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The growth and saponin production of *Platycodon grandiflorum* (Jacq.) A. DC. (Chinese bellflower) hairy roots cultures maintained in shake flasks and mist bioreactor

Natalia Urbańska[1], Joanna Giebułtowicz[2], Olga Olszowska[1], Wojciech J. Szypuła[1]*

[1] Department of Pharmaceutical Biology and Medicinal Plants Biotechnology, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland

[2] Department of Bioanalysis and Drug Analysis, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland

Abstract

The growth and saponin accumulation were measured in two lines of transgenic hairy roots of *Platycodon grandiflorum*, Pl 6 and Pl 17, cultured for 8 weeks in 250-ml shake flasks containing 50 ml of hormone-free woody plant medium supplemented with 40 g/l sucrose and in the Pl 17 line cultured for 12 weeks in a 5-l mist bioreactor containing 1.5 l of the same medium. With both methods, the growth of transgenic hairy roots was assessed as both fresh and dry weight and the biomass growth was correlated with the conductivity and sucrose uptake. The accumulation of saponins was measured and compared with that in roots derived from the field cultivation. The saponin concentrations were significantly higher in the two hairy root lines cultured in shake flasks [6.92 g/100 g d.w. (g%) and 5.82 g% in Pl 6 and Pl 17, respectively] and the line cultured in the bioreactor (5.93 g%) than in the roots derived from the field cultivation (4.02 g%). The results suggest that cultures of *P. grandiflorum* hairy roots may be a valuable source for obtaining saponins.

Keywords: *Platycodon grandiflorum*; Chinese bellflower; hairy roots; saponins; mist bioreactor

Introduction

Hairy, or transgenic roots are characterized by a rapid and stable growth, which importantly can be initiated and maintained in a hormone-free medium. They often synthetize approximately the same and in some cases even greater amounts of secondary metabolites compared to their mother plants [1,2] and the rate of production remains stable. This makes hairy roots of medicinal plants a good source of valuable compounds and obviously their large-scale culture in bioreactors holds immense potential for the pharmaceutical industry [3].

Platycodon grandiflorum (Jacq.) A. DC. (Campanulaceae) is an ornamental plant growing wild in northern Asia, in China, Korea, Japan and east Siberia. For culture it needs light, medium moisture, organically rich, well-drained loams and full sun to part shade.

Platycodi radix, the root of *P. grandiflorum*, has been used as food and in traditional oriental medicine to treat bronchitis, asthma and other pulmonary diseases. Triterpene saponins, called platycosides, are responsible for the medicinal value of this plant material. They have demonstrated a broad spectrum of therapeutic effects, such as antitumor [4–7], anti-inflammatory [8,9], hepatoprotective [10], antiatherosclerotic [11] and antinociceptive [12]. Additionally, these compounds were found to be a potent adjuvant of specific cellular and humoral immune responses, with the potential use in vaccine production [13–15]. Finally, saponins from *Platycodi radix* stimulated osteoblast differentiation via *RUNX2*, a principal osteogenic master gene for bone formation [16].

Although the extensive pharmacological studies of these valuable compounds were undertaken, to date the sum of saponins, not the chosen, in hairy root cultures of *P. grandiflorum* was determined only by our research team [17]. Besides, this is the first report of the growth of *P. grandiflorum* transgenic roots cultured in a mist bioreactor and their yield of saponins. The method of *Platycodi radix* saponins determination published in "Chinese pharmacopeia" [18] exhibited as no suitable for quantitative analysis of saponins from *P. grandiflorum* transgenic roots. Therefore, the authors elaborated their own method [17].

The compounds mentioned above are the main chemicals found in *Platycodi radix*. They are typical composed of oleaene backbones with two side chains: one is a 3-O-glucose linked by glycosidic bond, and the other is a 28-O-arabinose-rhamnose-xylose-apiose linked by an ester bond [19]. To date, more than 55 triterpenoid saponins have been isolated from *Platycodi radix* [20]. Examples are platycodins A–I and polygalacins D and D2. According to WHO monographs

* Corresponding author. Email: wszypula@wum.edu.pl

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standards [21] the content of saponins should exceed 2%. An effective HPLC method to analyze 18 platycosides from three-year-old Chinese bellflower roots was developed. Amongst them platycoside E showed the highest content (2.00 mg/g), followed by polygalacin D2 (1.77 mg/g) and 3″-O-acetylplatyconic acid A (1.35 mg/g) [22]. Considerably smaller amounts of 7 platycosides in the wild and hairy roots of *P. grandiflorum* received Kim et al. [23]. It could be due to the other origin or age of the plants. No information is given about that in the article. Authors developed hairy roots of *P. grandiflorum* with inserted gene of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. This enzyme catalyzes the synthesis of triterpenoids. The highest level of total platycosides (1.60 ±0.2 mg/g) was detected in the T7 line, what was 2.5-fold higher than that of the controls.

