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BRIEF COMMUNICATION

Production of saponins from *Panax ginseng* suspension and adventitious root cultures

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Abstract

Biomass growth and ginsenoside production in cell suspension and adventitious roots of *Panax ginseng* C.A. Meyer cultures cultivated both in Erlenmayer flasks and a 3 dm^3 bioreactor were studied. The maximum content of ginsenosides was found in the suspension culture cultivated in the bioreactor (4.34 % dry mass), however the saponin content was limited to two major ginsenosides, Rb_1 and Rg_1. The production of ginsenosides in adventitious roots was lower (1.45 or 1.72 % dry mass), nevertheless, the full range of ginsenosides was detected.

Additional key words: callus, suspension cultures, bioreactor, ginsenosides.

Panax ginseng C.A. Meyer (*Araliaceae*) is a very important medicinal plant. Biologically active compounds – ginsenosides are extracted from its roots. Natural sources of wild growing plants in mountain areas, from Nepal to Manchuria and from eastern Siberia to Korea, have been overexploited and are therefore very limited. The current supply of ginseng mainly depends on field cultivation, which takes place mainly in Japan, Korea and north China but is a long and laborious process. Ginseng plants need 5 - 7 years of growth prior to harvest and the content of ginsenosides is low.

Application of bioreactors for *in vitro* cultivation of plant cell as well as organ cultures for the production of biologically active secondary metabolites was reported in many recent papers (Eeva *et al*. 2003, Shin *et al*. 2003/4). *In vitro* mass production in large-scale systems seems to be a potentially more efficient alternative for the production of ginseng bioactive components. It was found that the full range of ginsenosides that had been obtained from the roots of native plants could be isolated from adventitious roots cultivated in liquid Schenk and Hildebrandt media (SH) supplemented with 24.6 µM indole-3-butric acid (IBA) (Choi *et al*. 2000). Apart from ginsenosides as the main bioactive compounds, some polyacetylen compounds, which have been reported to have cytotoxic activity, were detected in the *Panax ginseng* roots (Newall *et al*. 1996).

The aim of our study was to establish *in vitro* cultures producing ginsenosides of the same quality as those found in native ginseng plants and to scale up its cultivation in a laboratory bioreactor.

Callus cultures were initiated from one-year-old roots of *Panax ginseng* grown in Czech Agricultural University in Prague (obtained from private *Panax* farm at Semenovka, Ukraine). Murashige and Skoog (1962, MS) medium was supplemented with 3 % of sucrose, 10 % of coconut water and different concentrations of dichlorophenoxyacetic acid (2,4-D) or only with 2,4-D without coconut water. Callus cultures were cultivated at 24 °C in dark and subcultured every four weeks.

Cell suspension cultures were initiated from callus cultures and cultivated in 500 cm^3 flasks with 200 cm^3 MS medium supplemented with 4.52 µM 2,4-D and 10 % coconut water. Erlenmeyer flasks were clutched on a

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Abbreviations: IBA - indole-3-butric acid, NAA - naphthalene acetic acid, 2,4-D - 2,4-dichlorphenoxyacetic acid, SH medium - Schenk and Hildebrandt medium, MS medium - Murashige and Skoog medium.

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platform shaker (*Innova 2100*, *New Brunswick Scientific*, New Brunswick, USA) (125 rpm) at 24 °C in the dark. Cells were subcultured every 14 d.

Adventitious roots of *Panax ginseng* were isolated from plantlets regenerated from somatic embryos and rooted in 1/3 strength solid MS medium. Separated roots were transferred to liquid MS medium supplemented with 1 µM naphthalene acetic acid (NAA) and cultivation was held in 500 cm^3 Erlenmayer flasks on a rotary shaker (125 rpm) for 20 d. Roots were subcultured every four weeks. Formation of adventitious roots was achieved in liquid SH medium supplemented with 24.6 µM IBA (Choi *et al.* 2000).

Suspension culture was inoculated into the bioreactor (*Applicon*, Schiedam, The Netherlands), total volume 3 dm^3) by filling it with 0.5 dm^3 of corresponding liquid medium (see above) under sterile conditions and with 0.5 dm^3 of suspension culture. The cells were cultivated for 4 weeks at 25 °C and 25 % oxygen saturation in the dark while mixing using "marine impeller" (60 rpm). After the first and second week of cultivation, 0.5 dm^3 of fresh medium was added to the bioreactor tank up to a total volume of 2.0 dm^3 in order to decrease cell density which caused cell sedimentation and death.

Adventitious roots were inoculated into the bioreactor by filling it with 1.5 dm^3 of corresponding liquid medium (see above) and 20.5 g of roots. After one month of cultivation, 0.5 dm^3 of fresh medium was added up to a total volume of 2.0 dm^3 in the bioreactor tank. The cultivation lasted 8 weeks. Adventitious roots grew slower than the suspension; the cultivation period was therefore doubled, following the double subcultivation period of standard cultivation in Erlenmeyer flasks.

