Plant Cell Tiss Organ Cult (2017) 131:51–64
DOI 10.1007/s11240-017-1261-0

ORIGINAL ARTICLE

Bioreactor shoot cultures of *Rhododendron tomentosum* (*Ledum palustre*) for a large-scale production of bioactive volatile compounds

Anna Jesionek[1] · Adam Kokotkiewicz[1] · Paulina Wlodarska[1] · Bozena Zabiegala[2] · Adam Bucinski[3] · Maria Luczkiewicz[1]

Received: 16 March 2017 / Accepted: 2 July 2017 / Published online: 12 July 2017

Abstract *Rhododendron tomentosum* Harmaja (*Ledum palustre*), a peat bog plant from Ericaceae family, has been used in traditional medicine as the anti-arthritis agent. Although modern researches confirm its anti-inflammatory properties, it remains threatened by habitat degradation and possibilities to collect this endangered species from its natural environment for further biological activity studies are limited. Therefore, *R. tomentosum* liquid in vitro cultures were established as the alternative source of that valuable plant material. Schenk–Hildebrandt medium with 24.60 µM 2-isopentenyladenine and 592.02 µM adenine provides intensive growth and proper morphology of the obtained microshoots. The *R. tomentosum* biomass was scaled up using the various bioreactors (immersion, temporary immersion and spraying systems) for better growth and improved volatile oil production. The largest biomass accumulation (fresh weight = 250 g l^{-1}, growth index = 280, dry weight = 20 g l^{-1}) and essential oil content (0.5% v/m) were achieved with application of commercially available RITA® bioreactor. GC/MS analysis revealed the high content of *p*-cymene (6.9%), alloaromadendrene (5.5%), shyobunone (8.2%) and ledene oxide (II) (13.0%) in the volatile fraction obtained from RITA® system. The biomass growth parameters and production profile in terms of essential oil and selected terpenoid compounds were determined during the 2 month period. The influence of culture conditions and bioreactor construction on the growth and volatile oil production in *R. tomentosum* biomasses was discussed.

Keywords Bioreactor · Essential oil · GC analysis · *Ledum palustre* · Liquid in vitro cultures · *Rhododendron tomentosum*

Abbreviations

AD	Adenine
BF	Blue filter
DMRT	Duncan's multiple range test
DW	Dry weight
EO	Essential oil
FW	Fresh weight
GC/MS	Gas chromatography/mass spectrometry
Gi	Growth index
IBA	Indole-3-butyric acid
2iP	2-Isopentenyladenine
MV	Magenta™ vessel
PE	Extract from peat
PGR	Plant growth regulator
SGB	Spray glass bioreactor
SH_{Rt}	Modified Schenk and Hildebrandt (medium)
TIGB	Temporary immersion glass bioreactor
TIS	Temporary immersion systems
TDZ	Thidiazuron
TRIA	Triacontanol
ZEA	Zeatin

Communicated by Ming-Tsair Chan.

✉ Maria Luczkiewicz
mluczc@gumed.edu.pl

[1] Department of Pharmacognosy, Medical University of Gdansk, al. gen. J. Hallera 107, 80-416 Gdansk, Poland

[2] Department of Analytical Chemistry, Gdansk University of Technology, ul. Narutowicza 11/12, 80-233 Gdansk, Poland

[3] Department of Biopharmacy, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, ul. dr A. Jurasza 2, 85-089 Bydgoszcz, Poland

Introduction

Rheumatoid arthritis, a chronic joint disease, affects about 1% of the world population (Kumar et al. 2016). The anti-arthritic treatment is frequently based on plant remedies, which are cheaper, less toxic and more available for patients than synthetic pharmaceuticals, but comparably effective (Kumar et al. 2016; Setty and Sigal 2005). One of the groups of phytocompounds modulating the pro-inflammatory signaling pathways are terpenes, the components of essential oils. Their effects on the arachidonic metabolism, cytokines production and pro-inflammatory gene expression were reported (Laev and Salakhutdinov 2015; Miguel 2010).

Rhododendron tomentosum Harmaja (formerly known as *Ledum palustre* L.), an aromatic swamp plant from the family Ericaceae, has been used for ages in folk medicine to treat joint disorders (Dampc and Luczkiewicz 2013). Modern researches confirm anti-inflammatory, antioxidant and analgesic activity of its volatile fraction, indicating its potential as therapeutic agent against arthritis (Ahmad et al. 2013; Baananou et al. 2015; Zhang et al. 2010). However, in central Europe *R. tomentosum* is an endangered species because of wetlands' degradation as well as over-harvesting of above-ground shoots by humans, who traditionally use it as a repellent (Jesionek et al. 2016). A large intraspecific variety of the specimens is an additional problem due to the fact that chemical composition of the essential oil is considerably related to the natural habitat, the phase of the vegetation period and the age of the discussed plant (Dampc and Luczkiewicz 2013). Taking into account the mentioned factors, providing the alternative, constant source of this valuable plant material which produces stable amounts of terpenoids seems to be indispensable to conduct further studies on its medicinal properties.

In our previous work, we established stationary microshoot cultures of *R. tomentosum*, capable of accumulating essential oil (Jesionek et al. 2016). In contrast to other plant metabolites, isolation of the measurable quantity of the volatile fraction for its analysis requires large amount of plant biomass. This was particularly important in case of the obtained in vitro cultures where the synthesis of essential oil in the microshoots decreased threefold in comparison with aged shoots of the maternal plant (Jesionek et al. 2016). Therefore, the objective of the current study was at first to scale up in vitro biomass production and subsequently to obtain the higher essential oil yield. To reach the goal, *R. tomentosum* liquid shoot cultures were established to provide the best growth parameters and afterwards, the various bioreactors (continuous immersion, temporary immersion and spraying systems) were applied. As shown by literature data (Grzegorczyk and Wysokinska 2008; Watt 2012), liquid cultures have a better access to nutrients and growth regulators compared with stationary ones, which may result in higher production of primary and secondary metabolites, including essential oils. Moreover, the agitated liquid culture serves as a preparatory step for introducing microshoots to large-scale installations, allowing to investigate the influence of the miscellaneous cultivation factors on biomass accumulation (Grzegorczyk and Wysokinska 2008). According to many reports, the controlled microenvironment, in terms of nutrients and gas transfer in bioreactors, is associated with higher multiplication rate, acceleration of biomass growth and improvement of secondary metabolites productivity (Georgiev et al. 2014; Jang et al. 2016; Paek et al. 2005; Scheidt et al. 2009; Yan et al. 2011; Zobayed et al. 2004). However, it should be noted that so far there are only a few articles concerning essential oil accumulation in bioreactor-grown microshoots (Fulzele et al. 1995; Hilton et al. 1995; Tisserat and Vaughn 2008).

Materials and methods

Reagents and general procedures

All reagents used for plant in vitro culture experiments were from Sigma-Aldrich (St. Louis, US-MO). Water was purified with the Elix/Synergy system (Merck KGaA, Darmstadt, Germany). Unless otherwise stated, in vitro cultures were incubated at $24 \pm 2\,^{\circ}C$ under white fluorescent light (16/24 h photoperiod, 40 µmol m^{-2} s^{-1}, TLD 35 W/33 tubes, Philips, Amsterdam, the Netherlands).

Agitated cultures

Initiation of agitated culture

The plant material obtained at the elongation stage of the micropropagation protocol of *R. tomentosum* (Jesionek et al. 2016) after 28 day growth on the stationary Schenk-Hildebrandt medium (Schenk and Hildebrandt 1972) modified with the addition of 0.5 g l^{-1} ammonium nitrate (SH_{Rt}) and supplemented with 24.60 µM 2-isopentenyladenine (2iP), was used for the liquid culture initiation. 3 g microshoots were transferred to 250 ml Erlenmeyer flasks closed with silicone foam stoppers (Carl-Roth, Karlsruhe, Germany) which contained 30 ml liquid medium with the same composition, but excluding agar. The shoot culture was agitated using the orbital shaker (120 rpm, 25.4 mm stroke, INNOVA 2300, New Brunswick Scientific, Enfield, CT, USA) and subcultured at 4-week intervals for 3 months prior to further experiments.

Optimization of culture conditions

For optimization of the culture medium composition, 3 g microshoots taken on 28 day of growth cycle from the initial agitated liquid culture were immersed in 250 ml Erlenmeyer flasks in 30 ml SH_{Rt} medium supplemented with different plant growth regulators (PGRs) (Table 1a). 2iP, indole-3-butyric acid (IBA), thidiazuron (TDZ) and adenine (AD) were added to the growth medium prior to autoclaving while zeatin (ZEA) and triacontanol (TRIA) were sterile-filtered (syringe filters, pore diameter 0.2 µm, Cronus, Maisemore, UK). Due to the limited solubility in water, TRIA was first dissolved in stock solution containing chloroform and Tween 20 and then diluted with water, according to the procedure described by Tantos et al. (2001). The pH of the studied media was adjusted to 5.6.