Chemical investigation on *Platycodi radix* revealed that, apart from saponins, in this plant material phytosterols B-amyrin and α-spinasterol [23], polycaetylene lobetyolin (0.019%) [24], phenylpropanoid esters [25], a mixed compound of n-tetracosanoic acid (lignoceric acid), n-hexacosanoic acid (cerotic acid), n-octacosanoic acid and α-monopalmitin [26], polysaccharides named PGPN, PGPA1, PGPA2 and PGPA3 [27], arabinogalactan PGAW1 [28], homogalacturonan PGA4-3b [linear poly-(1→4)-α-D-galactopyranosyluronic acid] [29] and polyunsaturated fatty acids: linoleic acid (20%), palmitic acid (7%), stearic acid (<1%) and oleic acid (<1%) [30] are present.

Aerial parts of *P. grandiflorum* contain no saponins but phenolics, particularly luteolin-7-O-glucoside and apigenin-7-O-glucoside [31]. From seeds flavoplatycoside, grandoside and four flavonoids: (2R,3R)-taxifolin, quercetin 7-O-glucoside, luteolin-7-O-glucoside and quercetin 7-O-rutinoside were isolated [32]. Flowers of *P. grandiflorum* comprise the following constituents: apigenin, apigenin-7-O-B-D-glucopyranoside, apigenin-7-O-(6″-O-acetyl)-B-D-glucopyranoside, luteolin, luteolin-7-O-B-D-glucopyranoside, luteolin-7-O-(6″-O-acetyl)-B-D-glucopyranoside, isorhamnetin-3-O-neohesperoside, 4-O-caffeoylquinic acid, chlorogenic acid methyl ester, 4-O-B-D-glucopyranosylcaffeic acid, lobetyolin, cordifolioidyne C, isomultiflorenyl acetate, B-sitosterol glucoside and α-spinosterol [33].

In vitro *P. grandiflorum* plants regeneration was elaborated by some scientists, including Urbańska et al. [34]. The aim was achieved by two ways: by somatic embryogenesis [35] or by two-step action: repeated shoot-section and following rooting of the developed new shoots [34,36,37]. In both methods developed plantlets, which grew to maturity in the soil.

Somatic embryogenesis was conducted as follows: mature zygotic embryos formed embryogenic calluses on Murashige and Skoog (MS) medium [38] supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 4.52 μM). From calli cell suspension culture was established using the same, but liquid, medium and the same phytohormone at the same dose. Following transfer to solid MS basal medium, cells gave rise to somatic embryos.

The MS medium was chosen as well for proliferation of shoots. For good proliferation rate medium should be supplemented with benzyladenine (BA; 4.4 μM) [36], with 2-isopenthenyladenine (2iP; 4.92 μM) and indole-3-acetic acid (IAA; 2.85 μM) [37], or with kinetine (KIN; 0.01 mg/l) and α-naphtaleneacetic acid (NAA; 0.01 mg/l) [34]. The highest rate of rooting of obtained shoots was induced on MS medium containing 4.90 μM or 9.80 μM indole-3-butyric acid (IBA) [37], 0.49 μM IBA [36] or on half-strength MS medium with IBA (0.05 mg/l) and saccharose (40 g/l) [34]. The medium enrichment on saccharose at a dose 40 g/l profitably influenced on the plantlets development, both shoots and roots [34]. The root, shoot and leaf thickness was found to be increased, while the length of shoots was decreased.

Urbańska elaborated the best conditions for maintenance the culture of non-transformed roots of *P. grandiflorum* (data not published). As the explants the plantlets growing by three passages on half-strength MS (MS/2) medium with IBA (1 mg/l) and saccharose (40 g/l) were used. The roots were cut from the shoots and their growth was tested on the following liquid media: MS/2, Gamborg medium (B5) [39] and woody plant medium (WPM) [40] full or half-strength media all with NAA (1 mg/l) and saccharose (40 g/l), the last mentioned also with IBA (1 mg/l) instead of NAA. The best for growing proved to be WPM medium with IBA (1 mg/l) and saccharose (40 g/l).

Besides *P. grandiflorum*, the other plants, which accumulate triterpene saponins in their roots, occur in the nature. They belong to genus *Panax* and *Glycyrrhiza*. For both genuses in vitro culture of different cells and tissues have been established successfully.

