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ORIGINAL ARTICLE

The influence of liquid systems for shoot multiplication, secondary metabolite production and plant regeneration of *Scutellaria alpina*

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Abstract To establish an effective system for shoot multiplication and bioactive metabolite (baicalin, wogonoside, luteolin, luteolin-7-glucoside and verbascoside) production, shoot tips of *Scutellaria alpina* were grown in various culture systems: 0.35 and 0.7% agar solidified medium, liquid stationary culture, and 5 L sprinkle bioreactor. *S. alpina* appeared very sensitive to immersion in the culture medium, and support was needed to achieve shoot proliferation in the liquid stationary culture. For this purpose, bacterial nanocellulose and polyurethane foam or steel mesh in bioreactor system as support were used. The liquid nanocellulose-supported culture system gave the greatest shoot proliferation rates (36.1 ± 4.9 within 5 weeks). Shoots multiplied in the liquid supported media had greater fresh and dry weights and accumulated higher levels of metabolites. The most evident differences in metabolite content were observed when liquid supported cultures were compared with culture on 0.7% agar solidified medium. The study presents for the first time the use of the liquid culture systems together with bioreactor culture for in vitro *S. alpina* shoot multiplication and production of therapeutically valuable metabolites.

Keywords Bacterial nanocellulose (BNC) · Bioreactor · Flavonoids · Liquid culture · Polyurethane foam (PU) · *Scutellaria alpina* · Verbascoside

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Introduction

Scutellaria, a genus of the *Lamiaceae* family, comprises over 300 species of annual or perennial herbs distributed in East Asia, Europe and United States. Of the *Scutellaria* species, *S. baicalensis* is officially listed in Japanese and Chinese Pharmacopeia and is one of the most widely-employed drugs for the treatment of hypertension, infectious disease, inflammatory and allergic diseases (Shang et al. 2010). The pharmacological activities of the plant depend on the presence of flavones, such as baicalin, wogonoside and luteolin (Shang et al. 2010). Besides *S. baicalensis*, about 50 other *Scutellaria* species are known to produce important compounds, one such species being *S. alpina*: an annual plant distributed throughout the mountain regions of central and southern Europe and cultured as garden species for its attractive flowers (Yamamoto 1991).

Our previous work describes in vitro *S. alpina* shoot regeneration and plant formation. The accumulation of baicalin, wogonoside, luteolin, luteolin-7-glucoside and verbascoside in in vitro plant material was also determined (Grzegorczyk-Karolak et al. 2015, 2016). Those studies were conducted using solid Murashige and Skoog (MS) (1962) medium. However, it is possible to improve shoot proliferation by developing liquid-based systems (Ascough et al. 2004; Russowski et al. 2006). The use of liquid media allows homogenous dispersion of the nutrients and better absorption of medium components by the explants, and the cultures that grew well in the liquid medium can be scaled up for the bioreactor system. The main disadvantage of liquid culture is hyperhydricity, a physiological abnormality of tissues causing anatomical deformities and failure of shoot development and rooting (Prasad and Gupta 2006). In order to overcome this problem, various supporting materials such as cotton fibers, glass seeds,

cellulose fibers, vermiculite or polypropylene membrane can be used (Ichimura et al. 1995; Kirdmanee et al. 1995; Afreen-Zobayed et al. 2000; Prasad and Gupta 2006). The support was placed on the bottom of a culture vessel so that the explants grown in the liquid culture have only partial contact with the medium.

The present work examines how liquid cultures affect the multiplication rate of *S. alpina* shoots, and the accumulation of secondary metabolites (flavones and verbascoside) in them. Our findings indicate that shoot tips excised from in vitro shoots of *S. alpina* and cultured in agitated and stationary (without agitation) systems failed to produce shoots, resulting in necrosis of explants (data not shown). Therefore, a liquid stationary culture was used for *S. alpina* shoot multiplication with bacterial nanocellulose or polyurethane foam acting as supporting material; the production of bioactive compounds in the cultures were compared to those in shoot cultures on solid (0.7% agar) and semi-solid (0.35% agar) media. The study also examines the effect of liquid cultures on further stages of *S. alpina* micropropagation i.e. shoot rooting and plantlet acclimatization. Since biotechnological production of secondary metabolites in in vitro cultures requires scaling-up with the use of a bioreactor, the present study evaluates the growth, flavone and verbascoside content in *S. alpina* shoots grown in 5 L nutrient sprinkle bioreactor, where shoots were supported on stainless steel, and liquid medium was delivered to shoots as droplets.