Further modifications of cultivation conditions referred to SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD (Table 1b). The spectrum of daylight was modified by covering the Erlenmeyer flasks with the foil bluelight filter (BF) (119 dark blue, ca. 3% visible light transmission, Lee Filtres, Andover, UK). Furthermore, the mineral content of SH_{Rt} medium was changed by replacement of the pure water with the aqueous peat extract (peat: aqua 1:5), commercial one (PE) (garden peat AB, Hollas, Paslek, Poland) as well as the extract from the peat collected at the maternal plant's natural habitat in Miszewko near Gdansk in Poland (PE_{NH}). One more modification was to adjust the pH of the liquid medium to 5.0 which is natural for PE.

The microshoots from the PGR-free SH_{Rt} medium as well as from the initial agitated liquid culture (SH_{Rt} medium with 24.60 µM 2iP) were the controls during the PGR optimization experiments while for the culture conditions study the plant material from SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD served as the control group. Each experiment was conducted with three replicates. On the 28th day of the growth cycle the plant material was harvested and the growth parameters (fresh weight (FW), growth index (Gi), dry weight (DW)) as well as microshoots' morphology (length, colour, vitality) were estimated (Table 1).

Table 1 The effect of various modifications of liquid SH_{Rt} medium composition in terms of PGRs (**a**), the application of BF as well as the change of pH and enrichment with PE (**b**) on the growth parameters and morphology of *R. tomentosum* microshoots after 28 days' cultivation in Erlenmeyer flasks

PGRs (µM)	Other modifications	FW (g l^{-1})[a]	Gi[a]	DW (g l^{-1})[a]	Microshoots' length (mm)[b]	Microshoots' morphology
(a)						
–	–	204.16a ± 14.05	99.36a ± 18.98	16.33a ± 1.12	10–25	Browning shoots with callus and some roots
2iP (24.60)	–	212.72a ± 16.41	104.03ab ± 11.12	17.01a ± 1.31	15–30	Green, juvenile
2iP (49.20)	–	207.52a ± 16.86	106.03ab ± 10.86	16.60a ± 1.34	10–25	With browning changes at the base
2iP (24.60) + IBA (4.92)	–	225.61ab ± 24.99	121.59abc ± 18.20	18.04ab ± 1.99	15–25	Green, juvenile, strongly vertical
2iP (24.60) + TDZ (1.00)	–	246.30b ± 21.10	128.46bd ± 18.09	19.70bc ± 1.68	10–30	Procumbent with browning changes
2iP (24.60) + TRIA (0.02)	–	221.62ab ± 22.15	122.69abc ± 19.85	18.34ab ± 0.87	10–25	Green, short
2iP (24.60) + ZEA (13.68)	–	245.03b ± 12.80	129.92bd ± 10.29	17.97ab ± 0.27	15–30	Green, with callus
2iP (24.60) + AD (592.02)	–	251.15b ± 26.50	140.91cd ± 25.75	19.50bc ± 1.12	20–30	Vivid green, juvenile, dense, strongly vertical
(b)						
2iP (24.60) + AD (592.02)	BF	221.87ab ± 9.91	112.44ab ± 1.73	18.46ac ± 0.09	20–40	Long, fragile, pale
2iP (24.60) + AD (592.02)	PE_{NH}	251.64b ± 3.98	150.47cd ± 16.17	20.08bc ± 0.03	20–45	Vivid green, juvenile, dense, strongly vertical
2iP (24.60) + AD (592.02)	PE	248.66b ± 5.92	150.56cd ± 6.36	20.60c ± 0.03	25–45	Vivid green, juvenile, dense, strongly vertical
2iP (24.60) + AD (592.02)	PE[c]	250.83b ± 6.94	152.63d ± 2.94	20.06bc ± 0.55	25–45	Vivid green, juvenile, dense, strongly vertical

[a]The data represent the mean (±SD) of three replicates; values followed by various letters are significantly different at $p < 0.05$ (one way ANOVA, Duncan's Multiple Test)

[b]The values indicate the length range the measured microshoots fall into

[c]pH of the medium was maintained 5.0 instead of 5.6

Optimization of the origin and quantity of plant inoculum

To assess the impact of the origin (agar-gelled or agitated cultures) and the quantity of plant inoculum on *R. tomentosum* biomass growth, 2 and 3 g microshoots from stationary and liquid SH_{Rt} media supplemented with 24.60 µM 2iP were immersed in 30 ml liquid SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD and shaken in Erlenmeyer flasks (Table 2). The inoculum [g]/liquid medium [ml] ratio was 1:15 or 1:10, respectively.

On the 28th day of the growth cycle the plant material was collected and the growth parameters (FW, Gi, DW) were compared (Table 2). Each experiment was conducted with three replicates.

Bioreactor systems

Five experimental in vitro culture systems were compared: (I) continuous immersion system: Magenta™ vessel GA-7 (MV) (Sigma-Aldrich, St. Louis, US-MO) modified with a stainless steel net (1 × 1 mm) at 1 cm above the bottom (Fig. 1c); (II) temporary immersion systems (TIS): (a) RITA® bioreactor (Vitropic, St. Mathieu de Treviers, France) (Georgiev et al. 2014; Watt 2012) (Fig. 1d), (b) PLANTFORM bioreactor (Plant Form AB, Sweden & TC propagation Ltd., Ireland) (Welander et al. 2014) (Fig. 1e), (c) temporary immersion glass bioreactor (TIGB) (Fig. 1f); (III) spray glass bioreactor (SGB) (according to Jaremicz et al. 2014, with modifications) (Fig. 1g). Systems I, IIa i IIb are commercially available while IIc and III are assembled by the authors of the paper (Fig. 2). The TIGB (Fig. 2b, c) and SGB (Fig. 2d) consisted of a cylindrical glass vessel (length 20 cm, diameter 12 cm), equipped with sterile air supply (Fig. 2a) and stainless steel net (1 × 1 mm) for microshoots immobilisation. Additionally, SGB was connected with a peristaltic pump (MasterFlex L/S, Cole-Parmer, Vernon Hills, USA). All bioreactors were steam sterilized (121 °C, 0.1 MPa, 22 min).

In all systems, *R. tomentosum* microshoots grown on stationary SH_{Rt} medium with 24.60 µM 2iP for 28 days (Fig. 1a) were inoculated into liquid SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD at 1:15 (m/v) ratio. All TIS and spray bioreactor were attached to an air humidifier and an air pump (IPX4 ACO-9602, Hailea, China) via a filter disc (diameter 60 mm, pore diameter 0.22 µm, Cole Parmer, US-IL). Additionally, SGB was connected with a peristaltic pump (MasterFlex L/S, Cole-Parmer, Vernon Hills, USA). The microshoots in MV were constantly immersed in the medium while immersion in TIS took place every 85 min for 5 min (air flow 0.5 l min^{-1}). The same cycle was maintained for SGB (medium dispersion rate 100 ml min^{-1}), using the 1.5 size TN-type hydraulic nozzle (Spraying Systems Co, Wheaton, US-IL).

The microshoots from the agitated liquid culture were used as the control (Fig. 1b). In the control group, *R. tomentosum* microshoots grown on stationary SH_{Rt} medium with 24.60 µM 2iP for 28 days (Fig. 1a) were inoculated into Erlenmeyer flask containing liquid SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD at 1:15 (m/v) ratio.

Each experiment was conducted with three replicates. After 28 days, the growth parameters were evaluated (FW, Gi, DW) (Fig. 3), the content of the essential oil was determined in the dried biomasses (Fig. 4) and the obtained volatile fractions were subjected to GC/MS analysis (Table 3).