For many years the ability to increase secondary metabolites production by colchicine is used in the practice. For this purpose, the seeds of *Glycyrrhiza glabra* var. *glandulifera* were treated with colchicine. The amount of glycyrrhizic acid, the main biologically active saponin among that produced by licorice (*Glycyrrhiza*), in callus formed from the plantlets grown from seeds treated with colchicines increased [41]. The other way to enhance the amount of glycyrrhize acid is the employment of elicitors. Methyl jasmonate was most efficient in increment glycyrrhize acid production in *G. inflata* hairy roots. What is more, sucrose (6%) was optimal for growth and glycyrrhizic acid accumulation [42]. The ammonia/nitrate ratio and phosphate concentration influence glycyrrhizic acid accumulation as well. The content of glycyrrhize acid in adventitious roots of *G. uralensis* reached the optimum (0.47 mg/g) at an ammonia/nitrate ratio of 15:15 and when 0.625 μM phosphate was applicated [43]. The method of elicitation to improve glycyrrhizic acid productiveness was applied in the roots of 65-day old plants of *G. glabra*. Treatment of plantlets with 0.1–2 mM of methyl jasmonate or with 0.1 and 1 mM salicylic acid enhanced the production of glycyrrhizic acid, as compared to the controls [44]. There are the other ways for increasing the amount of glycyrrhizic acid. In the hairy roots of *G. uralensis* with inserted gene of squalene syntase, the enzyme, which plays a regulatory role in the biosynthesis of triterpene saponins, the highest glycyrrhizic acid content was obtained [45]. Finally, it worth to add that the scale-up of the suspension culture of *G. inflata* was established successfully in a bioreactor with a low-shear impeller [46]. The balloon-type bubble bioreactor can be utilized as well to provide a suspension culture [47].

Elicitation as the method for increase *Panax ginseng* saponins, known as ginsenosides, was used in hairy root culture of this species [48]. Jasmonic acid in the range 4.8–23.8 μM strongly improved total ginsenosides production. The same elicitor at 2.0 mg/l increased significantly ginsenoside yield in adventitious root culture of *P. ginseng* in flasks and balloon type airlift bioreactor [49]. The other elicitor, vanadate at a dose 50 μM, was used successfully to improve ginsenoside production in suspenson cultures of *P. ginseng* [50].

Initial sucrose concentration and inoculum size influence ginseng saponin production by suspension culture of *P. ginseng*. The maximum saponin production of 275 mg/l was achieved at 6 g/l of inoculum size and 60 g/l initial medium sucrose [51]. Temperature and light quality influence ginsenoside production by hairy root culture of *P. ginseng* in the bubble bioreactor. Ginsenoside production was optimal under 20°C/13°C day (12 h)/night (8 h) cycle. Ginsenoside accumulation was optimum in the cultures grown under fluorescent light [52]. Comprehensive review of influence of various factors on ginsenoside production published Wu and Zhong [53].

Material and methods

Hairy root culture induction

Hairy roots of *Platycodon grandiflorum* were established using *Agrobacterium rhizogenes* ATCC 15834 [17]. The bacteria were grown on yeast mannitol broth (YMB) solid medium [54] for 120 h at 25°C, in the dark. Next, single colonies were inoculated into yeast extract broth (YEB) liquid medium [55] with acetosyringone (0.2 mg/l) 72 h at 25°C, in the dark, with continuous shaking. The bacterial cultures were diluted (1:4) with YEB liquid medium before transformation. The leaves and stems of 50 micropropagated plants developed from the seeds and next growing for three passages on the MS/2 medium with IBA (1 mg/l) and saccharose (40 g/l) were directly wounded with a sterile needle containing bacterial suspension. The explants were cultured on phytohormone-free MS solid medium with sucrose (40 g/l), for 96 h at 25°C, in the dark, and then transferred to the same medium with the addition of Claforan® (500 mg/l). The roots emerged at the wounding site after 15 days of incubation under 40 μmol m^{-2} s^{-1} fluorescent lights for 18 h per day (Fig. 1).

To prove the transformed nature of hairy roots PCR analysis was done. Total genomic DNA was extracted from hairy roots using DNeasy Plant Mini Kit (Qiagen, USA). Primers for detecting *rolA* gene (5′-CGT TGT CGG AAT GGC CCA GAC C-3′ and 5′-CGT AGG TCT GAA TAT TCC GGT CC-3′), *rolB* gene (5′-CGT TGT CGG AAT GGC CCA GAC C-3′ and 5′-CGT AGG TCT GAA TAT TCC GGT CC-3′) and *rolC* gene (5′-TGT GAC AAG CAG CGA TGA GC-3′ and 5′-GAT TGC AAA CTT GCA CTC GC-3′) were used in amplification process. Primers amplified a 248-bp fragment of *rolA* coding region, 652-bp fragment of *rolB* coding region and a 490-bp fragment of *rolC* coding region respectively. Primers for detecting the *virC* gene (5′-ATCATTTGTAGC GACT-3′ and 5′-AGCTCAAACCTGCTTC-3′) outside the T-DNA of Ri plasmid were also used to eliminate the possibility of *A. rhizogenes* contamination of the hairy roots lines. The negative controls were DNA from non-transformed roots, clone number 26 and positive control was *A. rhizogenes*, strain ATCC 15834 respectively. The amplification conditions for *rolA* and *rolC* were as follow: 2 min melting at 94°C followed by 35 cycles of a 1 min melting at 94°C, a 1 minute annealing at 55°C and 1 min elongation at 72°C and final elongation for 5 min at 72°C. The amplification conditions for *rolC* were as follow: 3 min melting at 94°C followed by 30 cycles of a 1 min melting at 94°C, a 1 minute annealing at 55°C and 1 min elongation at 72°C and final elongation for 5 min at 72°C. PCR products were visualized by electrophoretic separation on 1% (w/v) agarose gels in 1× TBE buffer and staining with ethidium bromide.