Materials and methods

Plant material and culture conditions

Aseptic shoot culture of *S. alpina* was maintained on MS agar (0.7%) medium supplemented with 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L BAP (6-benzylaminopurine) and sucrose (3%). The pH of the medium was maintained at 5.6–5.7 prior to autoclaving. The medium was autoclaved at 121 °C, at a pressure of 0.1 MPa for 17 min. The shoots were grown under a 16-h photoperiod (light intensity 40 μM/m^2 s) at 26 ± 2 °C and were subcultured at 5-week intervals. The same culture conditions were used for all experiments described in the work. The shoot tips (1 cm length) from 5-week-old shoots (subculture 26–28) were used as the explants to study *S. alpina* shoot multiplication.

Multiple shoot induction in the different culture system

Shoot proliferation of *S. alpina* was studied in the following culture systems:

- stationary (without agitation) liquid culture. In order to avoid complete explant submersion in the liquid medium, the stationary liquid culture was supported by polyurethane foam (EuroFoam, Zgierz, Poland) (PUL system) or bacterial nanocellulose (BNCL system). The BNC membranes were obtained from cultivation of the *Gluconacetobacter xylinus* strain E25 in the Institute of Technical Biochemistry, Lodz University of Technology. The detailed characterization of cellulose is as according to Krystynowicz et al. (2002). The support measured 5 × 5 × 0.5 cm for bacterial nanocellulose and 5 × 5 × 0.7 cm for polyurethane foam. The support material was placed on a bottom of culture vessel. For the experiments, 300 mL glass culture jars containing 30 mL of liquid medium were used. The vessels with support and medium were autoclaved. Two explants [initial fresh weight (wt) about 0.017 g per each] were placed in each culture vessel;
- solid (0.7% agar) (AS) and semi-solid (0.35% agar) (SS) cultures. The cultures were performed in 300 mL glass jars containing 30 mL of medium. Two explants (initial fresh wt about 0.017 g per each) were incubated in each vessel;
- in a 5 L nutrient sprinkle bioreactor (BL system) consisting of a growth chamber made of glass, which was used as a reservoir for the medium. The explants were placed on stainless steel mesh (with a 5 mm pore size) 15 cm above the bottom of the growth chamber (Fig. 1). The medium was sprayed with polypropylene spraying nozzle situated at the bottom of the growth chamber by a peristaltic pump (type CMF 10 Chemap). The dispersed medium was sprayed over the plant material and returned to the medium reservoir. The circulation rate of the medium was 60 ml during a 40 s delivery, with 2 min breaks between each delivery. Before starting the cultivation, the bioreactor was sterilized by autoclaving for 17 min at 121 °C. The bioreactor was inoculated with 15 shoot tips (more than 0.5 cm in length) derived from the 5-week-old shoots: fresh wt 0.325 ± 0.021 g and dry wt 0.033 ± 0.0002 g.

MS medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BAP were used for evaluation of shoot multiplication in all tested culture systems. At the end of 5 weeks of culture, the percentage of explant-regenerated shoots, the multiplication rate (number of shoots per explant), average length of shoots and their hyperhydricity (%) were estimated. Culture growth was also determined by measuring fresh and dry wt (g/explant). Experiments were repeated three times and each series counted 8–16 explants.

Fig. 1 *Scutellaria alpina* shoots grown for 5 weeks in MS liquid medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BAP in the nutrient sprinkle bioreactor. *Bar* 1 cm

Rooting of shoots and plantlet acclimatization ex vitro

The shoots of *S. alpina* (>1 cm in length) multiplied in different culture systems (BNCL, PUL, AS, SS) were transferred individually into half-strength MS medium (1/2 MS) solidified with 0.7% agar. The rooting medium composition was chosen on the basis of the results of our previous work (Grzegorczyk-Karolak et al. 2016). Means for rooting percentage, root length and root number were calculated from three replications (ten shoots/replicate) and were recorded after 4 weeks of culture. Subsequently the plantlets were transplanted into pots containing a sterilized mixture of sand, peat and soil (3:3:4 v/v/v). The plants were kept in the greenhouse at a controlled temperature (at 26 ± 2 °C) and under natural light. After 12 weeks, the survival rate was recorded as the percentage of plants that had survived.