Biomass growth and essential oil production profile in RITA® bioreactor compared with agitated culture

For *R. tomentosum* microshoot culture maintained in RITA® bioreactor, the biomass growth and the essential oil production profile were determined in a 63 day experiment. 13.3 g microshoots from the stationary agar culture (SH_{Rt} medium with 24.60 µM 2iP, 28 day of cultivation) were transferred to RITA® bioreactor and immersed every 85 min for 5 min in 200 ml liquid SH_{Rt} medium supplemented with 24.60 µM 2iP and 592.02 µM AD. The biomass was collected at 7 days intervals after total unloading

Table 2 The effect of the origin and the quantity of plant inoculum on the growth parameters of *R. tomentosum* microshoots after 28 days' cultivation in agitated Erlenmayer flasks, in liquid SH_{Rt} medium supplemented with 24.60 µM 2iP and 592.02 µM AD

Origin of inoculum	Inoculum [g] /liquid medium [ml] ratio	FW (g l^{-1})[c]	Gi[c]	DW (g l^{-1})[c]
Stationary[a]	1:10	254.21a ± 10.77	163.41a ± 10.18	20.10a ± 0.89
	1:15	272.52a ± 12.30	323.64b ± 7.80	18.34ab ± 1.09
Liquid[b]	1:10	251.15a ± 26.50	140.91a ± 25.75	19.50a ± 1.12
	1:15	224.65a ± 23.31	165.47a ± 13.17	16.78b ± 1.35

[a]Inoculum from stationary SH_{Rt} medium with 24.60 µM 2iP after 28 days' cultivation

[b]Inoculum from liquid SH_{Rt} medium with 24.60 µM 2iP after 28 days' cultivation

[c]The data represent the mean (±SD) of three replicates; values followed by various letters are significantly different at $p < 0.05$ (one- way ANOVA, Duncan's Multiple Test)

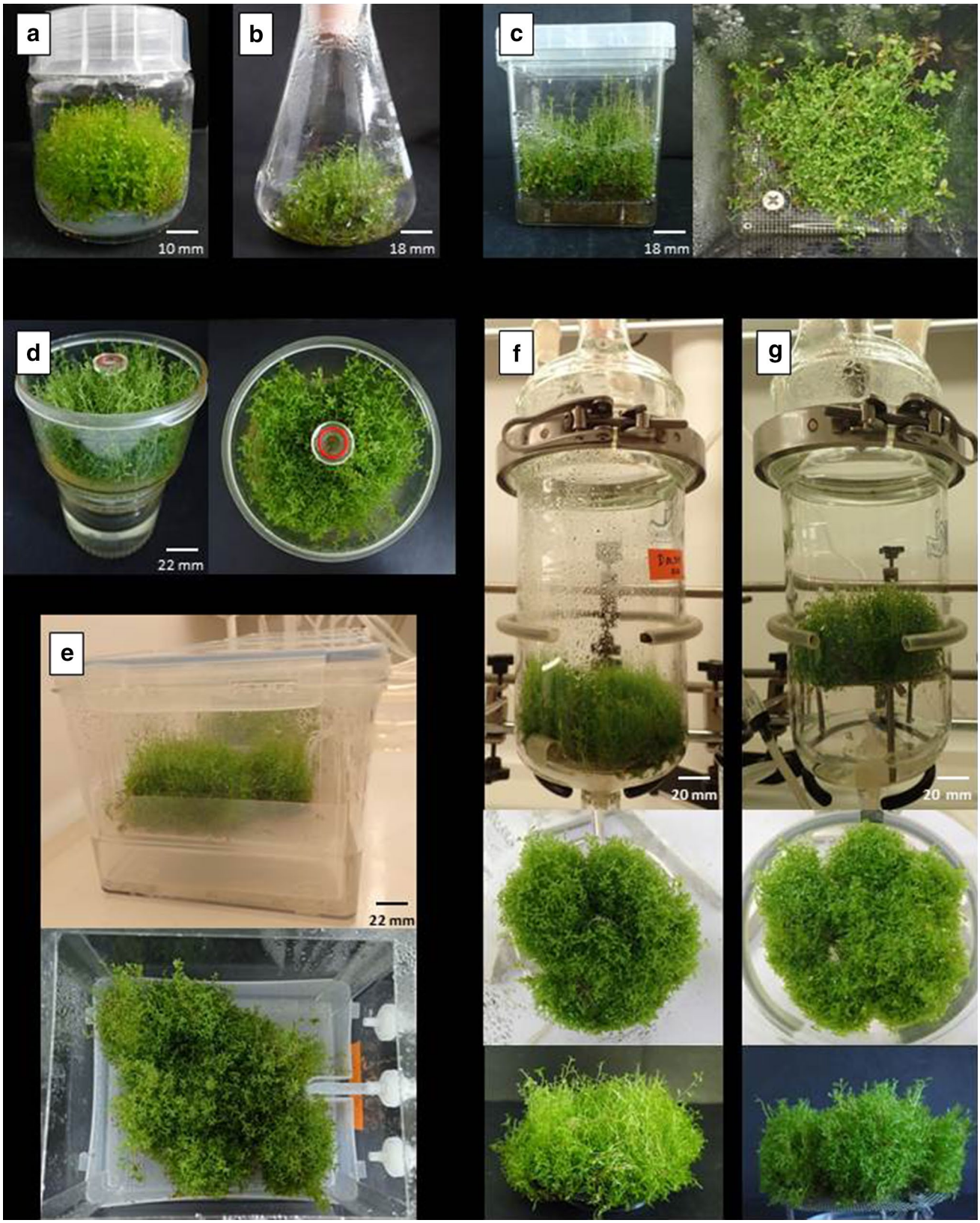


Fig. 1 *Rhododendron tomentosum* microshoots in the studied in vitro systems, after 28 days of cultivation: on agar-gelled SH_{Rt} medium supplemented with 24.6 µM 2iP: **a** baby food culture jar (plant inoculum); on liquid SH_{Rt} medium supplemented with 24.6 µM 2iP and 592.02 µM AD: **b** Erlenmeyer flask; **c** Magenta™ vessel GA-7; **d** RITA® bioreactor; **e** PLANTFORM bioreactor; **f** TIGB; **g** SGB

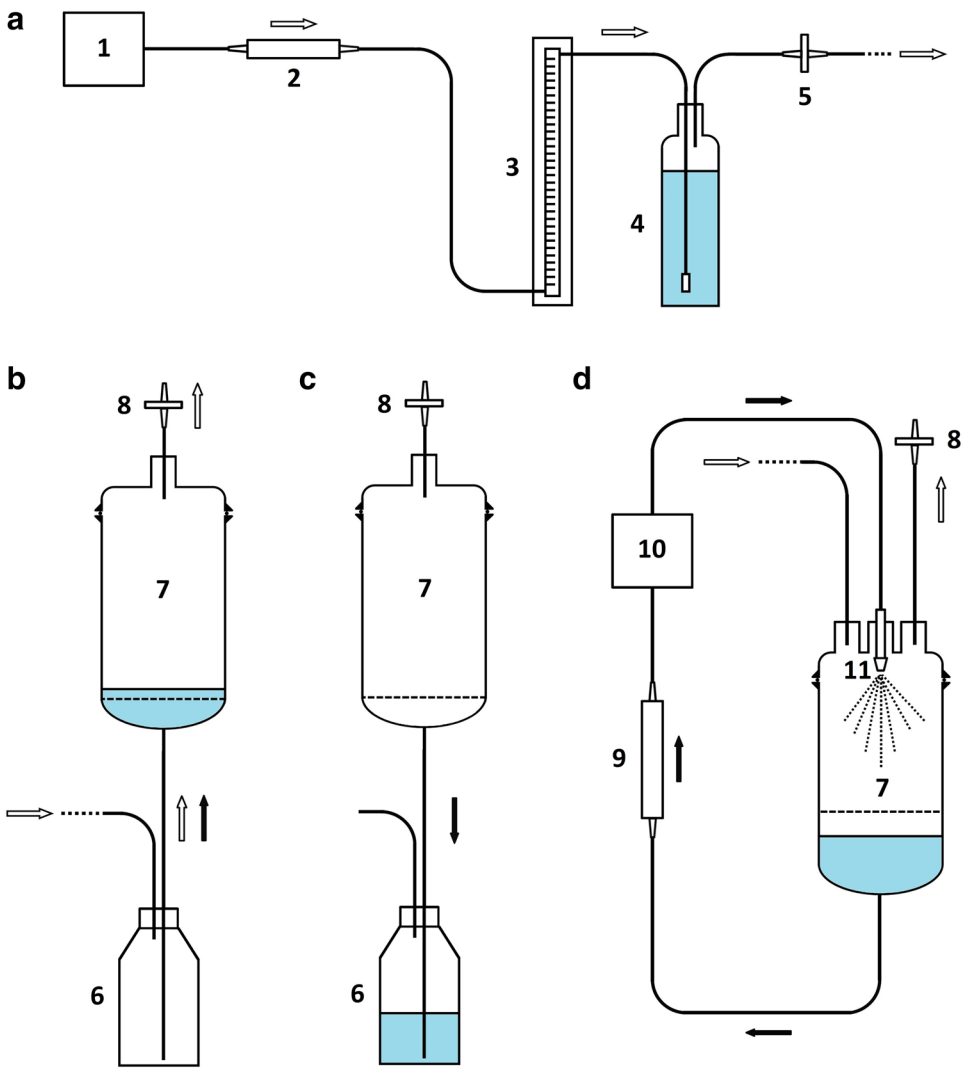


Fig. 2 Schematic diagrams of custom-made bioreactor systems used in the study: **a** air supply (common for all systems), **b** TIGB (immersion phase), **c** TIGB (medium withdrawal phase), **d** SGB; *1* air pump, *2* air prefilter, *3* flowmeter, *4* bottle washer (air humidifier), *5* inlet PTFE filter (0.22 µm), *6* medium reservoir, *7* growth vessel with stainless steel mesh support, *8* outlet PTFE filter (0.22 µm), *9* polypropylene fiber filter, *10* peristaltic pump, *11* spray nozzle. *White* and *black arrows* indicate air and growth medium flow, respectively

