documented (Dampc and Luczkiewicz, 2013). However, natural resources of *R. tomentosum* are limited due to exploitation, drainage and destruction of peatlands for forestry or agriculture (Ejankowski, 2010; Čížková et al., 2013).

In accordance with the Global Strategy for Plant Conservation, 75% of threatened plant species should be stored in *ex situ* collections and 20% should be available for recovery programs by 2020 (CITES Plants Comitee, 2011). Plant micropropagation and *in vitro* preservation have been recognized as important tools for *ex situ* conservation and reintroduction trials of rare and endangered plants (Fay, 1992; Sugii and Lamoureux, 2004; Dubova et al., 2010). The above approach enables effective production of pathogen-free plants which can be introduced into their natural habitat after ecosystem restoration.

The aim of the presented work was to develop an efficient micropropagation protocol of *R. tomentosum*. To the best of the authors' knowledge, this is the first report on *in vitro* propagation of the marsh tea. The established procedure included shoot initiation, multiplication, elongation, rooting and *ex vitro* acclimatization. The identity of mother plant was confirmed based on unique morphological characters and at molecular level by comparing the DNA sequence of 23 and 24 exons with intervening intron of nuclear *RBP2-I* gene to well described and documented material originating from sole molecular phylogeny of the genus by Goetsch et al. (2005). Random Amplified Polymorphic DNA (RAPD) method was employed to assess genetic fidelity of 16 randomly chosen regenerants. Furthermore, the chemical composition of the mother plant, *in vitro* shoots and progeny plants was evaluated by GC/MS in terms of essential oil content.

MATERIALS AND METHODS

PLANT MATERIAL

The aerial parts of the *R. tomentosum* plants were collected in early June from the peatland in Miszewko, approximately 20 km east of Gdansk in Poland (54°24'33" N, 18°21'46" E). Regional Director for Environmental Protection in Gdansk had granted permission to collect the protected species plant material. Voucher specimen (no 14252) was deposited in the herbarium of Department of Biology and Pharmaceutical Botany (GDMA), Medical University of Gdansk, and is available upon request.

CULTURE MEDIA AND CULTURE CONDITIONS

Schenk-Hildebrandt (SH) (Schenk and Hildebrandt, 1972; modified with the addition of 0.5 g l^{-1} ammonium nitrate), Anderson's Rhododendron (AR) (Anderson, 1978) and Woody Plant (WP) media (Lloyd and McCown, 1981), characterized by different mineral composition and pH value (Table 1), were used in the current study. The media were supplemented with plant growth regulators (PGRs) at various concentrations (Tables 2–5). All media reagents were from Sigma-Aldrich (Sigma-Aldrich, St. Louis, US-MO). Water was obtained with the use of Elix/Synergy water purification system (Merck KGaA, Darmstadt, Germany).

R. tomentosum in vitro cultures were incubated in a growth room at 24 ± 2°C under a 16/8h photoperiod at 40 μmol m^{-2} s^{-1} white fluorescent light (TLD 35W/33 tubes, Philips). Unless otherwise stated, sub-culturing was carried out at 4 weeks intervals.

CULTURE INITIATION

Explants obtained from the maternal plant (nodal shoot segments, axillary buds and leaves) were dipped in ca. 1:1000 detergent solution ('Ludwik' washing-up liquid, INCO, Gora Kalwaria, Poland) for 1 min, washed with running tap water for 20 min and rinsed twice with distilled water. Surface sterilization was carried out using 0.1% (w/v) mercuric chloride for 15 min, followed by washing three times with sterilized, double-distilled water. The explants were then transferred onto PGR-free or cytokinin-supplemented (2iP and/or TDZ) SH, AR and WP media (Table 2). The response of the explants was recorded 60 days after inoculation. The newly developed microshoot clusters were subcultured for further multiplication.

TABLE 1. Composition of growth media employed in the study (Schenk and Hildebrandt, 1972; Anderson, 1978; Lloyd and McCown, 1981).

Constituent	Concentration (mg l^{-1}) in the medium		
	Schenk-Hildebrandt (SH)	Anderson's Rhododendron (AR)	Woody Plant (WP)
NH_4NO_3	500*	400	400
KNO_3	2500	480	–
KH_2PO_4	–	–	170
K_2SO_4	–	–	990
NaH_2PO_4	300	330.6	–
$CaCl_2$	151	332.2	72.5
$Ca(NO_3)_2$	–	–	386
$MnSO_4$ x H_2O	10	16.9	22.3
$MgSO_4$	195.4	180.7	180.7
$ZnSO_4$ x $7H_2O$	1	8.6	8.6
H_3BO_3	5	6.2	6.2
KI	1	0.3	–
$CoCl_2$ x $6H_2O$	0.1	0.025	–
$CuSO_4$ x $5H_2O$	0.2	0.025	0.25
Na_2MoO_4 x $2H_2O$	0.1	0.25	0.25
$FeSO_4$ x $7H_2O$	15	55.7	27.85
Na_2EDTA x $2H_2O$	20	74.5	37.3
Adenine sulphate	–	80	–
Thiamine	5	0.4	1
Nicotinic acid	5	–	0.5
Pirydoxine	0.5	–	0.5
Glycine	–	–	2
myo-Inositol	500	100	100
Saccharose	30000	30000	20000
Agar	6000	6000	6000
pH**	5.7	4.9	5.2

* concentration modified from the original work
** the pH of the media was adjusted to the respective value prior to autoclaving

TABLE 2. The effect of cytokinins on *in vitro* shoot culture initiation from *R. tomentosum* leaf fragments, after 60 days of cultivation.

Medium	Cytokinins used (μM)			
	(–)	2iP (9.84)	TDZ (1.00)	2iP (9.84) + TDZ (1.00)
SH	–	–	+	+
AR	–	–	–	+
WP	–	–	+	+

+ microshoots established; – no microshoots formed

TABLE 3. The effect of the medium composition on *R. tomentosum* shoot multiplication, after 30 days of cultivation.

Medium	PGRs (µM)	FW (g l^{-1})*	Gi**	Microshoot length (mm)***	Microshoot morphology
SH	–	255.79a±44.86	204.31a±27.70	15–20	Long, but fragile
	2iP (9.84) + TDZ (1.00)	276.28a±50.90	343.72b±32.43	10–15	Vivid green, juvenile
AR	–	140.79b±11.50	126.71c±6.24	5–10	Loose clusters with browning changes
	2iP (9.84) + TDZ (1.00)	170.90ab±5.63	180.84ad±17.05	5–10	Shoot reddening
WP	–	171.74ab±19.57	170.03ad±21.50	10–20	Long, juvenile
	2iP (9.84) + TDZ (1.00)	207.23ab±21.60	160.55cd±24.92	10–15	Shoot-tip necrosis

* the data represent the mean (±SD) of four replicates (4 explants each), values followed by various letters are significantly different at $p < 0.05$ (one-way ANOVA on Ranks, Dunn's test)
** the data represent the mean (±SD) of four replicates (4 explants each), values followed by various letters are significantly different at $p < 0.05$ (one-way ANOVA, DMRT)
*** the values indicate length range the measured shoots fall into

TABLE 4. The effect of different PGR combinations of SH medium on the elongation of *R. tomentosum* microshoots, after 30 days of cultivation.