Phytochemical analysis

The five-week-old shoots of *S. alpina* (250 mg dry wt) from different culture systems were lyophilized, powdered and extracted three times with mixture of methanol: water 7:3 (v/v) (30 mL) at 15 min in the ultrasonic bath. The extracts were combined and evaporated under reduced pressure. For UPLC analysis, the dry residue was dissolved in 2 mL methanol. The analysis was performed using an Agilent Technologies 1290 Infinity UPLC apparatus equipped with a diode array detector (DAD) and Zorbax Eclipse Plus C18 column (100 × 3 mm; 1.8 µm Agilent Technologies) according to Grzegorczyk-Karolak et al. (2015).

Baicalin, wogonoside, verbascoside, luteolin and luteolin-7-glucoside were detected at 320 nm. The compounds were identified by comparing their retention times, UV spectra and mass spectra with those of standard compounds (Grzegorczyk-Karolak et al. 2015). Verbascoside was provided by Phytoplan, baicalin was purchased from Sigma-Aldrich, wogonoside from ChemFace (China), and luteolin and luteolin-7-glucoside from Roth. The quantification of these compounds was achieved using calibration curves (Grzegorczyk-Karolak et al. 2015). The experiments were performed in triplicate. The compound contents were expressed as mg/g of dry wt.

Statistical analysis

Means and standard errors were calculated using EXCEL 2010. The analysis of variance test was performed with STATISTICA 10.0 software using the Kruskal–Wallis test. The significance level was assumed to be 5%.

Results and discussion

Shoot multiplication

Several authors have reported the value of liquid media for shoot multiplication (Russowski et al. 2006; Pati et al. 2011; Cavallaro et al. 2014). However, *S. alpina* shoot tip explants placed in liquid culture, either agitated or stationary, did not derive shoots, and tissue necrosis was observed, probably caused by the continuous contact between the plant tissue and the liquid medium. In addition, Savio et al. (2011) suggest that the limited gas exchange between tissue and environment may account for the low rate of multiplication observed for completely submerged *Hypericum perforatum* shoots in liquid culture. Also, *Scutellaria latifolia* and *S. costaricana* plantlets grown in an immersed liquid culture had a lower multiplication ratio than those on agar culture (Tascan et al. 2010).

In the present study, the highest multiplication rate of *S. alpina* shoots after 5 weeks was achieved in the liquid culture supported with bacterial cellulose (36.1 ± 4.9 for 93% responding explants), which was 50–70% more than observed in the other tested culture systems. However, cultures on agar-based media gave better results with respect to the percentage of responding explants (Table 1). The propagation ratio in the BNCL system was at least 45% higher than those observed in our earlier report, where *S. alpina* shoots were proliferated on 0.7% agar solidified MS

Table 1 The effect of different culture systems on *S. alpina* shoot multiplication, rooting and plant acclimatization

Stage of culture	Parameter	Culture system			
		BNCL	PUL	AS	SS
Shoot multiplication	Explants forming shoots (%)	93.5	77.8	100.0	88.2
	Multiplication rate	36.1 ± 4.9^{a}	24.1 ± 1.9^{b}	21.4 ± 4.1^{b}	23.4 ± 4.3^{b}
	Mean shoot length (cm)	1.54 ± 0.08^{b}	1.96 ± 0.11^{b}	1.29 ± 0.08^{c}	2.11 ± 0.12^{a}
	Hyperhydricity of shoots (%)	0.6	0	0	6.8
	Fresh weight (g/explant)	0.78 ± 0.073^{a}	0.62 ± 0.102^{b}	0.19 ± 0.02^{d}	0.53 ± 0.059^{c}
	Dry weight (g/explant)	0.075 ± 0.007^{a}	0.050 ± 0.008^{c}	0.019 ± 0.002^{d}	0.056 ± 0.006^{b}
	% dry wt	9.64	8.02	9.98	10.62
Rooting	Shoots forming roots (%)	84.0	72.0	92.0	91.5
	Root number	1.93 ± 0.15^{c}	2.60 ± 0.22^{b}	3.89 ± 0.16^{a}	2.91 ± 0.20^{b}
	Root length (cm)	0.90 ± 0.1^{b}	0.97 ± 0.2^{b}	1.72 ± 0.3^{a}	1.29 ± 0.15^{b}
Plant acclimatization	Survival rate (%)	95	96	98	95