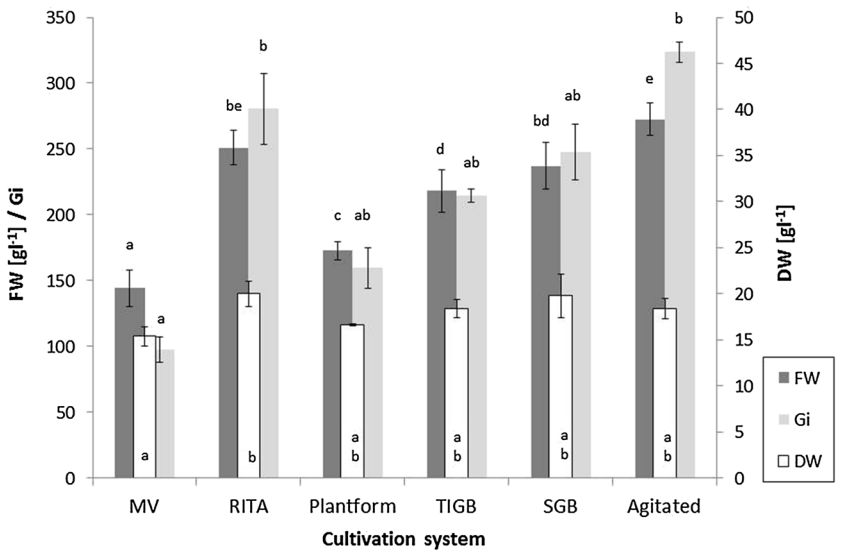


Fig. 3 Growth parameters of *R. tomentosum* microshoots, cultivated in the studied bioreactor systems for 28 days. Values are the means of three replicates; data followed by *different letters* are significantly different at $p < 0.05$ (FW–DMRT, Gi and DW–Dunn's test)

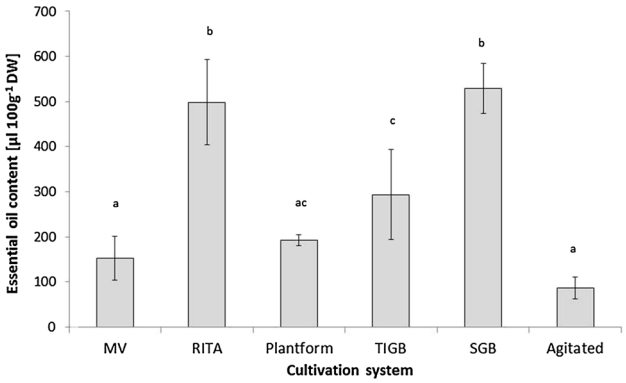


Fig. 4 Essential oil content in *R. tomentosum* biomasses obtained in the studied bioreactor systems, after 28 days of cultivation. Values are the means of three replicates; data followed by *different letters* are significantly different at $p < 0.05$ (DMRT)

of the culture vessel to determine the growth parameters (FW, Gi, DW) and to establish the volatile fraction content (starting from 14 day of the growth cycle). The obtained results were compared with the microshoots from the agitated liquid culture where 2 g inoculum from the stationary agar culture (SH_{Rt} medium with 24.60 µM 2iP, 28 day of cultivation) were immersed in 30 ml SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD in Erlenmeyer flasks (Figs. 5, 6). For *R. tomentosum* agitated culture, the biomass was collected also at 7 days intervals during 63 days, but the essential oil content was determined only in 28 day of cultivation. Each experiment was conducted with three replicates. The GC/MS analysis of volatiles was performed (Fig. 7).

Determination of growth parameters

Fresh weight (FW) was measured by weighing the harvested plant material after cleaning it from the liquid medium. Gi was calculated with the formula: $Gi = (FW_x - FW_0)/FW_0 \times 100$ where Gi is the growth index and FW_0 and FW_x is the fresh weight of the inoculum and the fresh weight of the microshoots after x days of cultivation (usually 28 days unless otherwise stated), respectively. The obtained biomasses were dried for 24 h at 30 °C in the drying chamber with forced convection (FD 115, Binder, Tuttingen, Germany) to determine dry weight (DW).

Determination of essential oil content

For the determination of volatile fraction content, the dried biomasses obtained in the examined bioreactors and in RITA® system during the essential oil production profile study, as well as the microshoots from agitated liquid culture grown for 28 days in the optimized SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD were subjected to hydrodistillation (5 g, 100 ml of distilled water, 3 h) in the Deryng apparatus (Polish Pharmacopoeia 2002). The isolated volatile fractions were collected in 1.0 ml of hexane:diethyl ether mixture (1:1 v/v) (Butkienė et al. 2008) which was afterwards evaporated from the samples under a stream of nitrogen gas. The essential oil content in the studied plant materials was presented as a mean value from the results of three hydrodistillations (Figs. 4, 6).

GC/MS analysis of essential oils

For GC/MS analysis, Gas Chromatograph 7890A coupled with Mass Selective Detector 5977A (Agilent Technologies, Santa Clara, US-CA) was used. The essential oils obtained from *R. tomentosum* in vitro cultures were diluted with ethyl acetate (1:80) and 1.0 µl of the prepared volatile fractions solutions was introduced on the DB-5ms 30 m × 0.25 mm × 0.25 µm capillary column (Agilent J&W) with Agilent Autosampler Systems 7693 (a split ratio 1:10, the injector temperature 250 °C). The carrier gas was helium and the constant flow rate was applied (1.1 ml min^{-1}). The oven temperature increased 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 GC/MS analysis results were compared with the data from NIST Library 11.0. and are presented in Table 3 and Fig. 7 as a mean values from two replicates of the experiment.

Statistical analysis

The collected data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT, $p < 0.05$) or Dunn's Test ($p < 0.05$), when equal variance test failed. The analyses were conducted with SigmaPlot 11.00 (Systat Software, San Jose, US-CA).

Results and discussion

Agitated cultures

In this study the *R. tomentosum* agitated liquid cultures were established from microshoot clusters growing on stationary SH_{Rt} medium with 24.60 µM 2iP by their immersion in the medium with the same composition, excluding agar. As shown by previous research (Jesionek et al. 2016), the SH_{Rt} medium enriched with 24.60 µM 2iP gave the best results in multiplication and elongation of *R. tomentosum* microshoots, providing their high vitality. However, the growth parameters obtained in the initial agitated liquid culture were too low to receive sufficiently high amount of plant material for essential oil

Table 3 The chemical composition of *R. tomentosum* essential oils obtained from various in vitro bioreactor systems and the agitated culture after 28 days' cultivation in liquid SH_{Rt} medium supplemented with 24.60 µM 2iP and 592.02 µM AD

