Chapter 40

Cultivation of root cultures of *Panax ginseng* in different bioreactors and in temporary immersion - Comparison of growth and saponin production

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Abstract: Different systems of large-scale cultivation of multiple adventitious roots of Panax ginseng C. A. Meyer were compared to cultivation in Erlenmeyer flasks. Adventitious roots were isolated from plantlets regenerated from somatic embryos and cultivated separately in liquid media. Multiplication of adventitious roots was performed in liquid Schenk and Hildebrandt (1972) medium containing 3 % sucrose, and 24.6 µmol indole-3-butyric acid.
The highest saponin content of 28.51 mg g^{-1} of dry weight was found in adventitious roots cultivated in the RITA® temporary immersion system (TIS). The best production of biomass was achieved in RITA® vessels and standard Erlenmeyer flasks placed on rotary shaker, followed by the Applikon 3-litre bioreactor and a simple airlift reactor. Saponin production in Erlenmeyer flasks was 10.07 mg g^{-1} of the dry weight while the production in the Applikon 3-litre bioreactor was only 3.60 mg g^{-1}. Other bioreactor systems tested showed neither significant saponin production nor high biomass production.

Key words: bioreactor, saponin, *Panax ginseng*, adventitious roots

Abbreviations: IBA – indole-3-butyric acid; MS – Murashige and Skoog (1962); NAA – naphthalene acetic acid; SH – Schenk and Hildebrandt (1972); TIS – temporary immersion system

1. Introduction

Panax ginseng C. A. Meyer (*Araliaceae*) is an herbaceous plant, which in Oriental medicine has a strong reputation since ancient times for being a tonic, regenerating, and rejuvenating, even though its pharmacological activity has not been fully elucidated. Ginseng grows wild in mountain areas, from Nepal to Manchuria, and from eastern Siberia to Korea but has been

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overexploited. Recently it was reported that ginsenosides and polyacetylenes isolated from ginseng roots have cytotoxic activity (Newal et al., 1996).

The current supply of ginseng mainly depends on field cultivation, which is a long and laborious process. Native ginseng plants need 5 – 7 years growth prior to harvest and the content of ginsenosides is low. *In vitro* mass production in large-scale systems seems to be potentially a more efficient alternative for the production of the bioactive components of ginseng.

In our previous study we compared saponin production in different ginseng tissue cultures namely callus, suspension culture and adventitious roots. We found a full range of ginsenosides distributed analogous as in the roots of native plants in adventitious roots cultivated in liquid Schenk and Hildebrandt medium supplemented with 24.6 µmol IBA while the saponin content in callus as well as in cell suspension cultures was limited to major ginsenosides, Rb1 or Rg1 (Langhansová et al., 2002; Langhansova et al., 2003b).

The aim of this study was to establish *in vitro* cultures producing ginsenosides from adventitious roots. We compared different cultivation systems to find a way for more efficient large-scale production.

2. Material and methods

2.1 Adventitious root induction

Adventitious roots of *Panax ginseng* were isolated from plantlets regenerated from somatic embryos and rooted on 1/3-strength MS medium (Langhansova et al., 2003a). Separated roots were transferred to liquid MS medium supplemented with 1 µmol NAA and cultivation was carried out in 500 ml Erlenmeyer flasks on a rotary shaker (125 rpm). Root proliferation occurred on liquid SH medium supplemented with 24.6 µmol IBA (Choi et al., 2000).

2.2 Bioreactor cultures

Adventitious roots cultures were cultivated in different bioreactors and temporary immersion systems (TIS) and in standard cultivation system in Erlenmeyer flasks:

1. Rocking temporary immersion system of our own construction (Figure 1b)
2. Simple airlift bioreactor (Figure 2b)
3. "Mafe" ½ l Bioreactor (Figure 3b)
4. RITA® – temporary immersion system (Figure 4b)
5. "Applikon" Bioreactor (Applikon, Netherlands) with total volume of 3 litre (Figure 5b)
6. Control: 250 ml Erlenmeyer flasks on a rotary shaker (125 rpm) (Figure 6b)

The roots were inoculated in culture vessels according to vessel volume (approx. 1 g per 100 ml) and cultivated for period of two months with the exception of simple airlift reactor where the roots were cultivated for three months. Cultivation was in dark at 24 ± 1 °C.

The Rocking TIS of our own construction is made of steel vessel (width 150 mm, length 240 mm, high 50 mm). Roots were placed on polyurethane foam floating loose in liquid medium in the reactor. Mixing was provided by rocking the reactor (Figure 1b).

The simple airlift bubble reactor made from a glass separation funnel was of a simple conical shape with gas-sparged mixing. The aeration was provided by air entering by a glass pipe from the top opening through a sparger at the bottom and as the air bubbles rise the biomass is lifted and the oxygen required is provided (Figure 2b).

"Mafe" (New Brunswick Scientific Co., INC., USA), a simple ½-litre bioreactor (i.d. 90 mm, height 160 mm) was assembled from an agitator, agitated by magnetic mixer, and a semipermeable silicone tubing system providing aeration. The culture was placed on the top of the supporting plate, which was perforated (10 holes (Ø 1 mm) per cm^2) and mounted 10 mm under medium level. The agitation ensured regular distribution of nutrients and dissolved gasses in the medium (Nepovim and Vanek, 1998) (Figure 3b).

RITA® (Vitropic s.a., France), temporary immersion system. Roots were placed on a polyurethane foam disc fixed in the upper container. The upper basket is mounted above a bell immersed in liquid medium. Flooding was set to 5 min h^{-1} and is provided by pressure applied to sterile air in the lower container which pushes the liquid medium into the upper container holding the roots for 5 min (Figure 4b).