For shoot multiplication: shoot tips were multiplied on/in MS medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BAP. System of culture: *BNCL* stationary liquid bacterial nanocellulose supported, *PUL* stationary liquid polyurethane foam supported, *AS* agar (0.7%) solid, *SS* semi-solid (0.35% agar). Explant initial fresh weight was about 0.017 g ± 0.001, and dry weight 0.0017 ± 0.00015 g. Data were recorded after 5 weeks

For rooting: data were recorded after 4 week culture on 1/2 MS agar free-growth regulator medium

For plant acclimatization: data were recorded after 12 weeks of growth in the greenhouse

Results are the means of three determinations ±SE. The means marked with the same letter in each row do not differ significantly according to the Kruskal–Wallis, at 5% significance level

media supplemented with various types of cytokinins at various concentrations (Grzegorczyk-Karolak et al. 2015).

The shoots multiplied in the BNCL system were healthy and good quality with proper length and very low hyperhydricity (0.6%). Due to the uniform refined 3-D structure of the microfibrils, their selective porosity (permeability to gases and metabolites) and remarkable water holding capacity (Bielecki et al. 2005), the support material can remove water vapour from the glass walls and reduce the humidity inside the culture vessel. In contrast, hyperhydricity occurred in around 7% of *S. alpina* shoots cultured on 0.35% agar solidified medium. The abnormal *S. alpina* shoots were completely eliminated when the agar concentration of the culture medium rose from 0.35 to 0.7% (Table 1). The observation is agreement with Saher et al. (2004), who note that the reduction of agar concentration from 0.8 to 0.58% resulted in more hyperhydric shoots of carnations. Shoot hyperhydricity in in vitro culture is known to be controlled by increasing the concentration of gelling agent, or by decreasing the concentrations of cytokinins or nitrogen ions (Sandal et al. 2001; Ivanova and Van Staden 2011).

The discovery that shoots of *S. alpina* in in vitro culture prefer partial contact between plant material and liquid medium enabled the selection of bioreactor type for shoot proliferation. For this purpose, a 5 L nutrient sprinkle bioreactor was used. In the bioreactor, the plant material (shoot tips of *S. alpina*) was supported on stainless steel and was sprayed by 1 L of liquid MS medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BAP: the circulation rate of the medium comprised repeated 40 s deliveries at intervals of 2 min. The type of bioreactor was earlier used for shoot culture of *Artemisia annua* (Liu et al. 1998) and *Salvia officinalis* (Grzegorczyk and Wysokińska 2010) as well as for the hairy roots of *Panax quinquefolium* (Kochan et al. 2012). After 5 weeks, 446.7 ± 19.78 shoots of *S. alpina* with an average length 1.56 ± 0.11 cm were harvested (Fig. 1) giving a multiplication rate of about 30 (Table 2).

The biomass of *S. alpina* shoots multiplied from a single shoot tip in the liquid culture supported with bacterial cellulose was also great; fresh wt grew 46-fold and dry wt increased 44-fold over inoculum values after 5 weeks of culture (Table 1). However, the biomass of the shoots grown in the bioreactor was higher; during the same period, fresh wt grew 56-fold (from 0.33 to 18.23 g) and dry wt 50-fold (from 0.033 to 1.64 g) (Table 2). The bioreactor culture seemed also to be valuable for shoot quality: only about 5% of multiple shoots showed features of hyperhydricity. The least biomass (11-fold over inoculum) was achieved from explants cultured on media solidified with 0.7% agar (Table 1). However, relative dry wt content (the ratio tissue dry wt to tissue fresh wt) was higher in solidified 0.7% agar medium, suggesting that shoots in the liquid supported systems contained more water.