Compound	RT	RI		(%) Content in the essential oils[a]					
				MV	TIS			SGB	Agitated liquid culture
		NIST	Study		RITA	Plantform	TIGB		
Thujene	5.69	924	920	–	0.3	0.2	0.3	–	0.2
α-Pinene	5.83	931	929	–	0.6	0.5	0.5	–	0.3
Camphene	6.13	947	946	–	0.2	0.1	0.1	–	–
Sabinene	6.55	969	971	0.3	1.7	1.5	1.7	5.1	1.1
β-Pinene	6.64	975	976	–	0.7	0.6	0.6	1.5	0.6
β-Myrcene	6.83	988	987	–	0.5	0.4	0.5	0.9	0.4
δ-2-Carene	7.38	996	1017	4.5	1.9	0.6	0.8	3.9	0.3
p-Cymene	7.53	1025	1027	0.9	6.9	5.6	5.5	17.9	7.8
β-Phellandrene	7.65	1031	1031	–	0.9	0.7	0.8	1.6	0.3
(Z)β-Ocimene	7.75	1034	1036	–	0.2	–	0.1	–	–
(E)β-Ocimene	7.96	1044	1047	0.1	0.3	0.2	0.2	0.5	0.1
γ-Terpinene	8.22	1059	1060	1.3	0.4	0.1	0.2	0.7	–
α-Terpinolene	8.83	1078	1092	–	0.4	0.3	0.3	–	0.3
Terpinen-4-ol	10.66	1177	1153	0.1	1.0	0.9	0.9	0.5	0.5
p-Cymen-8-ol	10.78	1187	1188	–	0.9	0.8	0.9	–	0.7
α-Terpineol	10.91	1190	1195	–	0.2	0.2	0.3	–	0.1
γ-Terpineol	11.91	–	1245	–	1.2	0.6	0.5	2.2	0.4
Geraniol	12.10	1256	1255	–	0.3	0.2	0.3	–	0.1
Piperitone	12.20	1228	1259	–	0.3	0.3	0.4	–	0.3
Bornyl acetate	12.82	1292	1291	0.2	1.9	1.9	1.7	0.2	1.7
Carvacrol	13.08	1299	1307	–	–	–	–	–	0.3
Citronellyl acetate	14.00	1331	1353	–	0.8	0.6	0.7	0.4	0.6
Geranyl acetate	14.58	1354	1384	1.1	2.4	1.9	2.2	6.4	2.4
β-Elemene	14.87	1398	1400	0.2	0.6	0.6	0.6	–	0.5
α-Gurgujene	15.25	1419	1421	–	0.2	0.2	0.2	–	0.1
Caryophyllene	15.44	1419	1431	–	0.2	0.2	0.2	–	0.2
β-Farnesene	15.94	1429	1459	–	0.7	0.9	0.6	0.6	0.9
Humulene	16.06	1455	1466	0.1	0.3	0.3	0.3	0.4	0.3
Alloaromadendrene	16.23	1462	1475	8.1	5.5	5.5	4.4	2.9	5.1
γ-Muurolene	16.42	1477	1486	–	0.2	0.2	–	–	0.2
Germacrene D	16.54	1482	1492	0.1	0.1	–	0.1	–	0.1
6-Epishyobunone	16.73	–	1504	7.3	4.3	4.1	3.9	3.7	4.4
α-Muurolene	16.83	1499	1509	1.0	0.5	0.6	0.5	0.3	0.5
Shyobunone	17.11	1510	1527	15.8	8.2	8.1	7.7	8.6	8.1
δ-Cadinene	17.23	1525	1533	3.1	4.1	3.0	2.5	3.8	2.1
Palustrol	18.06	1562	1582	–	0.3	0.2	0.1	–	0.1
Germacren D-4-ol	18.16	1576	1588	1.5	0.3	0.2	0.3	–	0.3
Methyl everninate	18.27	1583	1594	3.5	4.3	3.3	3.6	7.0	2.8
Ledene oxide (II)[b]	18.74	1682	1623	14.7	13.0	9.5	9.0	9.0	10.2
Dehydroxy-isocalamendiol	19.12	1616	1646	0.9	1.1	1.1	1.1	–	2.9
τ-Cadinol	19.23	1640	1654	2.9	2.6	3.0	2.3	–	1.9
epi-α-Murrolol	19.29	1644	1657	–	–	0.3	0.5	_	0.5
α-Cadinol	19.46	1654	1667	3.6	2.9	3.7	3.1	2.8	2.4
Isolongifolol	19.88	1712	1694	–	0.4	0.4	0.4	–	–
Cyclocolorenone	21.06	1744	1771	–	0.4	0.5	0.4	–	–
Total (%)	–	–	–	70.9	72.6	62.4	59.4	79.9	60.3
Monoterpene hydrocarbons	–	–	–	7.3	14.6	10.4	11.1	31.9	10.9
Oxygenated monoterpenes	–	–	–	1.4	8.7	7.2	7.5	9.5	6.8

Table 3 (continued)

Compound	RT	RI		(%) Content in the essential oils[a]					
				MV	TIS			SGB	Agitated liquid culture
		NIST	Study		RITA	Plantform	TIGB		
Sesquiterpene hydrocarbons	–	–	–	4.3	5.8	4.9	3.9	4.9	4.0
Oxygenated sesquiterpenes	–	–	–	35.4	24.4	24.4	23.1	21.9	23.3
Sesquiterpenes with aromadendrane skeleton	–	–	–	22.8	19.2	15.8	13.9	11.9	15.5

Data by GC/MS

[a]The data represent the means of two replicates

[b]Not clearly confirmed

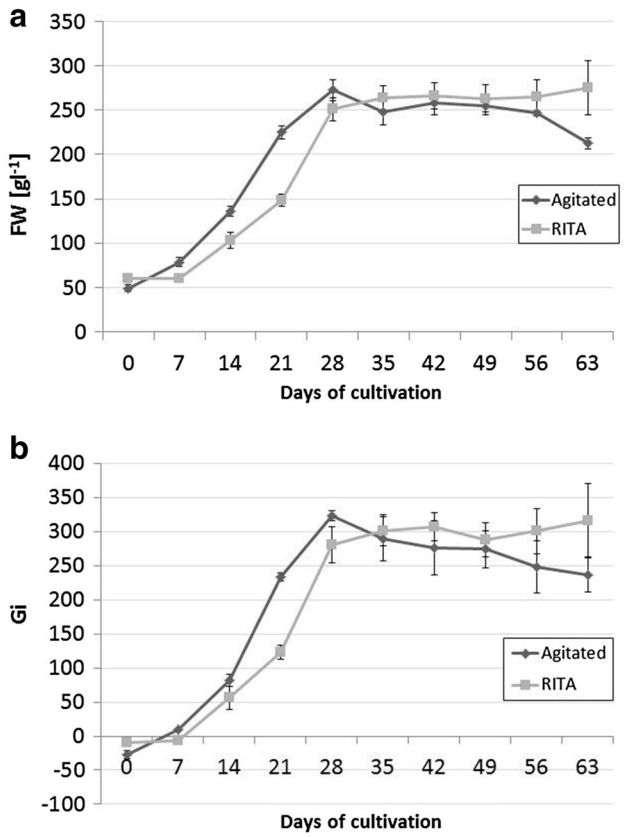


Fig. 5 Growth profile of *R. tomentosum* microshoots, cultivated in RITA® bioreactor, in comparison with the agitated liquid culture, during 63 days of cultivation. Values are the means of three replicates

extraction (Table 1). Therefore, attempts were undertaken to stimulate growth of the studied biomass by addition of other PGRs to the medium, simultaneously maintaining the content of 2iP as the basic cytokinin responsible for the proper microshoots' morphology.

The PGRs chosen for the experiment included 2iP at doubled concentration, AD, ZEA, TDZ, TRIA and IBA (Table 1a). 2iP is widely applied to control the growth of *Rhododendron* sp. (Eeckhaut et al. 2010; Mao et al. 2011; Tomsone and Gertnere 2003) whereas AD is the constituent of Anderson's medium which is commonly used in the micropropagation of the mentioned genus (Anderson 1978). According to the literature data, addition of ZEA as PGR usually intensifies shoots proliferation in Ericaceae family (Almeida et al. 2005; Eeckhaut et al. 2010). Small amounts of TDZ and TRIA are known to increase multiplication rate, and thus are frequently used in woody plant cultures (Guo et al. 2011; Huetteman and Preece 1993; Tomsone and Gertnere 2003; Tantos et al. 2001). Auxins, like IBA, are usually applied in combination with cytokinins to balance their effects and to elongate microshoots (Almeida et al. 2005; Mao et al. 2011; Tomsone and Gertnere 2003; Vejsadova 2008).

As presented in Table 1a, the enrichment of SH_{Rt} medium with the mixture of 2iP and above-mentioned PGRs improves growth parameters of the *R. tomentosum* liquid shoot culture. The highest values of FW, Gi and DW were determined for AD, ZEA and TDZ supplementation. However, TDZ caused undesirable adverse changes in the microshoots' morphology. Taking into account the comparable effects of AD and ZEA, the first one seems to be more suitable for large-scale cultivation systems due to its much lower price and the lack of callus-inducing effect. Surprisingly, the addition of IBA did not influence the length of microshoots. It is also worth noting that doubling 2iP concentration from 24.60 to 49.20 µM did not correlate with the biomass accumulation. It is in accordance with the research of Mao et al. (2011) which indicated that 2iP increased the number of *Rhododendron* sp. shoots only at concentrations up to 40 µM, whereas concentrations over 60 µM inhibited shoot formation.

After the selection of the most beneficial PGR composition for the *R. tomentosum* liquid culture, further modifications in the cultivation conditions were made (Table 1b). Based on the reports that blue light promotes microshoots formation (Rout et al. 2000), the daylight spectrum was changed with the foil bluelight filter, which decreased the light intensity and its range (Table 1b). Although in natural environment *R. tomentosum* prefers shaded forest or