Hairy root culture in shake flasks

Two hairy root lines (Pl 6, Pl 17) showing rapid growth were maintained in 250-ml Erlenmyer flasks with 50 ml of hormone-free WPM liquid medium with sucrose (40 g/l), which proved to be the best for optimal growth. The pH value was adjusted to 5.7 before autoclaving. The culture was incubated in the dark at 25°C on a rotary shaker (100 rpm) and subcultured every six weeks. The average inoculum size was 0.4 ±0.1 g fresh weight, which corresponded to 0.04 g

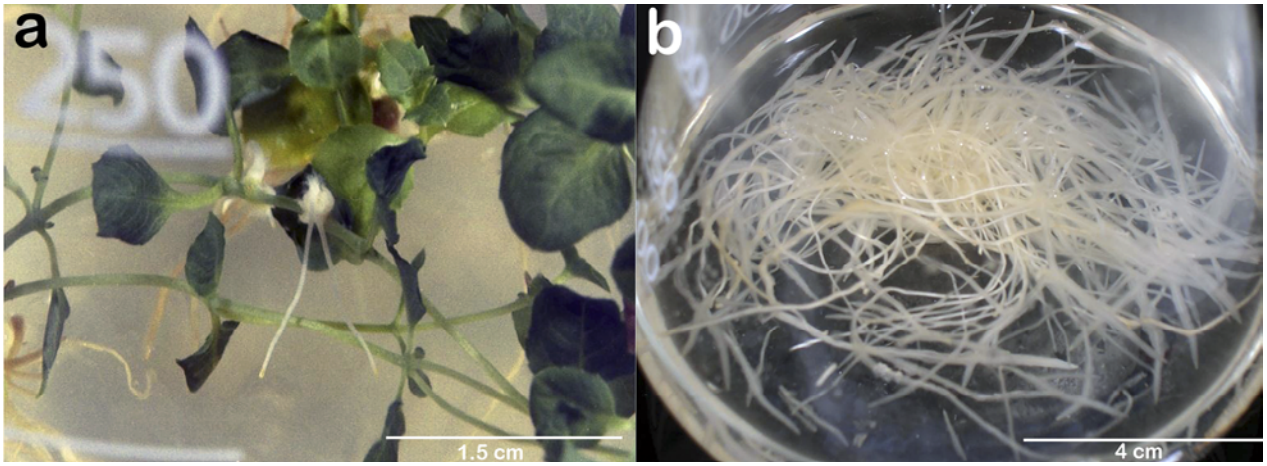

Fig. 1 **a** *Platycodon grandiflorum* hairy roots growing from inoculated site of plantlet, week 2 after inoculation. **b** Hairy roots on hormone-free WPM medium with sucrose (40 g/l). Culture week 2.

of dry biomass. After 14 passages, the transgenic line Pl 17, whose growth was superior to that the Pl 6 line, was used for studies in a bioreactor.

Hairy root culture in a bioreactor

The mist bioreactor used in this study measured 400 mm in height and 200 mm in diameter. It consisted of one 5 l-glass vessel fitted with a stainless steel mesh placed at the level of 150 mm from the bottom. The air inlet and the air outlet with filters were situated on the top of the column. The initial volume of the medium was 1.5 l. A peristaltic pump (MasterFlex® L/S, Cole Pomer, USA) maintained recirculation and dosing of the hormone-free WPM medium supplemented with 40 g/l sucrose through a polypropylene dispersal nozzle situated at the level of 70 mm from the bottom of the vessel. The operating time of the pump was 60 s with the medium supplied at 10 s intervals. The air flow rate was 0.4 l/min. The bioreactor was inoculated with four fragments of hairy roots. The inoculum size was 18 g fresh weight, which was proportional to the inoculum size 0.4 g in 50 ml of medium in the shake flasks. The roots grew at 23°C, in the dark, for 12 weeks. After 8 weeks of culture the medium was exchanged and 2 weeks later 1 l of the nutrient was added to the medium.

Measurement of root growth

The growth of transgenic roots was assessed as fresh and dry weight and by correlating the biomass growth with conductivity and sucrose uptake. For the fresh weight determination, the roots were gently pressed on filter paper to remove excess water, and weighed. The growth rate (*GR*) was calculated using the formula [56]: *GR* = final weight (g) – initial weight (g)/initial weight (g) × 100%.