PGRs (µM)	Number of microshoots per explant*	Microshoots longer than 10 mm (%)**	Microshoots longer than 20 mm (%)**
–	84.10a ± 22.76	64.12a	10.90a
2iP (9.84) + TDZ (1.00)	73.91a ± 22.12	7.26b	0.25b
2iP (9.84)	25.67b ± 9.33	46.32ab	10.39a
2iP (9.84) + IBA (4.92)	88.67ac ± 30.73	50.85a	8.36a
2iP (24.6)	107.83c ± 26.43	62.98a	12.91a

* the data represent the mean (±SD) of three replicates (4 explants each), values followed by various letters are significantly different at $p < 0.05$ (one-way ANOVA, DMRT)
** the data represent the mean (±SD) of three replicates (4 explants each), values followed by various letters are significantly different at $p < 0.05$ (one-way ANOVA on Ranks, Dunn's test)

SHOOT MULTIPLICATION

Three different media: SH, AR and WP, PGR-free or supplemented with 2iP and TDZ (Table 3), were tested for multiple shoot formation. Four explants per treatment (ca. 1.5 g fresh weight, obtained from microshoot clusters formed from leaves) were used and each experiment was repeated 4 times. The response of the shoots was recorded 30 days after inoculation. Due to the very large number of shoot primordia formed at the multiplication stage (Fig. 1b, c), shoot counting was considered impractical and prone to errors. Instead, shoot growth was expressed by FW (g l^{-1}) and Gi (%) values (Table 3). The growth index was calculated with the formula: $Gi = (FW_{30} - FW_0)/FW_0 \times 100$ where Gi is the growth index and FW_0 and FW_{30} is the fresh weight of the inoculum and the weight of shoots after 30 days of cultivation, respectively.

SHOOT ELONGATION

For shoot elongation, microshoot clusters formed at the stage of shoot multiplication (SH medium supplemented with 9.84 µM 2iP and 1.0 µM TDZ) were transferred onto the SH media, supplemented with different concentrations of 2iP, as well as 2iP/IBA combination (Table 4). PGR-free SH medium and

TABLE 5. The effect of the medium composition on rooting of *R. tomentosum* microshoots, after 45 days of cultivation.

Medium	Sucrose content (g l^{-1})	PGRs (μM)	Root induction (%)*	Number of roots per explant*		Microshoots longer than 20 mm (%)*
				Median	Range	
SH	30	–	32.35a	0ab	0–3.0	23.53a
AR	30	–	0a	0a	0–0	0a
WP	20	–	72.73ab	1.53b	0.8–2.0	45.45a
		IAA (5.71)	42.86a	1.20b	0–3.2	66.67a
		NAA (5.37)	94.74c	2.65c	2.2–3.0	68.42a
		IBA (4.92)	86.36b	3.94c	1.4–4.4	68.18a
	10	–	66.67b	2.10b	1.0–2.7	33.33a
½WP	20	–	100.00c	4.62c	4.4–6.0	47.62a
	10	–	94.11c	6.17c	1.6–7.0	52.94a
		IBA (4.92)	100.00c	10.20d	6.83–11.29	82.76b

* the data represent the results of four replicates (5 explants each); values followed by various letters are significantly different at $p < 0.05$ (Mann–Whitney U test)

SH medium supplemented with 9.84 μM 2iP and 1.00 μM TDZ served as the control groups. Four explants per treatment were used and each experiment was repeated 3 times. The total number of microshoots per explant and the percentage of the elongated microshoots were recorded after 30 days.

ROOTING

For root induction, microshoots elongated on SH medium supplemented with 24.6 μM 2iP (15–20 mm in length) were transferred individually onto the media with different basal composition (SH, AR or WP), strength (full- or half-strength: ½WP), sucrose concentration and PGR content (Table 5).

Besides the agar-solidified media, the perlite-liquid ½WP medium (6 g perlite per jar, saturated with liquid medium and autoclaved) was tested (Fig. 2). Five explants per treatment were used and each experiment was repeated 4 times. In both experiments, the percentage of responding shoots and/or the number of roots developed per explant were recorded after 45 days of cultivation.

The third, labor-saving approach, included immersion of the elongated microshoot clusters (obtained on SH medium supplemented with 24.6 μM 2iP) in 400 mg l^{-1} aqueous solutions of NAA (2.15 mM), IBA (1.97 mM) and IAA (2.28 mM) for 10 s, and transferring them directly to *ex vitro* conditions: the explants were placed in plastic pots filled with autoclaved soil mixture consisting of deacidified peat, perlite and gravel (3:1:1 v/v/v), and covered with glass beakers. Five explants per each treatment were used.

HARDENING AND ACCLIMATIZATION OF PLANTLETS

For hardening, 8 rooted plantlets (obtained on ½WP medium supplemented with 1% sucrose and 4.92 μM IBA) were cultured on PGR-free SH medium (SH0) for 14 days. For *ex vitro* acclimatization, the hardened plantlets were subsequently transferred to plastic pots filled with autoclaved mixture of deacidified peat, perlite and gravel (3:1:1 v/v/v), and maintained at 22 ± 2°C.

Alternatively, 12 rooted plantlets (obtained on ½WP medium supplemented with 1% sucrose and 4.92 μM IBA) were transferred directly to the soil mixture of acidic or deacidified peat, perlite and gravel (3:1:1 v/v/v), and grown at 22 ± 2°C (6 explants per modification).

The pots were covered with glass beakers to maintain high humidity. The regenerated plants were watered profusely twice a week, both into the pot and the saucer. After one month, the survival rate and the height of the regenerated plants were recorded (Fig. 3).