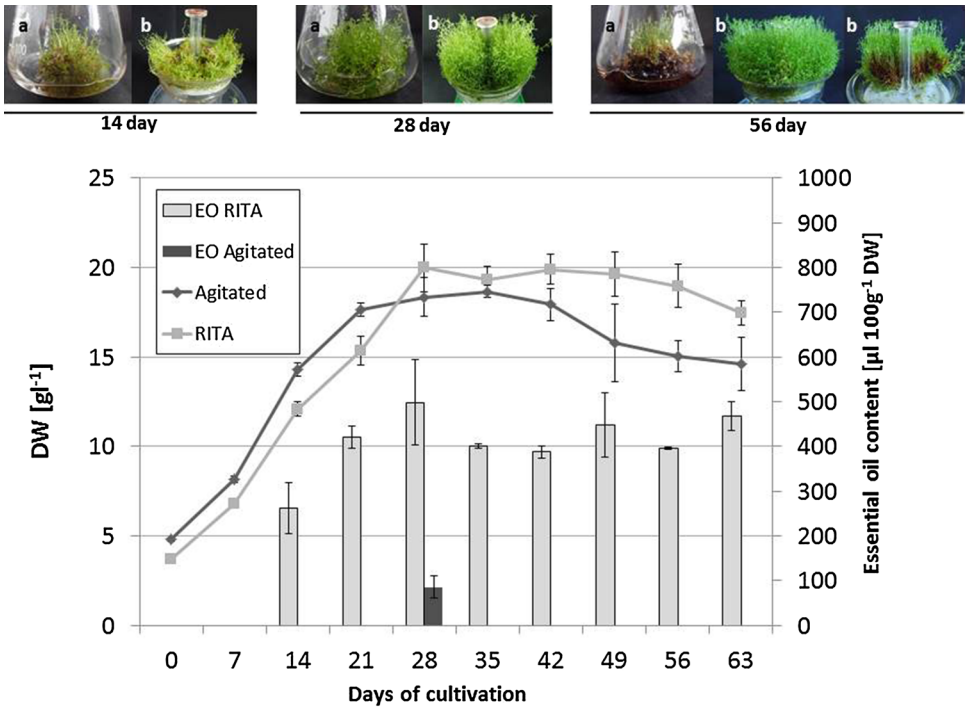
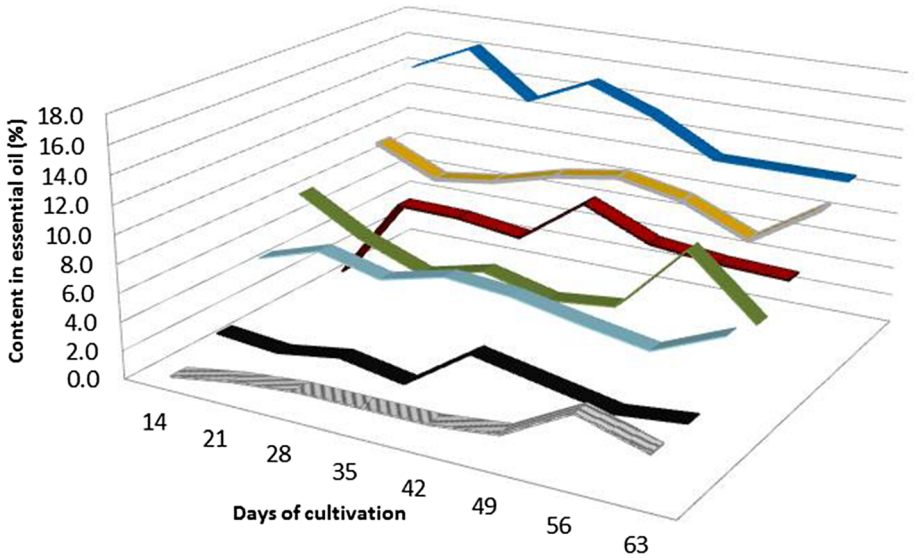


Fig. 6 Dry weight values and the essential oil production profile of *R. tomentosum* microshoots cultivated in RITA® bioreactor (**a**) in comparison with the agitated liquid culture (**b**), during 63 days of cultivation. Values are the means of three replicates



	14	21	28	35	42	49	56	63
γ-terpineol	0.1	0.8	1.2	1.2	1.2	1.7	4.2	2.8
sabinene	1.4	1.0	1.7	0.9	4.0	3.1	2.2	2.7
alloaromadendrene	5.2	6.6	5.5	6.6	6.2	5.3	4.5	6.5
methyl everninate	8.5	6.0	4.3	5.4	4.3	4.8	9.6	5.5
p-cymene	1.1	7.0	6.9	6.3	9.6	7.6	7.1	6.9
shyobunone	9.6	7.6	8.2	9.4	10.1	9.2	7.2	10.2
ledene oxide	14.3	16.3	13.0	15.0	13.3	10.8	10.8	10.8

Fig. 7 Content of the selected terpenoid compounds in the essential oils of *R. tomentosum* microshoots, cultivated in RITA® bioreactor for 63 days. Values are the means of two replicates

wetland habitats (Sellmer et al. 2003), in the liquid culture under low-intensity light the significant reduction in the biomass accumulation was observed.

Furthermore, it is well known that the establishment of *R. tomentosum* field crops is limited by the plant's specific requirements in terms of such soil factors as good drainage, moisture, high organic matter, low pH and sufficient nutrient availability (Sellmer et al. 2003; Shaver 1983). Moreover, the importance of the Ericaceae symbiosis with ericoid mycorrhizal fungi in utilizing mineral compounds as nitrogen and phosphorus is commonly emphasized (Perotto et al. 2002). Therefore, the effect of the supplementation of SH_{Rt} medium with undefined nutrients derived from the aqueous peat extracts was examined, using the horticultural soil (PE) as well as the peat collected from the natural habitat of maternal plant (PE_{NH}), which was additionally regarded as a source of microorganisms and the products of their metabolism, characteristic of the area. The pH of the medium was also decreased to provide more acidic environment, similar to natural conditions (pH 5.0) (Table 1b). The enrichment of the liquid medium with the peat extract improved the Gi parameter by about 7%, regardless of its origin. However, though noteworthy, this result was not statistically significant. For that reason as well as because of the expected difficulties in maintaining constant composition of the peat extract during all stages of the research, the SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD without any alterations to its mineral composition was selected to further experiments. As demonstrated, the change of pH did not influence the growth parameters of in vitro culture (Table 1b).

Finally, the optimal inoculation procedure (with respect to biomass origin and shoot/medium ratio) was specified (Table 2). Although initial immersion of 3 g microshoots in 30 ml liquid SH_{Rt} medium with 24.60 µM 2iP and 592.02 µM AD (1:10) resulted in the similar growth parameters regardless of the biomass origin (agar or liquid shoot culture), reducing the amount of inoculum to 2 g (1:15) led to a different situation. Microshoots taken from the liquid culture showed strong tendency to drown under the medium surface after subculturing, probably as a consequence of the hindered stem verticalization under mechanical stress. In turn, along with lower amount of inocular biomass from stationary culture, Gi of the obtained plant material increased almost twice (from 163.41 to 323.64). The ratio of mass to volume 1:15, using the plant inoculum from agar-gelled culture, was thus selected to further experiments.

Bioreactor systems

For scaling up the *R. tomentosum* shoot culture, the various bioreactor types were used: MV as an continuous immersion system, RITA®, PLANTFORM and TIGB as temporary immersion systems (TIS) and SGB as a gas-phase system (Fig. 1). The largest accumulation of biomass was achieved in RITA® and SGB (FW = 250 and 240 g l^{-1}, Gi = 280 and 250, respectively), probably due to the high moisture content inside the culture vessel (Fig. 3). However, the obtained FW and Gi values were slightly lower than in the microshoots shaken in the Erlenmayer flasks. It can be explained by the tissue hyperhydricity in the liquid agitated culture. This assumption can be confirmed by the DW values obtained in RITA® and SGB bioreactor systems (DW = ca. 20 g l^{-1}) which were the highest from the studied biomasses, even in comparison with the shaken microshoots, presumably thanks to the less hydrated cells (Fig. 3). From the two systems mentioned above, application of RITA® seems to be more profitable for conducting the large-scale studies because it is relatively cheap, light and easy to handle in contrast to expensive, heavy and prone to breakage bioreactors made of glass. Moreover, RITA® containers can be connected in series for scaling up purposes and occupy little space in a growth room.

As many authors demonstrated, TIS, in which microshoots are covered by the capillary medium film after periodic flooding, have positive effect on the proliferation rate, providing the most natural environment, low mechanical stress, improved gas exchange and nutrients transfer (Alvard et al. 1993; Georgiev et al. 2014; McAlister et al. 2005; Zobayed et al. 2004). Notwithstanding, two of the studied TIS had some disadvantages which presumably contributed to their less effectiveness compared to RITA®: largely unused headspace volume despite higher medium consumption per vessel in PLANTFORM bioreactor and the separation of the medium reservoir from the culture vessel in TIGB, which hindered its handling and probably decreased to some extent the level of moisture inside. This was not in the case SGB, where the medium remained on the bottom of the culture vessel and was temporarily sprayed as a mist onto the biomass surface.

MV, in which microshoots were permanently submerged in the liquid medium without forced air supply and lack of mechanical stress, was characterized with the lowest growth parameters, probably due to asphyxia which outweighed the benefits of better tissue-medium contact (as compared to agar culture) (Watad et al. 1996).