The dry weight was determined after lyophilization. The conductivity (expressed in miliSiemens, mS) was determined using a conductivity meter (model CC-317, Elmetron, Poland; sensitivity 0.5%). The sucrose concentration (expressed as percentage) was measured using a saccharimetr (model RR 10, PZO, Poland; sensitivity 0.1%). The pH was determined using a pH-meter (model N5170, ELWRO, Poland; sensitivity 0.01).

The contents of three flasks of the transgenic hairy root line Pl 17 were harvested and analyzed for biomass, conductivity and sucrose concentration at 7 days intervals during the 8-week experiment. Samples of the medium from bioreactor obtained with a sampler were tested for conductivity and sucrose uptake from the beginning of week 8.

The convenient formula to describe the inverse relationship between biomass and conductivity during the shake flask culture was $\Delta m = a\,\Delta k + b$ where Δm (g) was the increase of dry biomass of hairy roots and Δk (mS/cm) was the decrease in conductivity. The a and b values were found to be 0.20 (0.03) and −0.08 (0.07) respectively. The correlation coefficient was 0.95 ($P = 0.0001$).

The relationship between biomass and sucrose level was described by the formula $\Delta m = a\,\Delta s + b$ where Δm (g) was the increase of dry biomass of hairy roots and Δs (%) was the decrease of sucrose level. The a and b values were found to be 0.30 (0.03) and −0.23 (0.06), respectively. The correlation coefficient was 0.98 ($P = 0.0001$).

Determination of saponins accumulation

The saponin content was determined in the hairy roots lines Pl 17 and Pl 6 cultivated in flasks and in the line Pl 17 cultured in the bioreactor and compared with the accumulation of saponins in 6-year-old roots from the field cultivation. Mother plant used as a control was derived from in vitro culture. Plantlets before transfer to the soil were micropropagated as mentioned above [34]. The 6-year-old roots were chosen for the experiment because the saponin content increases with the age of the plant.

Dried, pulverized roots (2 g) were put in a Soxhlet apparatus, soaked in methanol (150 ml), allowed to macerate for 17 hours and next refluxed for 6 hours, left overnight, and finally filtered. The filtrate was evaporated in vacuum to afford a dark brown residue, which was dissolved in methanol (50 ml). The methanol solution was poured into water (1/1, v/v) and then methanol was removed in vacuum. The aqueous residue was successively extracted 10 times with diethyl ether (4/1, v/v) and subsequently 5 times with butanol saturated with water (4/1, v/v). The butanol layers were combined and evaporated in vacuum. The residue was dissolved in methanol (10 ml). The methanol solution was poured into diethyl ether (35 ml). The resulting precipitates of the crude saponins were collected by filtration and dried at 105°C to constant weight.

The mean value and standard deviation represent the replicate of six determinations. Normal distribution and homogeneity of variances of the data were checked with the Shapiro–Wilk test and the Leaven's test respectively. Since in any of the analyzed groups there was not enough evidence to reject null hypothesis ($P > 0.05$), subsequent statistical evaluation of significance was performed by ANOVA test. Post-hoc Dunnett's test was used for individual comparison of groups with the control group. Otherwise LSD test (least significant differences) was used. Calculations were performed using the STATISTICA version 9.0 software.

Results

Induction of hairy roots

Infections of *Platycodon grandiflorum* leaf explants with *Agrobacterium rhizogenes* ATCC 15834 resulted in formation of hairy roots (Fig. 1). An analysis of amplification products obtained in PCR confirmed the presence of the *rolA*, *rolB* and *rolC* genes in genomic DNA of hairy roots. PCR analysis result for the *P. grandiflorum* are shown in Fig. 2.

Studies in shake flask cultures

Platycodon grandiflorum hairy roots grew in liquid WPM medium without growth regulators and demonstrated a typical transformed phenotype. Hairy roots tended to proliferate root hairs, which appeared to be white and healthy (Fig. 3). During the culture of the transgenic line Pl 17 the phases of growth typical of hairy roots were observed: lag phase, exponential phase and stationary phase. The exponential phase started from week 2 of growth and lasted until week 7. The growth rate after this phase was 3770%.

During this phase the considerable biomass increase was correlated with a marked decrease in the sucrose level in the