Table 2 Biomass production and shoot multiplication of *S. alpina* in the nutrient sprinkle bioreactor

Parameter	
No of shoot	
Initial	15
Final	446.7 ± 19.78
Multiplication rate	29.7 ± 1.32
Shoot length (cm)	1.56 ± 0.11
Shoot fresh weight (g/L)	
Initial	0.325 ± 0.021
Final	18.23 ± 0.52
Shoot dry weight (g/L)	
Initial	0.033 ± 0.002
Final	1.64 ± 0.091
Hyperhydricity shoots (%)	4.8
Metabolite content (mg/g dry weight)	
Verbascoside	6.95 ± 0.17
Baicalin	15.18 ± 0.13
Wogonoside	4.08 ± 0.09
Luteolin	5.15 ± 1.16
Luteolin-7-glucoside	1.45 ± 0.04
Metabolite production (mg/L)	
Verbascoside	11.4 ± 0.28
Baicalin	24.9 ± 0.21
Wogonoside	6.7 ± 0.15
Luteolin	8.45 ± 1.90
Luteolin-7-glucoside	2.4 ± 0.07

Data were recorded after 5 weeks of culture. Results are the means of three determinations ±SE

Quantification of flavones and verbascoside in multiple shoots

In all tested *S. alpina* shoot samples, baicalin, wogonoside, luteolin, luteolin-7-*O*-glucoside and verbascoside were detected by UPLC-DAD examination. The concentrations of bioactive metabolites in the liquid cultures (BNCL, PUL) were significantly higher than those in shoots cultured on agar solidified medium (Fig. 2). The most evident differences were observed occurred in baicalin concentration. Its level in the shoots grown in the BNCL and PUL systems (about 15 mg/g dry wt) was twice as great than in those grown on the medium solidified with 0.7% agar (7.12 ± 0.25 mg/g dry wt). These levels were also significantly higher than those found in the leaves of in vitro grown *S. alpina* and other eight *Scutellaria* species cultivated on 1/2 MS with 0.275% gelrite solidified medium in a previous study (Nishikawa et al. 1999).

Multiple shoots cultured in the supported liquid systems accumulated more baicalin than those of field-grown *S. alpina*, in which the flavone concentration reached 2.17 mg/g dry wt (Zgórka 2006) or 1.29 mg/g dry wt (Grzegorczyk-Karolak et al. 2016). It can be seen that liquid shoot cultures of *S. alpina* produced also much greater baicalin amount than the leaves of *S. baicalensis, S. lateriflora, S. tomentosa* and *S. wrighti* grown in a greenhouse (Islam et al. 2011). Only the leaves of *S. racemosa* plants contained a similar concentration of the flavone (15.21 mg/g dry wt) as in vitro cultivated *S. alpina* shoots. A similar trend was observed with respect to wogonoside content: *S. alpina* culture grown in the BNCL system accumulated more wogonoside than the aerial parts of aseptic plantlets of *S. alpina* and other *Scutallaria* species; however, the wogonoside concentration in *S. incana* was similar to that found in shoots cultivated in our study (Nishikawa et al. 1999).

The contents of baicalin, wogonoside and luteolin in the shoots of *S. alpina* grown in the nutrient sprinkle bioreactor reached 15.18, 4.08, 5.15 mg/g dry wt, respectively (Table 2): approximately similar to the content in *S. alpina* shoots multiplied in the liquid nanocellulose supported culture (BNCL). However, the concentrations of verbascoside (6.95 mg/g dry wt) and luteolin-7-glucoside (1.45 mg/g dry wt) in the shoots grown in the bioreactor were significantly lower than those achieved in the BNCL system and more comparable to those found in the shoot cultures on medium solidified with 0.7% agar (AS culture). The efficiency of the sprinkle bioreactor for cultivating *S. alpina* was demonstrated when examining the production of secondary metabolites in mg per bioreactor (1 L of medium). For the shoot culture in the bioreactor system, the respective yields for baicalin, wogonoside, luteolin, luteolin-glucoside and verbascoside were 24.9, 6.7, 8.5, 2.4 and 11.4 mg/L.