The essential oil content in biomasses obtained in RITA® and SGB was clearly higher than in other bioreactor systems and the control groups, reaching 0.5% (v/m) (Fig. 4). This amount was comparable with the volatile fraction content in the aged shoots of maternal plant (Jesionek et al. 2016). It indicates that *R. tomentosum* has great potential to be used in large-scale installations for production of terpenoid compounds. There are few reports which refer to successful accumulation of secondary metabolites in shoot

cultures cultivated in various types of bioreactors: dibenzocyclooctadiene lignans (Szopa et al. 2017), cardiotonic glycosides (Perez-Alonso et al. 2009), flavonoids (wogonin, baicalein, baicalin) (Zobayed et al. 2004), polyphenols (Jain et al. 2012), xanthones and benzophenone derivatives (Kokotkiewicz et al. 2015), indole alkaloids (vindoline, catharanthine) (Yingjin and Zongding 1994), isoquinoline alkaloids (galanthamine) (Georgiev et al. 2012), artemisinin (Liu et al. 2003), phenolics and flavonoids (Jang et al. 2016). However, only few semi-technical sources of volatile oils based on bioreactor-grown microshoots have so far been reported. These include in vitro shoot cultures of *Artemisia annua* maintained in a bubble column bioreactor, *Mentha spicata* microshoots grown in TIS, as well as transformed shoots of *M. piperita* and *M. citrata* in a mist bioreactor (Fulzele et al. 1995; Hilton et al. 1995; Tisserat and Vaughn 2008).

The volatile fractions obtained from the bioreactor-cultured *R. tomentosum* microshoots were characterized with the unique chemical composition (Table 3). About 70% of total compounds were identified. Methyl everninate and ledene oxide (II), revealed in a large amount (2.8–7.0% and 9.0–14.7%, respectively) in the studied in vitro biomasses, were absent in the maternal plant according to previous research (Jesionek et al. 2016). On the other hand, major volatile constituents of the intact plant were either not found (ledol) or confirmed only in trace quantities (palustrol) in the microshoots. It can be assumed that some metabolic pathways of the aromadendrane-related sesquiterpenes were not fully developed at this stage of the biomass cultivation under in vitro conditions. Additionally, it was shown that the essential oils from TIS-cultivated microshoots were characterized with the similar qualitative and quantitative terpenoids content which did not differ significantly from the volatile fraction isolated from the agitated liquid culture. Oxygenated sesquiterpenes (23.1–25.1%) and sesquiterpenes with aromadendrane skeleton (13.9–19.2%) constituted the majority of total compounds. The most abundant terpenes in TIS (>5%) were *p*-cymene, alloaromadendrene, shyobunone and ledene oxide (II). The volatile fractions obtained from immersion and spray systems included two times less compounds (22 and 23, respectively) than TIS (44). Monoterpene hydrocarbons predominated (31.9%) in the SGB-derived essential oil which contained substantial amounts of *p*-cymene (17.9%) and sabinene (5.1%). However, the alloaromadendrene level significantly decreased in SGB-grown shoots. The highest percentage of alloaromadendrene (8.1%), shyobunone (15.7%) and δ-2-carene (4.5%) were observed in MV.

Given the obtained results, RITA® –TIS which stimulated to the largest extent the *R. tomentosum* microshoots growth and production of essential oil rich in potentially bioactive terpenes—was selected for further experiments.

Biomass growth and essential oil production profile

The biomass growth and the essential oil production profiles in RITA® bioreactor were determined during the 2-month cultivation, in parallel to the agitated liquid culture (Figs. 5, 6). The growth profiles of both cultures were similar and were characterized with the presence of the particular development phases. The phase of logarithmic and linear growth lasted until 28 day of cultivation, changing then into plateau phase. Although the growth rate of the agitated liquid culture was faster and both FW and Gi values achieved after 4 weeks were higher than in RITA®, the largest biomass accumulation defined by DW was obtained in TIS (ca. 20 g l^{-1}). This difference was caused probably by the excessive hydration of the shaken microshoots. The above could also contribute to the more visible decay phase in the case of the agitated liquid culture (Fig. 6a), while the clear decrease in DW of in vitro cultures in RITA® was noticed only after 2 months and FW and Gi were still high (Fig. 6b).

The content of the essential oil in RITA® microshoots rised along with the biomass growth till 28 day of cultivation when it reached 500 µl 100 g^{-1} DW, exceeding five times the volatile fraction level in the agitated culture (Fig. 6). Although after 2 months of cultivation DW started to decline, the production of terpenoid compounds was still stable. This phenomenon is beneficial from the technological perspective, enabling the use of the proposed system as an alternative source of *R. tomentosum* essential oil for biological activity studies. However, it should be taken into account that chemical composition of the obtained volatile fraction may be subject to change over a period of days.

The percentage content of the most abundant components in the essential oil from RITA® bioreactor were determined every 7 days during 2 months, starting from 14 day of cultivation (Fig. 7). Some of the selected compounds were reported to possess bioactive properties: anti-inflammatory (*p*-cymene: Bonjardim et al. 2012; sabinene: Valente et al. 2013), nociceptive (*p*-cymene: Bonjardim et al. 2012), cytotoxic (γ-terpineol: Wu et al. 2014; alloaromadendrene; Mah et al. 2013) and insecticidal (shyobunone: Chen et al. 2015). Although shyobunone and alloaromadendrene content despite small fluctuations remained at a fairly constant level during the entire biomass cultivation period, the concentrations of other compounds varied depending on the stage of experiment. The amount of ledene oxide (II) declined after 42 day of the growth cycle in parallel with γ-terpineol and sabinene increase. The results of our previous research suggested that γ-terpineol content can be correlated with the age of plant material (Jesionek et al. 2016). The highest amount of methyl everninate was observed at the beginning and at the end of cultivation period which indicates that this

compound might be produced in response to stress associated with initiation and dying of the in vitro culture. The biosynthesis of *p*-cymene was shown to be intensified only after 21 day of microshoots cultivation.

Due to the changes in the concentration of the volatile compounds during cultivation, it is recommended to select the proper biomass harvesting time to obtain the optimal content of the terpenoids in the *R. tomentosum* in vitro essential oil.

Conclusions

In conclusion, liquid shoot cultures of *R. tomentosum* were established for the first time, providing the alternative, renewable and continuous source of in vitro plant material for essential oil isolation. Scaling up to bioreactors improved the growth parameters and stimulated the essential oil production. It was proven that the type of in vitro cultivation system influences the primary and secondary metabolism of the studied biomass. The best effects were observed with application of RITA® bioreactor. Since the amounts of volatiles found in bioreactor-grown microshoots were comparable with maternal plant, it is possible that the developed system will enable the large-scale production of *R. tomentosum* essential oil rich in bioactive compounds.

Acknowledgements The authors are thankful to National Science Center in Poland for financial support (Decision No. UMO-2014/15/N/NZ7/03027).

Author contributions AJ carried out the in vitro experiments and the extraction of volatiles, analyzed the data and wrote the manuscript. AK and PW participated in the biotechnological experiments. BZ developed the analytical method for determination of essential oil chemical composition and analyzed GC/MS data. AB checked and corrected the manuscript. ML planned the study, analyzed the data and edited the manuscript. All co-authors agree on the contents of this manuscript and consent to submit it. The manuscript has not been submitted to any other journal for simultaneous consideration.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Ahmad M, Faraazi AA, Aamir MN (2013) The effect of Ocimum sanctum and *Ledum palustre* on serum uric acid level in patients suffering from gouty arthritis and hyperuricaemia. B Chem Soc Ethiopia 27:469–473

Almeida R, Goncalves S, Romano A (2005) In vitro micropropagation of endangered *Rhododendron ponticum* L. subsp. baeticum (Boissier & Reuter) Handel-Mazzetti. Biodivers Conserv 14:1059–1069

Alvard D, Cote F, Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion of explants. Plant Cell Tissue Organ C 32:55–60

Anderson W (1978) Tissue culture propagation of Rhododendrons. In Vitro 14:334

Baananou S, Bagdonaite E, Marongiu B, Piras A, Porcedda S, Falconieri D, Boughattas NA (2015) Supercritical CO_2 extract and essential oil of aerial part of *Ledum palustre* L.—Chemical composition and anti-inflammatory activity. Nat Prod Res 29:999–1005

Bonjardim LR, Cunha ES, Guimarães AG, Santana MF, Oliveira MGB, Serafini MR, Araújo AAS, Antoniolli AR, Cavalcanti SCH, Santos MRV, Quintans-Júnior LJ (2012) Evaluation of the anti-inflammatory and antinociceptive properties of p-cymene in mice. Z Naturforsch 67c:15–21

Butkienė R, Šakočiūtė V, Latvėnaitė D, Mockutė D (2008) Composition of young and aged shoot essential oils of the wild *Ledum palustre* L. Chemija 19:19–24

Chen H-P, Yang K, Zheng LS, You C-X, Cai Q, Wang C-F (2015) Repellant and insecticidal activities of shyobunone and isoshyobunone derived from the essential oil of Acorus calamus rhizomes. Pharmacogn Mag 11:675–681

Dampc A, Luczkiewicz M (2013) *Rhododendron tomentosum* (*Ledum palustre*). A review of traditional use based on current research. Fitoterapia 85:130–143