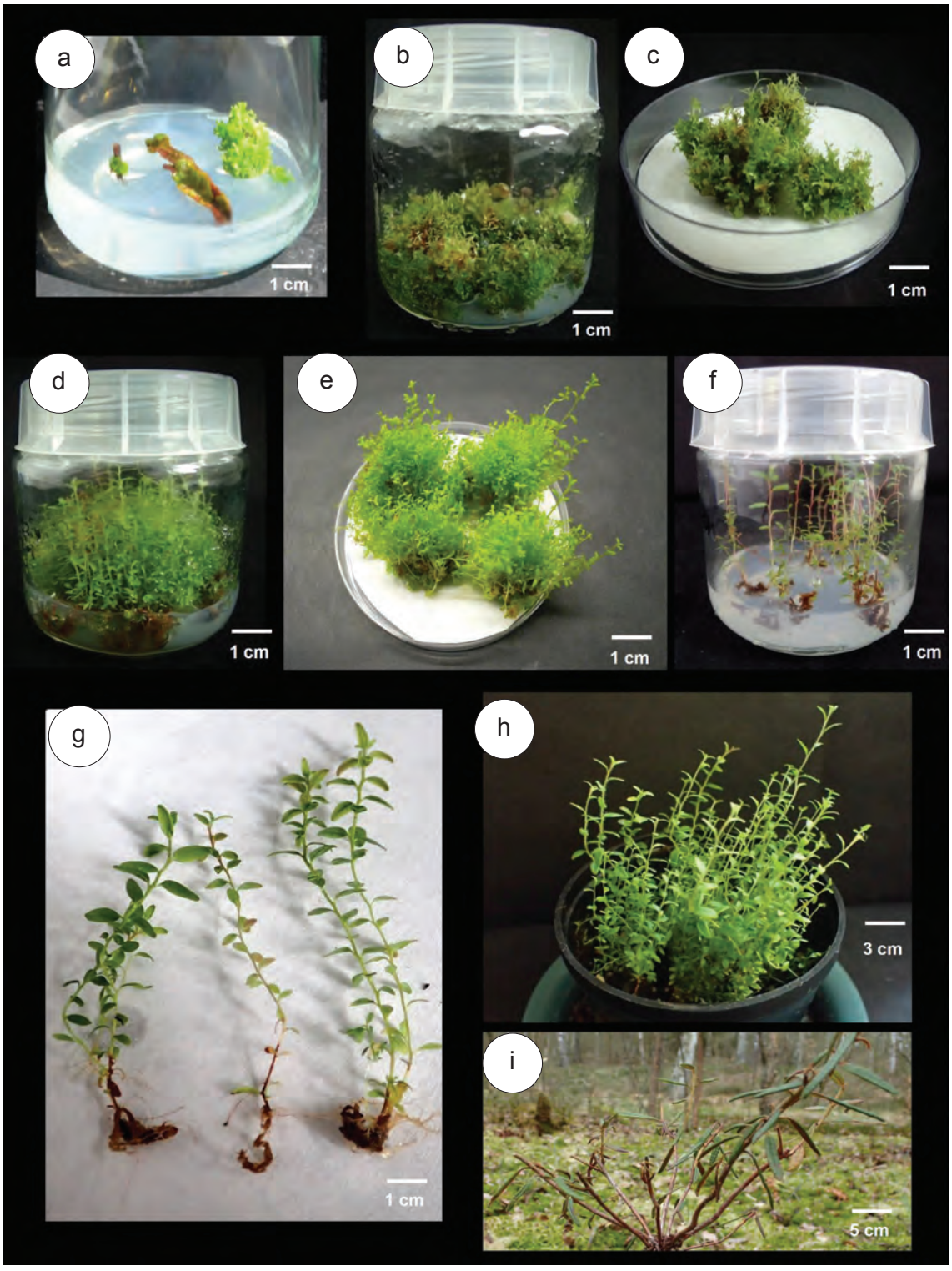


Fig. 1. Subsequent stages of *R. tomentosum* micropropagation: (**a**) initiation of *in vitro* shoot culture (SH medium supplemented with 9.84 μM 2iP and 1.0 μM TDZ); (**b**) and (**c**) shoot multiplication (SH medium supplemented with 9.84 μM 2iP and 1.0 μM TDZ); (**d**) and (**e**) shoot elongation (SH medium supplemented with 24.6 μM 2iP); (**f**) *in vitro* rooting (½WP medium with halved sucrose content, supplemented with 4.92 μM IBA); (**g**) rooted microshoots after hardening (PGR-free SH medium); (**h**) regenerated plants, acclimatized to *ex vitro* conditions; (**i**) maternal plant (Miszewko near Gdansk, Poland).

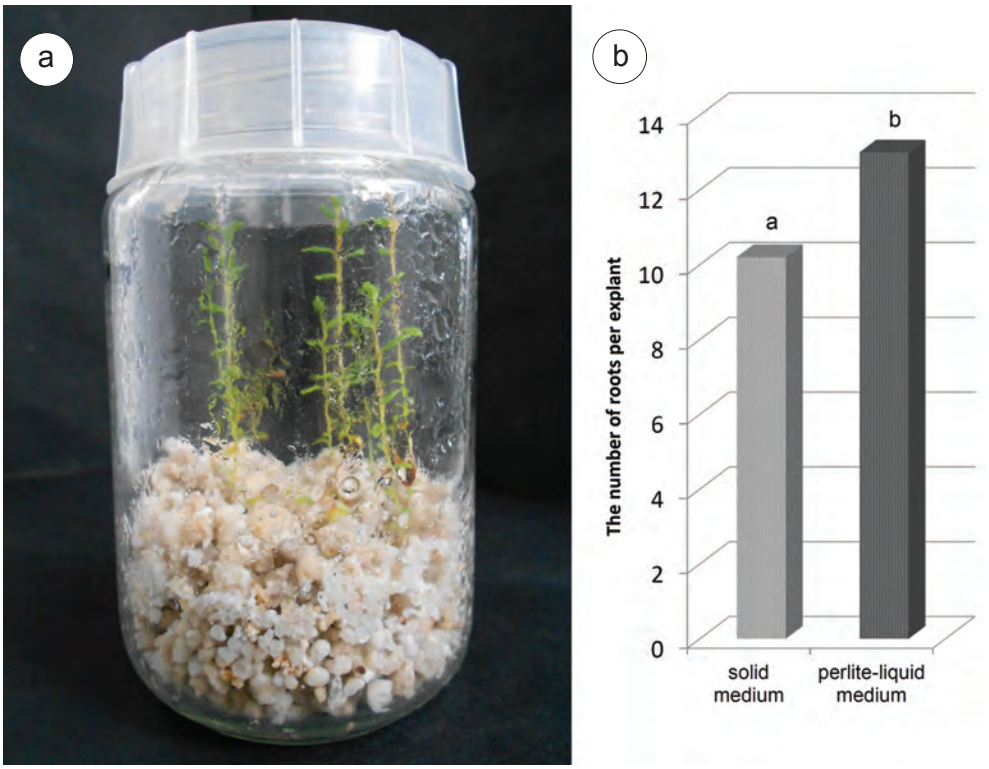


Fig. 2. (**a**) *R. tomentosum* microshoots rooted in the perlite-liquid ½WP medium*; (**b**) average number** of roots formed in the perlite-liquid and agar-solidified ½WP media*. (*) half-strength WP media supplemented with 10,000 mg l^{-1} sucrose and 4.92 μM IBA; (**) values are the medians of four replicates, each consisting of 5 explants; data followed by different letters are significantly different at $p < 0.05$ (Mann–Whitney U test).