It is likely that higher metabolite production in shoots grown in the liquid media was connected with the higher efficiency in the uptake of nutrients by the plant tissues in these conditions. Similar studies with other medicinal plant species have also revealed that the liquid media increased production of metabolites such as lignans in shoot-differentiating callus cultures of *Schisandra chinensis* (Szopa et al. 2016) and polyphenolic compounds in the *Hypericum perforatum* shoot liquid culture (Savio et al. 2011). The elimination of agar from the culture medium also resulted in substantial savings in cost. Pati et al. (2011) calculated a 5.2-fold reduction in cost associated with the use of liquid medium for *Catharanthus roseus* shoot multiplication.

The results shown in Fig. 2 also demonstrated that the concentration of agar in the culture medium influenced the level of flavones and verbascoside in the shoot biomass of *S. alpina*. The shoots multiplied on medium solidified with 0.35% agar (SS culture) produced more baicalin, wogonoside, luteolin, luteolin-glucoside and verbascoside than those grown on solidified 0.7% agar (AS) culture (significant difference at $p \leq 0.05$).

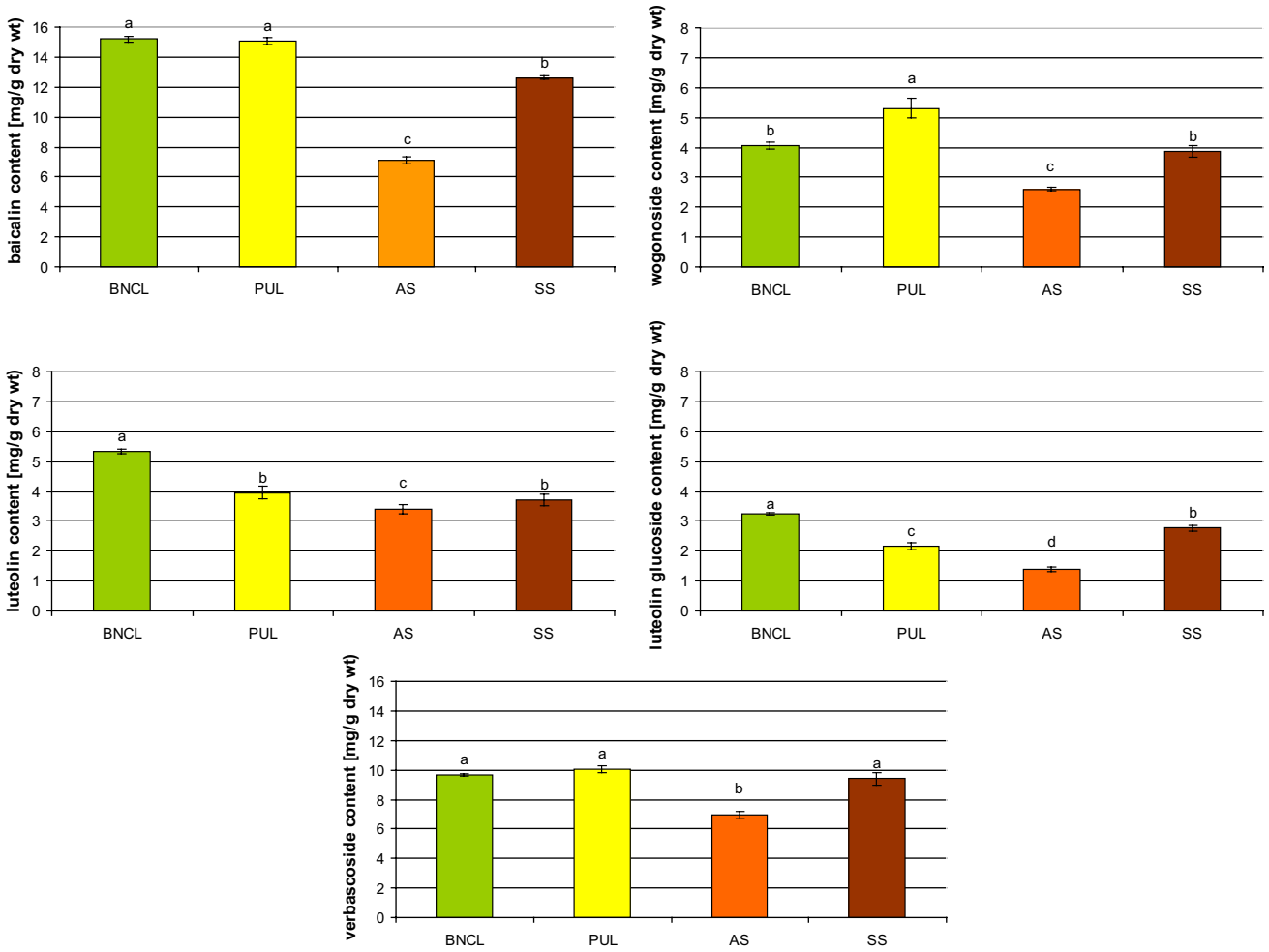

Fig. 2 Secondary metabolite contents in shoots of *S. alpina* cultivated in different systems. System of culture: *BNCL* stationary liquid cellulose supported, *PUL* stationary liquid polyurethane foam supported, *AS* agar (0.7%) solid, *SS* semi-solid (0.35% agar). All cultures grown on/in MS medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BAP for 5 weeks. Results are the means of three determinations ±SE. The means for the same metabolite marked with the same letter do not differ significantly according to the Kruskal–Wallis, at 5% significance level

Rooting of shoots and acclimatization ex vitro

The shoots of *S. alpina* multiplied in the different culture systems (BNCL, PUL, AS, SS) were separated and transferred to rooting medium (1/2 MS with 0.7% agar). Table 1 shows that the potential for root induction varied between the shoots derived on the agar-gelled media and the shoots from the liquid media with supported material, with the former being more suitable for rooting. Both agar-gelled cultures (AS and SS) gave better results in terms of frequency of root formation (about 92%) than liquid supported systems, but the number of roots per shoot and root length were significantly higher for the shoots obtained from