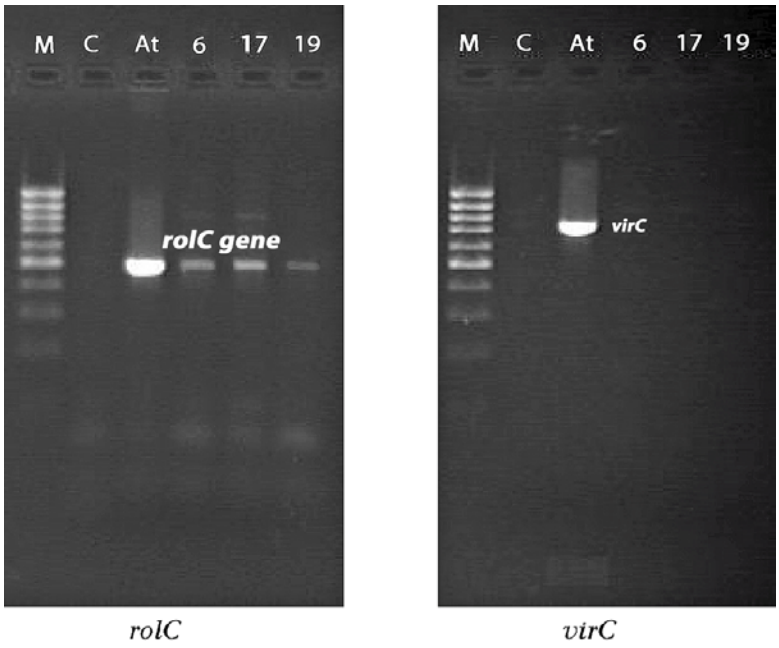

Fig. 2 Confirmation of transformation in hairy root lines by PCR reaction. The pictures shows agarose gel electrophoresis of PCR-amplified products performed with *rolC* primers and *virC* primers. Non transformed roots of *Platycodon grandiflorum*, clone No. 26 were used as negative control (C), while template for the positive (At) control was *Agrobacterium rhizogenes*, strain 15834. 100 bp DNA ladder was used for the estimation of DNA fragment size. No. 6, 17 and 19 corresponds to the transformed roots of *P. grandiflorum*.

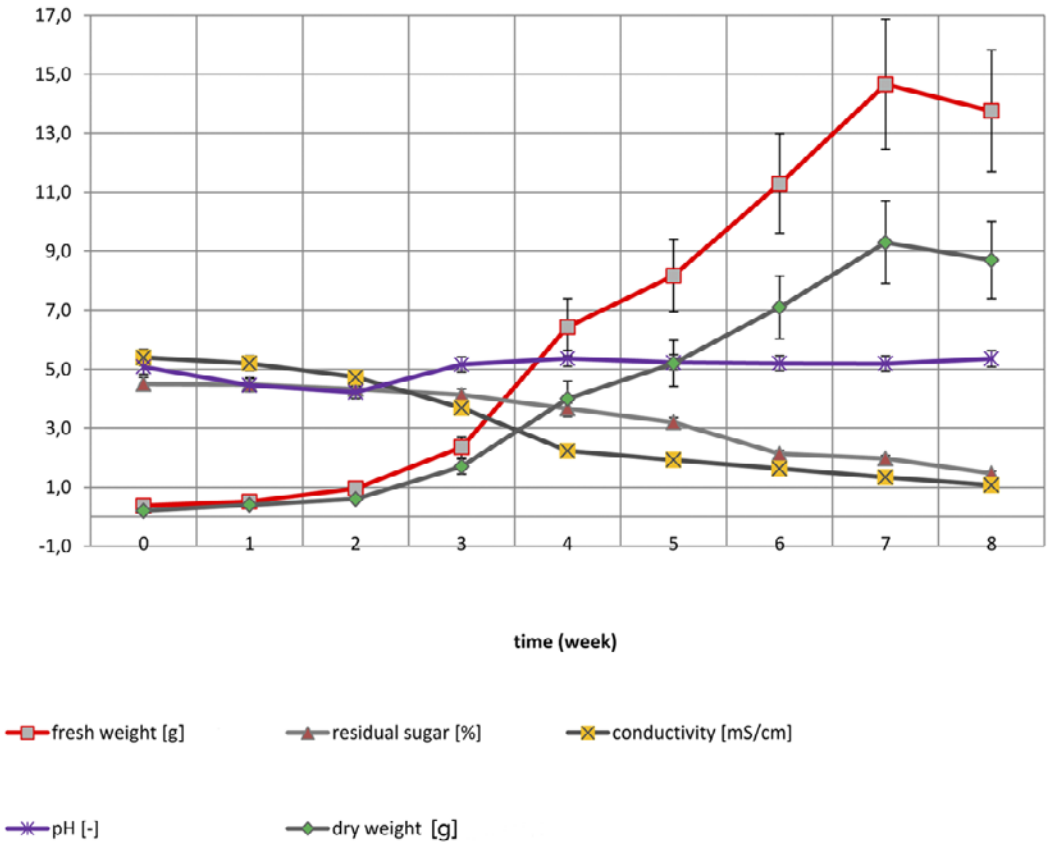

Fig. 3 Changes in medium conductivity and sucrose concentration in the medium and the biomass decrease during the culture of *Platycodon grandiflorum* hairy root line Pl 17 in shake flasks. Each point is the mean value of three estimates (three Erlenmeyer flasks from one passage).

medium (from 4.0 to 1.7%) and conductivity (from 5.30 to 1.24 mS/cm). The changes of hairy root biomass, conductivity, pH and sucrose level is shown in Fig. 3.