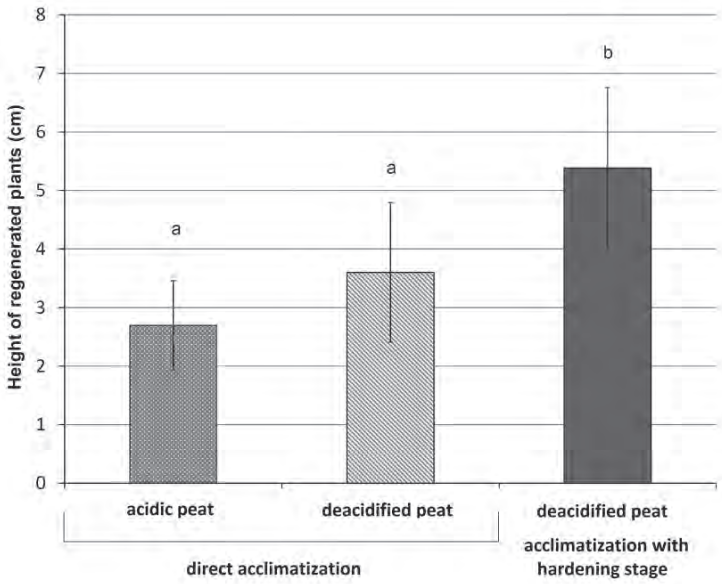


Fig. 3. Height of the regenerated *R. tomentosum* plants. Values are the means of 6 (direct acclimatization in acidic or deacidified peat) or 8 replications (acclimatization of hardened plantlets); data followed by different letters are significantly different at $p < 0.05$ (one-way ANOVA, DMRT).

DETERMINATION OF ESSENTIAL OIL CONTENT

The harvested *R. tomentosum* plant materials: I) shoots of the maternal plants (young and aged ones, collected separately and cut in 4–5 cm segments) (Fig. 1i), II) microshoots maintained on SH medium supplemented with 2iP (9.84 μM) and TDZ (1.00 μM), III) microshoots elongated on SH medium supplemented with 2iP (24.6 μM) and IV) two-month old regenerated plants (with roots removed) were air-dried at 22 ± 2°C.

The essential oils were isolated by hydrodistillation (3h) of the dried biomasses (ca. 10 g for the materials I, II and III, ca. 4 g for the regenerated plants) in the Deryng apparatus (Polish Pharmacopoeia VI, 2002), using 200 ml of distilled water. The volatile fractions were collected in 1.0 ml of hexane:diethyl ether mixture (1:1) (Butkiene et al., 2008) which was subsequently evaporated from the samples under a stream of nitrogen gas. The essential oil content in the examined plant materials was presented as the mean value from the results of two hydrodistillations (Fig. 4).

GC/MS ANALYSIS OF ESSENTIAL OILS

For GC analysis, the essential oils obtained from *R. tomentosum* microshoots at different stages of micropropagation as well as from maternal plant were diluted with acetone (1:100). Chromatographic analysis of the obtained essential oils was carried out on DB-5ms 30 m × 0.25 mm × 0.25 μm capillary column (Agilent J&W), using Gas Chromatograph 7890A coupled with Mass Selective Detector 5977A. 1.0 μl of the acetone solutions of the essential oil was injected in the split mode (1:10), applying Agilent Autosampler Systems 7693. The injector temperature was 250°C. The GC analysis parameters were as follows: carrier gas: helium, constant flow mode (1.1 ml/min), oven temperature increase: from 50 to 280°C, at a rate 7°C min^{-1}, the final GC temperature was held for 20 min. The single GC run time was 53 min. The spectra were compared with the data from NIST Library 11. GC/MS analysis results are presented in Table 6.

DNA ISOLATION, MOLECULAR IDENTIFICATION OF THE MOTHER PLANT AND RAPD ANALYSIS

For genetic studies, DNA was extracted from 16 randomly selected plantlets and the mother donor plant of *R. tomentosum* using standard CTAB protocol (Doyle and Doyle, 1987). Between 20 and 50 mg of fresh leaf tissue was used for single extraction reaction, resulting in high quality DNA yield (260/280 ratio > 1.8, 260/230 ratio > 1.7 and concentrations from 80 to 220 ng μl^{-1}).

For identification of the mother donor plant the target region comprising exons 23, 24 and intervening intron of nuclear *RBP2-I* gene was amplified in 25 μl with 20 ng of genomic DNA 1x PCR buffer with $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of forward (23F) and reverse (24R) primer [sequences given by Goetsch et al. (2005)] and 1 unit of *Taq* polymerase. The thermal profile of the reaction was 94°C for 4 min (initial denaturation) followed by 35 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 1 min 30 s, with a final elongation 72°C for 10 min. PCR product of 918 bp length was sequenced in both directions using commercial sequencing service for extra-long run (Genomed, Warszawa, Poland). Consensus sequence was generated with Sequencher (version 5.2.4, GeneCodes Corp.) and incorporated in the alignment stored in TreeBASE under M2277

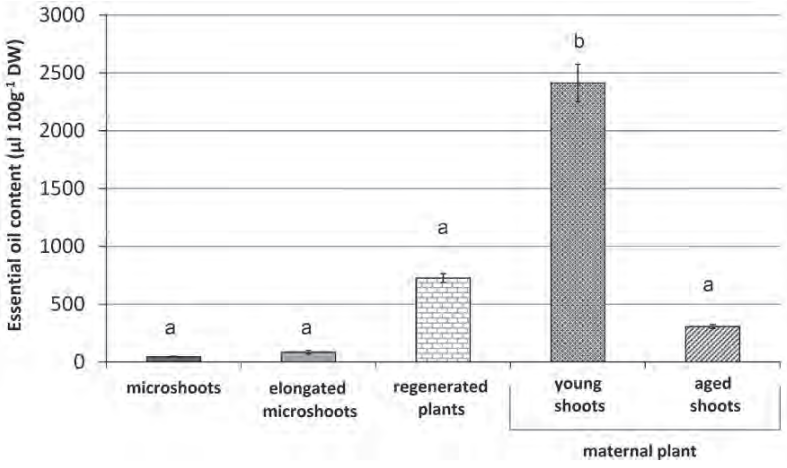


Fig. 4. Essential oil content of *R. tomentosum* microshoots, regenerants and maternal plants. Values are the means of 2 replications; data followed by various letters are significantly different at $p < 0.05$ (one-way ANOVA on Ranks, Dunn's test).