the medium solidified with 0.7% agar. The lowest rooting percentage was obtained for *S. alpina* shoots from the liquid medium supported with polyurethane foam; 72% of the shoots developed roots after 4 weeks of culture (Table 1). *Centaurium erythraea* shoots from liquid culture demonstrated a similar decrease in root formation (Piątczak et al. 2005). However, in that case, decreased shoot length was observed in the liquid medium. No such relationship was found in *S. alpina* shoot cultures.

When rooted shoots were transferred into pots and grown in the greenhouse, 95–98% of the plants survived after 12 weeks of acclimatization, without any clear differences between the plants derived from the shoots multiplied under different experimental conditions (Table 1).

Conclusions

Our results indicate that the combination of the use of liquid medium with support material is an efficient way to produce high quality shoots with high concentrations of verbascoside and flavones, particularly baicalin. Of the two liquid culture systems tested, the best results were obtained in the shoots cultured in the nanocellulose-supported liquid system. Compared with medium solidified with 0.7% agar, the BNCL culture gave higher values for shoot number (1.5×) and shoot biomass (4×), as well as baicalin (2×) and verbascoside (almost 1.5×) content.

The results present also the possibility of using of the a sprinkle bioreactor for *S. alpina* shoot propagation, where partial contact between dispersed liquid medium and plant material is maintained. Generally, with regard to the content of the analyzed metabolites (in mg/g dry wt), the use of a bioreactor gave similar or better results than culturing on 0.7% agar solidified medium, but these were sometimes lower than those found in the tested liquid culture systems. Therefore, further studies are needed to optimize the flow profile of the medium, particularly the duration and frequency of contact between medium and the plant material.

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Author contributions IG-K obtained in vitro cultures, conducted the in vitro study, regenerated plants, prepared samples, performed UPLC analysis and analyzed results. IG-K and HW described results. PR obtained bacterial nanocelulose membranes. HW and SB were responsible for verification of the paper.

Compliance with ethical standards

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

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