For detection of ginsenosides plant material was homogenized and extracted with 7 cm^3 methanol g^{-1} (f.m.) for 5 d at room temperature. The sample was then filtered and evaporated to dryness under vacuum. The residue of the extract was redissolved in distilled water and partitioned with diethyl-ether, and twice in *n*-BuOH saturated with water. The *n*-BuOH layer was concentrated *in vacuo* to obtain a crude saponin fraction (Sanada 1974). HPLC analysis of the *n*-BuOH soluble fraction was then used for the identification and quantification of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1. HPLC analyses were performed in a system consisting of two high pressure pumps (*SDS 020* a *SDS 030*, *DeltaChrom*, *Watrex*, Prague, Czech Republic) with a mixer and PDA detector (*MD 1510*, *Jasco*, Tokyo, Japan); the stainless steel column (250 × 4 mm) packed with reverse phase Si-C18, 7 µm (*Biospher*, Prague, Czech Republic); flow-rate 1 cm^3 min^{-1}. The injection volume was set up at 0.02 cm^3 in the autosampler (*AS300*, *TSP*, San Jose, USA). Eluents: A - 15 % acetonitrile and water, B - 100 % acetonitrile. Gradient elution profile: 0 - 40 min, 0 - 35 % B; 40 - 45 min, 35 % B. The peaks were monitored by UV detection at 203 nm (Soldati and Sticher 1980, Pietta *et al.* 1986, Petersen and Palmqvist 1990). Each ginsenoside was identified by comparing its retention time and UV spectra with authentic ginsenosides purchased from *Carl Roth GmbH & Co*., Karlsruhe, Germany. The ginsenoside content was expressed in mg.g^{-1}(f.m.). The presence of ginsenosides was additionally confirmed by LC-MS.

For statistical analysis *Statistica 7.0* (*StatSoft*, Inc., Tulsa, USA) software was used.

The production of particular ginsenosides as well as the total production in different tissue cultures and cultivation systems differed considerably from that of native roots (Table 1), where the total production of ginsenosides was found to be 3.31 % of dry mass. A particular ginsenoside profile similar to that in native roots was produced by adventitious roots cultivated in 250 cm^3 Erlenmayer flasks on a rotary shaker; the total ginsenoside content varied from 1.2 to 1.8 % of dry mass. After the cultivation of adventitious roots was scaled up in the 3 dm^3 *Applicon* bioreactor, the total production was

Table 1. Production of ginsenosides by different types of *Panax ginseng* cultures: native roots cultivated in field conditions (for 5 years), callus culture growing on agar media, root culture growing in 500 cm^3 Erlenmeyer flasks, cell suspension culture and root culture cultivated in 3 dm^3 *Applicon* bioreactor (all for 4 weeks). Means ± SD of three independent experiments.

Ginsenoside content [mg g^{-1}]	Culture type native roots	callus culture	root culture	bioreactor - cells	bioreactor - roots
Total	33.12 ± 4.86	12.78 ± 0.55	17.25 ± 0.14	43.42 ± 1.19	14.48 ± 0.44
Rg_1	12.20 ± 2.63	4.20 ± 0.30	8.32 ± 0.60	2.83 ± 0.20	6.68 ± 0.49
Re	4.12 ± 0.73	0	2.43 ± 0.15	0	2.52 ± 0.11
Rf	1.74 ± 0.02	0.30 ± 0.02	0.35 ± 0.02	0	0.26 ± 0.02
Rb_1	7.86 ± 0.22	5.39 ± 0.12	2.04 ± 0.04	35.34 ± 0.77	2.62 ± 0.11
Rb_2	2.94 ± 0.13	0.60 ± 0.02	2.05 ± 0.08	5.25 ± 0.21	1.51 ± 0.13
Rc	0.99 ± 0.51	0.87 ± 0.06	1.45 ± 0.09	0	0.51 ± 0.02
Rd	3.27 ± 1.07	1.41 ± 0.03	0.61 ± 0.01	0	0.38 ± 0.15
Growth [g g^{-1}(explant)]	-	4.01	5.23	1.45	0.97

lower (1.45 % in dry mass). On the contrary, production in callus and suspension cultures differed greatly – the total ginsenoside content reached 4.3 % of dry mass, but was limited only to Rb1 or Rb1 and Rg1 (Table 1).

For the production of individual ginsenosides (Rb1 and Rg1 in our arrangement), the suspension system offered better yields and, due to the undifferentiated plant system, less problems with the extraction of the compounds desired. This system therefore was advantageous for the production of individual compounds.

As an alternative of the collection of wild *Panax ginseng* production and/or its field cultivation, the *in vitro* adventitious root culture has distinct advantages. First, the ginsenoside composition is nearly identical with that found in the native root. Second, "real" roots are produced that can be utilised directly both for the preparation of extracts and as native root material. Additionally, as a general rule, and due to their genetic stability, organs are less submitted to erratic metabolite production than undifferentiated cells (Bourgaud *et al.* 2001).

The effect of scaling up the suspension and the adventitious root culture is measurable, regarding both the content of individual ginsenosides and the total ginsenoside production (Table 1). Generally speaking, the differences in production are small. They can be attributed to the effect of differences in the geometry of the cultivating vessels and the type of aeration which influence mass transfer limitations of oxygen and nutrients, as well as to the fact that inhomogeneous culture systems cause cell sedimentation and death (Steward *et al.* 1999). Growth is also greatly influenced by the sensitivity of plant cells to shearing (see Table 1). As compared to cell suspension cultures, organ cultures generally display a lower sensitivity to shear damage. This situation is well known from published data (*e.g.* Bourgaud *et al.* 2001) and makes it necessary to optimise each specific cultivation system.

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