TABLE 6. Chemical composition of *R. tomentosum* essential oils obtained from different types of plant material. Data by GC/MS.

Compound	RT	Kovats' RI		% Composition of the essential oils				
		NIST	Experimental	Microshoots	Elongated microshoots	Regenerated plants	Maternal plant	
							Young shoots	Aged shoots
α-Pinene	5.83	931	929	0.1	0.6	1.9	1.4	0.6
Camphene	6.13	947	946	–	0.1	1.3	0.9	0.5
Sabinene	6.55	969	971	0.5	1.2	2.0	2.3	0.2
β-Pinene	6.64	975	976	0.6	1.2	2.0	1.1	0.7
β-Myrcene	6.83	988	987	0.2	0.5	0.7	4.1	0.8
δ-2-Carene	7.38	–	1017	0.6	2.5	4.2	7.1	1.4
α-Terpinene	7.48	1017	1018	1.8	7.9	6.0	–	–
p-Cymene	7.53	1025	1027	4.7	4.6	5.2	5.1	4.9
Limonene	7.74	1032	1033	–	–	–	0.5	0.5
γ-Terpinene	8.22	1059	1060	1.1	3.6	3.7	2.7	0.9
Linalool	9.06	1100	1101	–	–	–	0.2	0.4
Terpinen-4-ol	10.66	1177	1153	0.4	1.0	0.9	1.4	0.8
p-Cymen-8-ol	10.78	1187	1188	0.6	0.5	0.3	0.7	0.7
α-Terpineol	10.91	1190	1195	0.4	0.3	0.2	0.3	0.2
Myrtenal	11.11	1196	1202	0.1	0.2	0.4	0.4	1.0
γ-Terpineol	11.91	–	1245	0.1	0.2	1.9	15.0	18.8
Geraniol	12.10	1256	1255	0.3	0.2	–	0.3	2.2
Bornyl acetate	12.82	1292	1291	1.0	2.0	4.7	1.8	2.1
Carvacrol	13.08	1299	1307	1.2	0.8	0.2	0.4	0.2
Citronellyl acetate	14.00	–	1353	0.6	0.6	0.3	0.6	0.9
Geranyl acetate	14.58	1354	1384	2.1	2.4	1.5	3.9	4.3
β-Elemene	14.87	–	1400	0.2	0.4	0.3	0.1	–
β-Farnesene	15.94	1429	1459	0.5	1.4	1.4	–	0.1
Alloaromadendrene	16.23	1462	1475	4.5	9.2	8.5	1.4	2.3
6-Epishyobunone	16.73	–	1504	3.2	5.9	4.6	1.8	1.9
α-Muurolene	16.83	1499	1509	0.2	0.3	0.2	0.3	0.1
Shyobunone	17.11	–	1527	5.1	7.9	6.1	6.3	4.2
δ-Cadinene	17.23	1525	1533	0.8	2.1	1.3	0.8	0.5
Palustrol	18.06	–	1582	0.2	0.3	0.3	11.5	15.7
Germacren D-4-ol	18.16	–	1588	1.0	1.2	1.1	–	–
Methyl everninate	18.27	–	1594	7.6	4.8	0.7	–	–
Ledol	18.72	1580	1603	–	–	–	9.6	12.1
Dehydroxy-isocalamendiol	19.12	–	1646	6.2	4.4	2.7	0.5	0.3
epi-α-Murrolol	19.29	1644	1657	0.7	0.5	0.2	0.4	0.4
α-Cadinol	19.46	1654	1667	2.0	2.1	0.9	4.1	1.6
Cyclocolorenone	21.06	–	1771	–	–	–	1.8	2.0

accession (Goetsch et al., 2005) using Mesquite software version 2.75 (Maddison and Maddison, 2015). The newly generated sequence is stored in GenBank (accession no. KY073138).

In total, 60 RAPD primers were screened for repeatable RAPD PCR products, and 11 of them that gave scorable and clear bands were selected for final analysis to assess genetic stability of the regenerants. RAPD PCR reaction was performed in total volume of 25 µl containing 20 ng of DNA, 1x PCR buffer with $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 µM primer and 1 unit of *Taq* polymerase. Amplification reactions were carried out in PTC-200DNA Engine cycler (MJ Research, USA) using temperature profile with 94°C 3 min for initial denaturation followed by 35 cycles of 1 min at 94°C, 2 min at 38°C, 2 min at 72°C, with a final elongation 7 min at 72°C. PCR products were separated on 1.2% agarose gel with addition of ethidium bromide at the final concentration of 0.4 µg ml^{-1}, in TAE buffor at 5 V cm^{-1}. Gels were stored in electronic format and analyzed manually. Each band was treated as a marker and scored as "1" when present and as "0" when absent, the intensity was not scored as variable. Binary matrix was further transformed to distance matrix in FAMD 1.31 Software (Schlüter and Harris, 2006) and genetic similarity between the mother donor plant and plantlets was measured by means of Jaccard's similarity coefficient (Fig. 5).

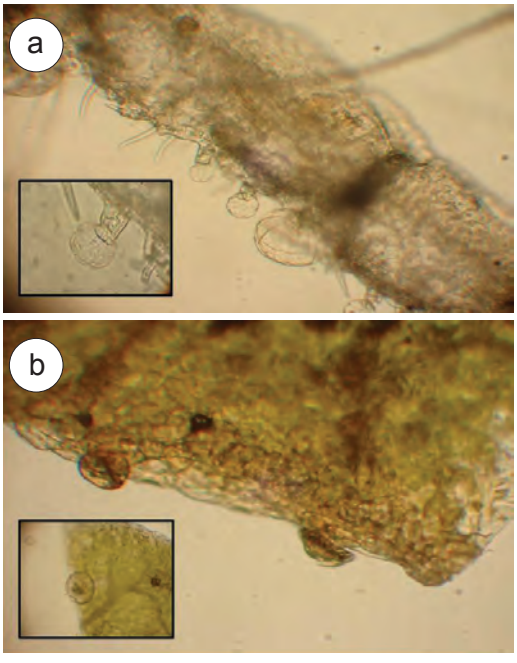


Fig. 5. Essential oil-secreting glandular hairs, seen in a cross-section of a leaf of: (**a**) intact plant; (**b**) *in vitro* rooted plantlet after hardening. Photographs were taken with TG-3 Olympus camera, using an optical microscope (magnification: 1:20, 1:40).