Eeckhaut T, Janssens K, De Keyser E, De Riek J (2010) Micropropagation of Rhododendron. In: Jain SM, Ochatt SJ (eds) Protocols for in vitro propagation of ornamental plants, methods in molecular biology, vol 589. Humana Press, New York, pp 141–152

Fulzele DP, Heble MR, Rao PS (1995) Production of terpenoid from *Artemisia annua* L. plantlet cultures in bioreactor. J Biotechnol 40:139–143

Georgiev V, Ivanov I, Berkov S, Ilieva M, Georgiev M, Gocheva T, Pavlov A (2012) Galanthamine production by *Leucojum aestivum* L. shoot culture in a modified bubble column bioreactor with internal sections. Eng Life Sci 12:534–543

Georgiev V, Schumann A, Pavlov A, Bley T (2014) Temporary immersion systems in plant biotechnology. Eng Life Sci 14:607–621

Grzegorczyk I, Wysokińska H (2008) Liquid shoot culture of *Salvia officinalis* L. for micropropagation and production of antioxidant compounds; effect of triacontanol. Acta Soc Bot Pol 77:99–104

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011) Thidiazuron: a multi-dimensional plant growth regulator. Afr J Biotechnol 10:8984–9000

Hilton MG, Jay A, Rhodes MJC, Wilson PDG (1995) Growth and monoterpene production by transformed shoot cultures of *Mentha citrata* and *Mentha piperita* in flasks and fermenters. Appl Microbiol Biotechnol 43:452–459

Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss Org 33:105–119

Jain N, Sharma V, Ramawat KG (2012) Shoot culture of Bacopa monnieri: standardization of explant, vessels and bioreactor for growth and antioxidant capacity. Physiol Mol Biol Plants 18:185–190

Jang H-R, Lee H-J, Shohael AM, Park B-J, Paek K-Y, Park S-Y (2016) Production of biomass and bioactive compounds from shoot cultures of *Rosa rugosa* using a bioreactor culture system. Hortic Environ Biotechnol 57:79–87

Jaremicz Z, Luczkiewicz M, Kokotkiewicz A, Krolicka A, Sowinski P (2014) Production of tropane alkaloids in Hyoscyamus niger (black henbane) hairy roots grown in bubble-column and spray bioreactors. Biotechnol Lett 36:843–853

Jesionek A, Kokotkiewicz A, Wlodarska P, Filipowicz N, Bogdan A, Ochocka R, Szreniawa-Sztajnert A, Zabiegala B, Bucinski A, Luczkiewicz M (2016) In vitro propagation of *Rhododendron tomentosum*—an endangered essential oil bearing plant from peatland. Acta Biol Cracov Bot 58:29–43

Kokotkiewicz A, Bucinski A, Luczkiewicz M (2015) Xanthone, benzophenone and bioflavonoid accumulation in Cyclopia genistoides (L.) Vent. (honeybush) shoot cultures grown on membrane rafts and in a temporary immersion system. Plant Cell Tissue Organ C 120:373–378

Kumar LD, Karthik R, Gayathri N, Sivasudha T (2016) Advancement in contemporary diagnostic and therapeutic approaches for rheumatoid arthritis. Biomed Pharmacother 79:52–61

Laev SS, Salakhutdinov NF (2015) Anti-arthritic agents: progress and potential. Bioorgan Med Chem 23:3059–3080

Liu C-Z, Guo C, Wang Y-C, Ouyang F (2003) Comparison of various bioreactors on growth and artemisinin biosynthesis of Artemisia annua L. shoot cultures. Process Biochem 39:45–49

Mah SH, Ee GCL, Teh SS (2013) Chemistry and cytotoxic activity of essential oil from the stem bark of Calophyllum soulattri. Asian J Chem 25:8831–8832

Mao AA, Kaliamoorthy S, Ranyaphi RA, Das J, Gupta S, Athili J, Yumnam JY, Chanu LI (2011) In vitro micropropagation of three rare, endangered, and endemic Rhododendron species of Northeast India. In Vitro Cell Dev Biol-Plant 47:674–681

McAlister B, Finnie J, Watt MP, Blakeway F (2005) Use of the temporary immersion bioreactor system (RITA®) for production of commercial Eucalyptus clones in Mondi Forests (SA). Plant Cell Tissue Organ C 81:347–358

Miguel MM (2010) Antioxidant and anti-inflammatory activities of essential Oils: a short review. Molecules 15:9252–9287

Paek KY, Chakrabarty D, Hahn EJ (2005) Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tissue Organ C 81:287–300

Perez-Alonso N, Wilken D, Gerth A, Jahn A, Nitzsche H-M, Kerns G, Capote-Perez A, Jimenez E (2009) Cardiotonic glycosides from biomass of *Digitalis purpurea* L. cultured in temporary immersion systems. Plant Cell Tissue Organ C 99:151–156

Perotto S, Girlanda M, Martino E (2002) Ericoid mycorrhizal fungi: some new perspectives on old acquaintances. Plant Soil 244:41–53

Polish Pharmacopoeia VI (2002) The determination of essential oil. Polish Pharmaceutical Society, Warsaw, p 151

Rout GR, Samantaray S, Das P (2000) In vitro manipulation and propagation of medicinal plants. Biotechnol Adv 18:91–120

Scheidt GN, Arakaki AH, Chimilovski JS, Portella ACF, Spier MR, Woiciechowski AL, Biasi LA, Soccol CR (2009) Utilization of the bioreactor of Immersion by bubbles at the micropropagation of *Ananas comosus* L. Merril. Braz Arch Biol Technol 52:37–43

Schenk R, Hildebrandt A (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Botany 50:199–204

Sellmer J, Bates R, Hoover G, Moorman G (2003) Ericacea (heath) family and their culture. In: The horticulture fact sheet series, college of agricultural sciences, The Pennsylvania State University, pp 1–7. http://extension.psu.edu/publications/xj0035. Accessed 24.11.2016

Setty AR, Sigal LH (2005) Herbal medications commonly used in the practice of rheumatology: mechanisms of action, efficacy and side effects. Semin Arthritis Rheum 34:773–784

Shaver GR (1983) Mineral nutrition and leaf longevity in *Ledum palustre*: the role of individual nutrients and the timing of leaf mortality. Oecologia 56:160–165

Szopa A, Kokotkiewicz A, Luczkiewicz M, Ekiert H (2017) Schisandra lignans production regulated by different bioreactor type. J Biotechnol 247:11–17

Tantos A, Mészáros A, Farkas T, Szalai J, Horváth G (2001) Triacontanol-supported micropropagation of woody plants. Plant Cell Rep 20:16–21

Tisserat B, Vaughn SF (2008) Growth, morphogenesis and essential oil production in *Mentha spicata* L. plantlets in vitro. In Vitro Cell Dev Biol-Plant 44:40–50

Tomsone S, Gertnere D (2003) In vitro shoot regeneration from flower and leaf explants in Rhododendron. Biol Plant 46:463–465

Valente J, Zuzarte M, Gonçalves MJ, Lopes MC, Cavaleiro C, Salgueiro L, Cruz MT (2013) Antifungal, antioxidant and anti-inflammatory activities of *Oenanthe crocata* L. essential oil. Food Chem Toxicol 62:349–354

Vejsadova H (2008) Growth regulator effect on in vitro regeneration of Rhododendron cultivars. Hortic Sci 35:90–94

Watad AA, Ahroni A, Zuker A, Shejtman H, Nissim A, Vainstein A (1996) Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. Sci Hortic 65:313–320

Watt MP (2012) The status of temporary immersion system (TIS) technology for plant micropropagation. Afr J Biotechnol 11:14025–14035

Welander M, Persson J, Asp H, Zhu LH (2014) Evaluation of a new vessel system based on temporary immersion system for micropropagation. Sci Hortic 179:227–232

Wu Z-L, Yin Z-Q, Du Y-H, Feng R-Z, Ye K-C, Wei Q, Hu Y, He L, Liao L, Wang Y (2014) γ- terpineol inhibits cell growth and induces apoptosis in human liver cancer BEL-7402 cells in vitro. Int J Clin Exp Pathol 7:6524–6533

Yan H, Yang L, Li Y (2011) Improved growth and quality of dioscorea fordii prain et burk and dioscorea alata plantlets using a temporary immersion system. Afr J Biotechnol 10:19444–19448

Yingjin Y, Zongding H (1994) A bioreactor for two-stage multiple shoot culture of Catharanthus roseus. Chin J Chem Eng 2:92–97

Zhang M, Zhao J-J, Wu W-B, Zhao Y, Zhu X-L, Qu W-J (2010) Analgesic and anti-inflammatory activities of extracts from *Ledum palustre* L. in Mice. Nat Prod Res Dev 22:326–329

Zobayed SMA, Murch SJ, Rupasinghe HPV, de Boer JG, Glickman BW, Saxena PK (2004) Optimized system for biomass production, chemical characterization and evaluation of chemo-preventive properties of Scutellaria baicalensis Georgi. Plant Sci 167:439–446