Post-hoc Dunett's test revealed that saponin concentration was significantly higher in both hairy roots lines ($P << 0.0001$ for line Pl 6, $P = 0.0005$ for line Pl 17) compared to normal roots derived from the field cultivation. The highest concentration was described for line Pl 6 ($0.0264 << P << 0.0001$). The mean values [g/100 g d.w. (g%)] of saponins concentrations for line Pl 6, line Pl 17 and field roots were 6.92 (0.88), 5.82 (0.84) and 4.02 (0.51) respectively.

Bioreactor studies

In our study, *P. grandiflorum* hairy roots grew well in a mist bioreactor. The roots were supported on a steel nest and sprinkled with nutrient WPM supplemented with 40 g/l sucrose. The roots occupied the whole space available and their growth was demonstrated by enhanced root length and lateral root induction (Fig. 4). After 8 weeks of culture the medium was replaced. The kinetic parameters from the medium poured out from the bioreactor provided evidence for considerable consumption of the mineral components and sucrose. The conductivity was decreased from 5.25 to 2.11 mS cm^{-1} and sucrose concentration decreased from 4.8 to 2.5%. In week 8 of culture a very rapid growth of *P. grandiflorum* hairy roots was observed which was correlated with fast exhaustion of the medium components. The conductivity decreased from 5.25 to 1.74 mS/cm and the sucrose concentration fell from 4.4 to 3.4%. The differences in conductivity and sucrose concentrations in the medium observed over the entire period of *P. grandiflorum* hairy root line Pl 17 culture in the bioreactor are shown in Fig. 5.

Fig. 4 *Platycodon grandiflorum* hairy root culture of Pl 17 line in 5-l mist bioreactor with liquid WPM medium supplemented with 40 g/l sucrose, cultivated for 12 weeks. After 8 weeks of culture medium was exchanged and 2 weeks later medium was refilled by 1 l of nutrient.

After culture day 71, when 1 l of medium was added, the conductivity and sucrose concentration did not change as much as described above. It was due to the necrosis of the hairy roots occupying the top of the mesh, which were not sufficiently sprinkled with the medium. The hairy roots from the bottom of the mesh continued to demonstrate enhanced length and lateral roots were induced.

After the culture of *P. grandiflorum* hairy roots in the bioreactor 591 g of fresh biomass was obtained, which corresponded to 36.6 g of dry weight (Fig. 6). The growth rate of the hairy roots was 3180%, comparable to the growth rate of hairy roots of the same line, when the exponential phase of growth in the shake flasks was completed. Moreover, comparable saponin contents were observed during both cultures of the hairy roots line 17 ($P = 0.80$). The mean saponin content of the hairy roots grown in the bioreactor was 5.93 g% (0.48).

Discussion

For the experiment WPM medium with increased up to 40 g/l amount of saccharose was chosen. Our previous study [34] showed that plantlets growing on MS medium with saccharose at a dose 40 g/l developed thicker roots compared to those cultivated on the MS medium with saccharose at a dose 30 g/l.

Hairy root growth can be easily monitored by measurement of the conductivity. An inverse relationship between biomass and conductivity was observed not only by our research team, but by Suresh et al. [57,58] as well. Authors [57] described this relationship occurred in hairy root culture of *Tagetes patula* by the same formula as used in *P. grandiflorum* hairy root shake flask cultures. The decrease in conductivity can be attributed to the intake of nutrients by growing hairy roots leading to a decrease in the number of ions.

Besides our experiment, an inverse relationship between biomass and sucrose level was observed by Sykłowska-Baranek et al. [59]. However, our results indicate that this correlation is weaker than that between biomass increase and conductivity decrease.

A nutrient mist bioreactor, used in this study, offers some advantages for growing hairy roots. It provides an environment with a low shear stress, where hairy roots receive rapid replenishment of nutrients and removal of toxic metabolites [60]. What is more, the dispersal of liquid medium in the gas phase could also facilitate better oxygen transfer due to its higher solubility in air than in aqueous medium [61,62]. A very similar type of bioreactor was used by Kuźma et al. [63]. In their study, *Salvia sclarea* hairy roots elicited by methyl jasmonate in bioreactor produced 9 and 3.8 times as much aethiopinone and salvipisone respectively, as elicited roots cultured in shake flasks. The nutrient sprinkle bioreactor