STATISTICAL ANALYSIS

The collected data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$) or Dunn's test ($p < 0.05$), except for the rooting stage of the micropropagation, where Mann-Whitney U test was used ($p < 0.05$). Analyses were conducted with SigmaPlot 11.00 (Systat Software, San Jose, US-CA).

RESULTS AND DISCUSSION

CULTURE INITIATION

Taking into account that *R. tomentosum* seeds are very small (ca. 1.7 × 0.3 mm) and thus sensitive to chemical sterilizing agents, and the fact that the examined plant reproduces predominantly through vegetative sprouting (Racine et al., 1987), we decided to initiate the *in vitro* cultures using the vegetative parts of wild growing plants (nodal shoot segments, axillary buds and leaves). Two months after sterilization, the shoot cultures were induced from all the examined explants. However, the effectiveness of surface sterilization of axillary buds and nodal segments (67 and 78%, respectively) was lower in comparison with the leaves (85%), presumably due to the smoother surface of this explant type. Given the above, leaf fragments of the maternal plant were selected as the most suitable for *R. tomentosum* micropropagation.

For *in vitro* shoot induction, Schenk-Hildebrandt (SH), Anderson's Rhododendron (AR) and Woody Plant (WP) media were selected. SH medium, supplemented with 9.84 µM 2iP and 1.0 µM TDZ, effectively stimulated microshoot formation in several plants, originating from various families, for instance *Cyclopia* and *Genista* spp. (Fabaceae) and *Caryopteris* spp. (Lamiaceae) (Luczkiewicz and Piotrowski, 2005; Kokotkiewicz et al., 2012; Luczkiewicz et al., 2015). Anderson's Rhododendron medium (AR) was described as suitable for *Rhododendron* micropropagation (Anderson, 1978; Eeckhaut et al., 2010). Woody Plant medium (WP) in turn is commonly used for *in vitro* propagation of ericaceous plants (Debnath, 2007).

As presented in Table 2, *in vitro* shoot cultures of *R. tomentosum* can be initiated on all SH (Fig. 1a), AR and WP media, supplemented with 2iP and TDZ. The experiment also demonstrated that TDZ is essential for microshoot induction. 2iP, on the other hand, failed to induce microshoots when applied as a sole PGR.

SHOOT MULTIPLICATION

The experiment included SH, AR and WP media compositions, characterized by different mineral content and pH value (Table 1). As demonstrated in previous reports, both 2iP and TDZ are widely applied for micropropagation of *Rhodododendron* (Eeckhaut et al., 2010; McCown, 2013), as well as other representatives of *Ericaceae*, most importantly of the genus *Vaccinium* (Debnath, 2007). Given the above, and considering the effects of our team (Luczkiewicz and Piotrowski, 2005; Kokotkiewicz et al., 2012; Luczkiewicz et al., 2015), indicating the versatility of TDZ as the agent for microshoot formation, we decided to apply 9.84 μM 2iP and 1.00 μM TDZ in all the media used. In this research addition of other cytokinins to the media was not taken into consideration.

Out of the media applied, SH medium provided the highest growth rate which was almost two times higher as compared to AR and WP (Table 3). The obtained shoots were healthy, without signs of necrosis (Fig. 1b, c), and easy to subculture. However, they were also relatively short and formed callus at the base of the explants. Considering the above, the multiplied shoots seemed to be unsuitable for direct rooting, and instead they were subjected to the elongation procedure.

SHOOT ELONGATION

Based on the results of previous micropropagation experiments involving the use of 2iP and TDZ (Luczkiewicz and Piotrowski, 2005; Kokotkiewicz et al., 2012), two experimental approaches were applied to elongate the previously-multiplied *R. tomentosum in vitro* shoots. These included removal of TDZ from the growth medium in order to reduce its shoot-inducing potential, as well as addition of auxin to stimulate shoot elongation. The microshoots cultivated on SH medium with 9.84 μM 2iP and 1.00 μM TDZ and on SH medium without PGRs were used as the control groups.

As it was observed already at the stage of multiplication, microshoots on PGR-free SH medium were to a large extent elongated (Table 4). Nevertheless, they were characterized by a strong tendency to wither. The removal of TDZ from the basal multiplication medium resulted in a significant increase in shoot length. However, at the same time, the number of microshoots was significantly decreased. The decline in the number of microshoots was not observed in the medium supplemented with 2iP/IBA mixture which also caused noteworthy shoot elongation in comparison to 2iP/TDZ composition. Interestingly, similar results were obtained with the medium supplemented with 24.6 μM 2iP only. The data indicate that in terms of microshoot formation, the higher amounts of 2iP (used as a sole PGR) can provide even better results than the 2iP/TDZ mixture, at the same time leading to a significant increase in shoot length. The relatively low growth of microshoots on SH medium with 24.6 μM 2iP (FW = 243.18 ± 32.93; Gi = 205.77 ± 26.79) can be explained with the beneficial reduction of callus at the base which, as a consequence, makes the biomass lighter. Given the obtained results, application of the above medium can be suggested not only for shoot elongation, but also for their multiplication, in order to simplify and quicken the micropropagation procedure (Fig. 1d, e).

ROOTING

The experiments involved three media compositions: SH, AR and WP. The last one was the most effective in root induction (Table 5) and was thus selected for further modifications aimed at improving the rooting rate of *R. tomentosum* explants. The media variants included the reduction of sucrose and mineral salts content (half-strength medium, ½WP) and the use of auxins – the strategies previously described by Anderson (1984) and Briggs et al. (1994) as effective for *in vitro* rooting of rhododendrons.

As presented in Table 5, both reducing the mineral salts content and the use of auxins (NAA or IBA) had a positive effect on the root induction in WP medium. Further improvement of explants rooting (100% response) was achieved by simultaneous halving of the concentrations of sucrose and mineral salts, and the application of IBA (which was shown to be superior to NAA in terms of the number of roots per explant). The above modification also positively affected shoot morphology by significantly increasing explant length (Table 5, Fig. 1f). In order to further improve rooting efficiency, the agar medium was replaced by perlite saturated with liquid ½WP medium. The described procedure, previously applied for other representatives of the genus (Bojarczuk, 1989), resulted in significant increase in the number of roots per explant (Fig. 2).

Unfortunately, the trials of direct *ex vitro* rooting, successfully adopted for several representatives of Ericaceae (Debnath, 2007), proved unsuccessful for *R. tomentosum*. Microshoot clusters immersed in auxin solutions and transferred to the soil mixture failed to form roots and subsequently withered.

HARDENING OF PLANTLETS AND ACCLIMATIZATION

Based on the available literature concerning propagation of ericaceous plants (Debnath, 2007; Matysiak and Nowak, 2008), the regenerated plant-

lets were acclimatized in the peat:perlite:gravel substrate. The use of glass covers was essential for maintaining high air humidity during the process, providing healthy plants without signs of withering. Modifications of the proposed acclimatization procedure (Fig. 3) did not affect the survival rate of plantlets (100% in each experiment). However, hardening of plantlets for 14 days prior to acclimatization significantly increased the average length of the regenerated plants (from 3.60 cm to 5.38 cm – Figs. 1g, h and 3).

ESSENTIAL OIL ANALYSIS

As shown in Fig. 4, there were significant differences in essential oil content between the examined *R. tomentosum* plant materials. Young shoots of *R. tomentosum* maternal plants were characterized by the highest amount of essential oil (2400 µl 100 g^{-1} DW), whereas aged shoots proved to be rather a poor source of volatiles (306 µl 100 g^{-1} DW). *R. tomentosum in vitro* shoots accumulated lower amounts of essential oil (< 100 µl 100 g^{-1} DW) in comparison to the intact plant. The above phenomenon was also observed in other species (Spencer et al., 1990) and presumably results from the morphological differences between the parent plant and the established *in vitro* culture (Fig. 5). Since organogenesis was shown to be positively correlated with the accumulation of volatiles (Mulder-Krieger et al., 1988), the probable cause of low essential oil content in *R. tomentosum* microshoots was the lack of developed secretory structures. Consequently, the regeneration process, associated with the development of intact plant morphology, resulted in substantial increase in essential oil content (725 µl 100 g^{-1} DW).

GC/MS analysis of the obtained volatile fractions demonstrated differences in the composition of essential oil from *R. tomentosum in vitro* cultures, regenerated plants and maternal shoots (Table 6). Some compounds, like methyl everninate, were present only in microshoots and regenerated plants' volatile fraction, while others were found solely in the ground material (for instance ledol). Alloaromadendrene was the predominant constituent in the microshoots (4.5–9.2%), while γ-terpineol (15.0–8.8%), palustrol (11.5–15.7%) and ledol (9.6–12.1%) occurred in large quantities in maternal plants. Other compounds, like *p*-cymene, were abundant in both *in vitro* cultures and maternal plants (4.6–5.2%). The observed differences in essential oil composition between the examined biomasses were probably caused by different conditions under which they were maintained (Thiem et al., 2011). The regenerated plants were cultivated under glasshouse conditions, whereas the mother plants grew in their natural bog habitat. As indicated by literature data, the composition of *R. tomentosum* volatile oil is strongly affected by environmental factors and age of the plant material (Dampc and Luczkiewicz, 2013). Moreover, the experiments by Hilton et al. (1995) demonstrated that the chemical composition of essential oil in *in vitro* shoot cultures can vary greatly with light intensity. Additionally, the present study reveals that the accumulation of some *R. tomentosum* volatiles is associated with organogenesis – the concentration of γ-terpineol and bornyl acetate increased in parallel with microshoot elongation and the age of plant material (Table 6).

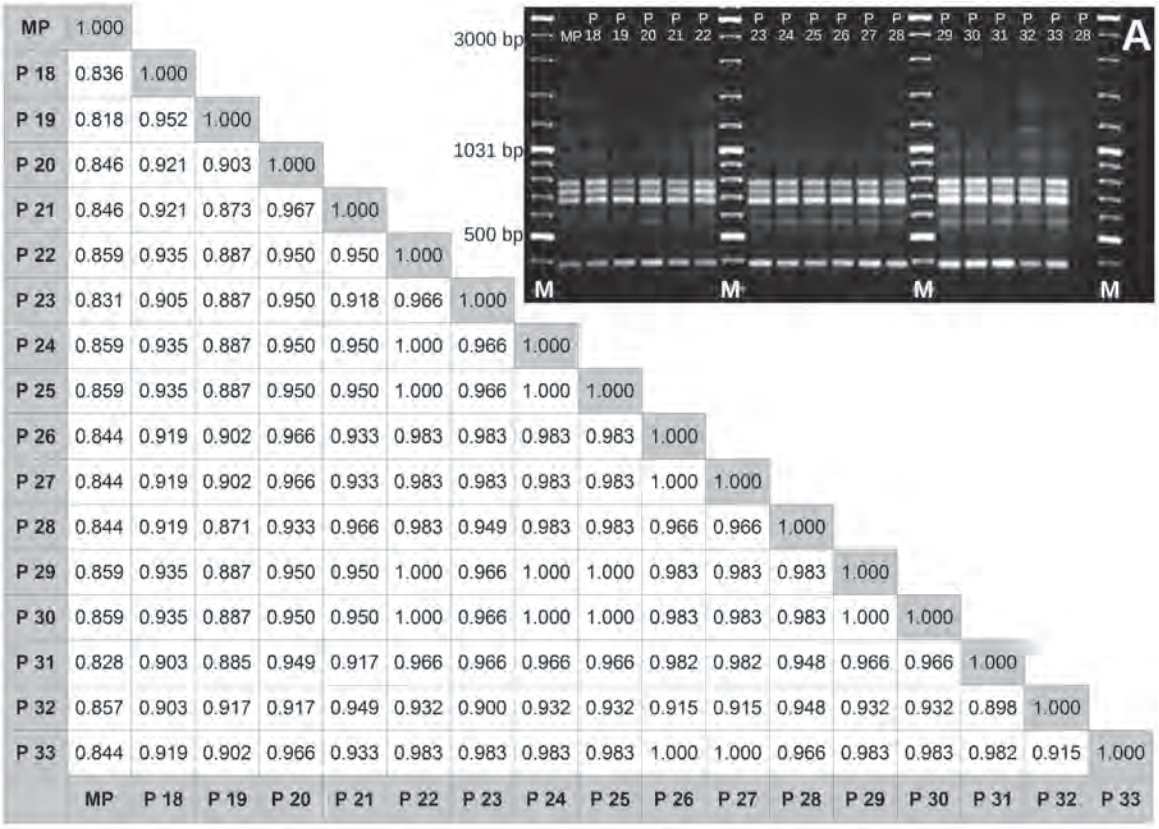
DNA ISOLATION, MOLECULAR IDENTIFICATION OF MOTHER PLANT AND RAPD ANALYSIS

BLAST search of our newly generated 918 bp sequence of exons 23, 24 and intervening intron of nuclear *RBP2-I* gene originating from the mother donor plant revealed 99% similarity both to *R. tomentosum* (voucher RSF 99/225 (WTU 357165) collected in South Siberia, Russia) and *R. hypoleucum* (voucher RSF 98/702 (WTU 357226) from Japan), which are sister taxa with high statistical support in Goetsch et al. study (Goetsch et al., 2005). Detailed comparison of the sequence to 88 *Rhododendron* accessions showed that the mother donor plant used in the present study shared specific substitutions and deletions at the following nucleotide positions: at 7356 Adenine not Guanine, at 7360 Guanine not Adenine, at 8063–8065 3 nt deletion, 8090 Cytosine not Guanine, and 8204 Guanine not Cytosine (coordinates from the TreeBASE No. M2277 alignment by Goetsch et al. (2005)). Since *R. hypoleucum* distribution is limited to Japan and Russia (Gibbs et al., 2011), this analysis together with morphological examination, confirmed taxonomic identity of the starting material as *R. tomentosum*.