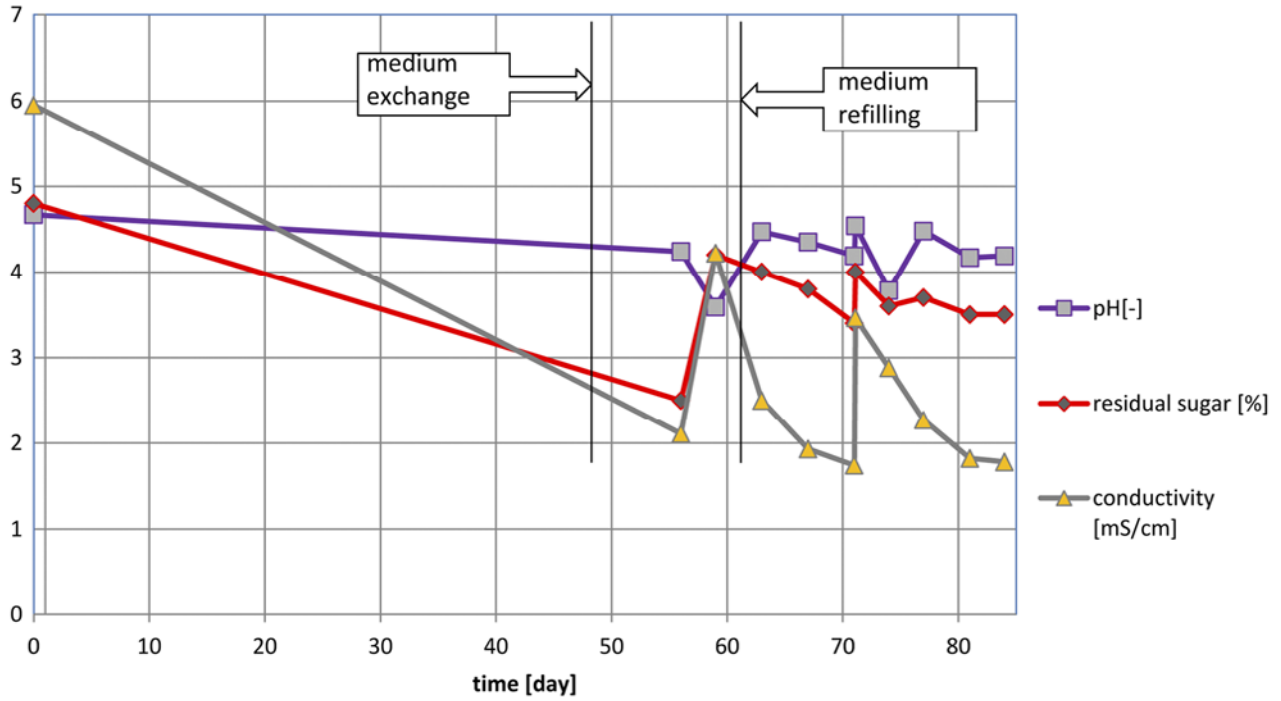

Fig. 5 Changes in medium conductivity and sucrose concentration in the medium during the culture of *Platycodon grandiflorum* hairy root line Pl 17 in bioreactor.

was used as well by Kochan et al. [64] to provide the culture of *Panax quinquefolium* hairy roots. The contents of six examined ginsenoside showed twofold higher level in hairy roots cultivated in bioreactor than in roots growing in the shaken flasks.

The medium exchange during *Platycodon grandiflorum* hairy root culture in the bioreactor considerably accelerated sucrose and nutrient consumption. These results were consistent with the experiment by Pawłowska and Chmiel [65]. They cultured *Paulownia tomentosa* hairy roots in a similar bioreactor using the same medium. After the entire medium exchange they observed a greater biomass increase and culture productivity than with the medium refilled with phosphate or ammonium nitrate only. The similar observations were done as well by Palazon et al. [66]. The medium exchange in the bioreactor was the requirement of the increased productivity of ginsenosides.

In the latter part of the *P. grandiflorum* hairy root culture in the bioreactor cell lysis in a population of roots put on the top of the mesh occurred, possibly because the culture medium did not remain in suitable contact with the cultured roots. Thus a modification of this bioreactor construction is required. Liu et al. [60] fitted a concentric draught tube with holes into their mist bioreactor which ensured better mist distribution compared to a standard mist bioreactor. The alternative can be the Wave bioreactor employment [66], in which the medium reach to any cell. The satisfactory results were received by Jeong and Park as well [67]. The most effective mass production of *Panax ginseng* hairy roots was achieved in several differently sized air bubble bioreactors compared to all other bioreactor types.

Fig. 6 *Platycodon grandiflorum* hairy root biomass of transgenic line Pl 17 after the culture period of 12 weeks in 5-l bioreactor with liquid WPM medium supplemented with 40 g/l sucrose.

Conclusions

Saponins obtained from the roots of *Platycodon grandiflorum* are valuable compounds showing a broad spectrum of pharmacological activities. It is noteworthy that the hairy root culture of *P. grandiflorum* presents weak haemolytic activity what makes this plant material safe in use [17]. Our results suggest that the hairy root culture is a good alternative to the field cultivation of the plant.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: concept of the study: NU, WJS; laboratory research and data analyses: NU, JG, WJS; writing of the manuscript: NU, JG, OO, WJS.

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