RAPD analysis with 11 decamer primers selected out of 60 produced 71 (54 monomorphic and 17 polymorphic) clear scoreable bands that gave 6.4 bands per primer with size range from 200 up to 2500 bp (Table 7). Jaccard similarity coefficients between the donor mother plant and 16 randomly selected plantlets varied from 0.818 to 0.859, while between the regenerants themselves from 0.873 to 1 (Fig. 6). Although those values suggest relatively high genetic similarity, both between the donor mother plant and planlets and between the regenerants themselves, the banding profile showed several polymorphic bands, with 4 occurring solely in the mother plant (primers RothC11, RothC8 and RAPD5). For the remaining primers polymorphic bands occurred among the regenerants, but never exceeding 2 band per individual. Such slight genetic variation can be treated as a natural phenomenon or

TABLE 7. List of RAPD primers, their sequences, number of bands and size of amplified fragments generated for *R. tomentosum* mother donor plant and 16 randomly selected regenerants.

Primer name	Sequence	No of bands (M, P [1])	Range of fragments (bp)
RothC2	5' GTGAGGCGTC 3'	10 (7 M, 3 P)	200–1500
RothC8	5' TGGACCGGTG 3'	3 (2M, 1 P)	400–1200
RothC11	5' AAAGCTGCGG 3'	7 (5 M, 2 P)	300–1200
RothC20	5' ACTTCGCCAC 3'	8 (5 M, 3 P)	300–1200
RH04	5' GAAACACCCC 3'	9 (6 M, 3 P)	700–2500
RH08	5' GGAAGTCGCC 3'	5 (5 M)	400–1200
RO20	5' ACACACGCTG 3'	6 (4 M, 2 P)	600–2000
RAPD5	5' GGGTAACGCC 3'	3 (2 M, 1P)	700–1200
RAPD6	5' GTGATCGCAG 3'	7 (6 M, 1 P)	600–1500
RAPD9	5' CAGCACCCAC 3'	9 (8 M, 1 P)	300–1500
RAPD14	5' AGGTGACCGT 3'	4 (4 M)	300–1000



	MP	P 18	P 19	P 20	P 21	P 22	P 23	P 24	P 25	P 26	P 27	P 28	P 29	P 30	P 31	P 32	P 33
MP	1.000																
P 18	0.836	1.000															
P 19	0.818	0.952	1.000														
P 20	0.846	0.921	0.903	1.000													
P 21	0.846	0.921	0.873	0.967	1.000												
P 22	0.859	0.935	0.887	0.950	0.950	1.000											
P 23	0.831	0.905	0.887	0.950	0.918	0.966	1.000										
P 24	0.859	0.935	0.887	0.950	0.950	1.000	0.966	1.000									
P 25	0.859	0.935	0.887	0.950	0.950	1.000	0.966	1.000	1.000								
P 26	0.844	0.919	0.902	0.966	0.933	0.983	0.983	0.983	0.983	1.000							
P 27	0.844	0.919	0.902	0.966	0.933	0.983	0.983	0.983	0.983	1.000	1.000						
P 28	0.844	0.919	0.871	0.933	0.966	0.983	0.949	0.983	0.983	0.966	0.966	1.000					
P 29	0.859	0.935	0.887	0.950	0.950	1.000	0.966	1.000	1.000	0.983	0.983	0.983	1.000				
P 30	0.859	0.935	0.887	0.950	0.950	1.000	0.966	1.000	1.000	0.983	0.983	0.983	1.000	1.000			
P 31	0.828	0.903	0.885	0.949	0.917	0.966	0.966	0.966	0.966	0.982	0.982	0.948	0.966	0.966	1.000		
P 32	0.857	0.903	0.917	0.917	0.949	0.932	0.900	0.932	0.932	0.915	0.915	0.948	0.932	0.932	0.898	1.000	
P 33	0.844	0.919	0.902	0.966	0.933	0.983	0.983	0.983	0.983	1.000	1.000	0.966	0.983	0.983	0.982	0.915	1.000

Fig. 6. Similarity matrices of *R. tomentosum* mother plant (MP) and 16 micropropagated plantlets (P1–P18) based on Jaccard's similarity coeficcient from RAPD data set. (**A**) RAPD products generated from the mother plant and 18 regenerants with RAPD9 marker. Lines: M – DNA size marker, MP – mother plant, P18 – P33 – micropropagated plants of *R. tomentosum*.

could be somaclonal variation induced during micropropagation procedure by various factors (hormones, nutrients, *in vitro* stress), which was previously shown in other studies (Devarumath et al., 2002; Guo et al., 2006).

CONCLUSIONS

The presented work is the first report of complete micropropagation of *R. tomentosum*, an endangered peatland species and a valuable medicinal plant. The established protocol can serve as an effective *ex situ* conservation tool as well as for the production of plant material for further *in vitro* experiments. Molecular biology studies demonstrated a high degree of uniformity between the regenerants and the maternal plants. GC/MS analysis showed that essential oil production was retained in the regenerated plants. However, further studies should be focused on the improvement of volatile oil production, taking into account temperature, light and soil conditions.

AUTHORS' CONTRIBUTIONS

A. Jesionek conducted the *in vitro* research, analyzed the data and wrote the manuscript. A. Kokotkiewicz and P. Wlodarska participated in the experimental work. N. Filipowicz and A. Bogdan carried out molecular analysis of the material. A. Bucinski and R. Ochocka checked and corrected the manuscript. A. Szreniawa-Sztajnert carried out GC analysis, B. Zabiegala developed the analytical method for essential oil determination and analyzed GC/MS data, M. Luczkiewicz conceptualized the study, participated in the experimental work, analyzed the data and edited the manuscript. All the authors read and approved the manuscript in its final form. The authors declare that they have no conflict of interest.

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