In the "Applikon®" (Applikon, The Netherlands) glass autoclavable bioreactor with a stirred tank, the aeration was provided by a steel pipe ending in a sparger at the bottom of a glass tank. Mixing was set to 60 rpm (Figure 5b).

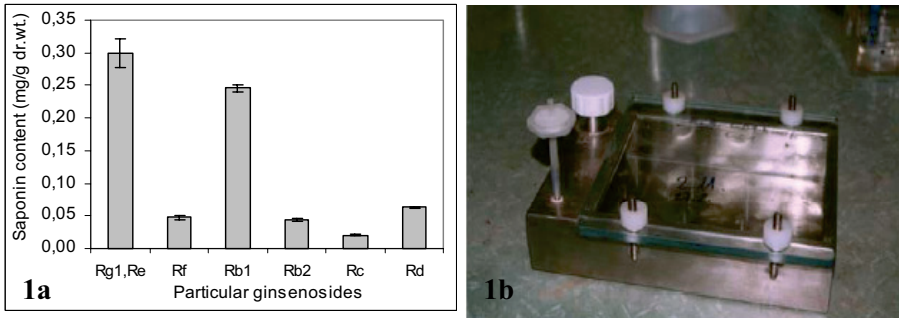

Figure 1: Results from (la) rocking TIS of our own construction (1b)

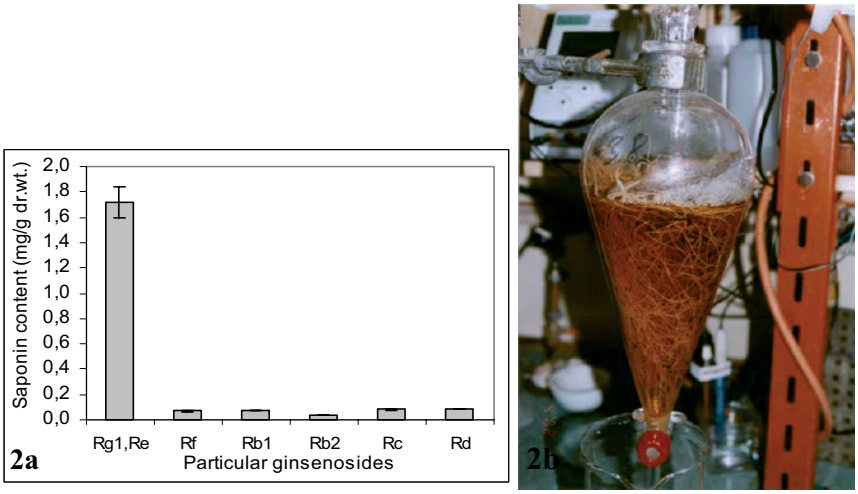

Figure 2: *Results from (2a) s*imple airlift bioreactor of conical shape made of separation funnel (2b)

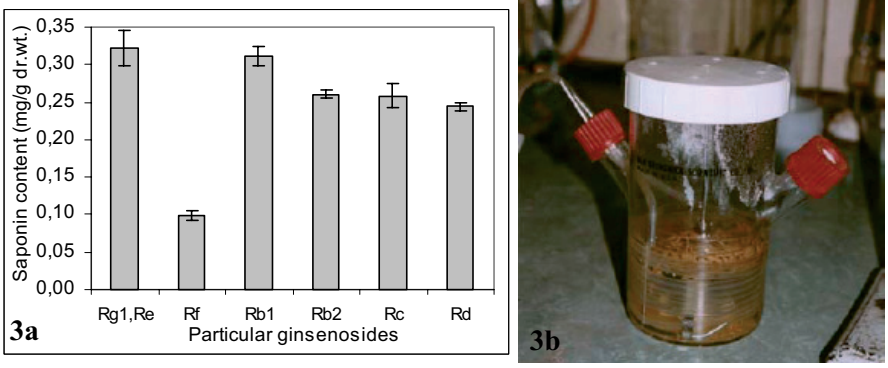

Figure 3: Results from (3a) MAFE, ½ litre Bioreactor (3b)

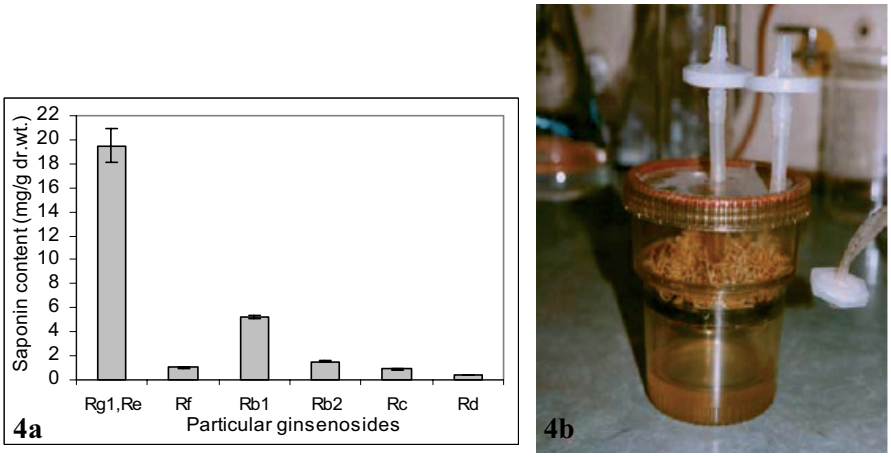

Figure 4: Results from (4a) RITA® – Temporary immersion system (4b)

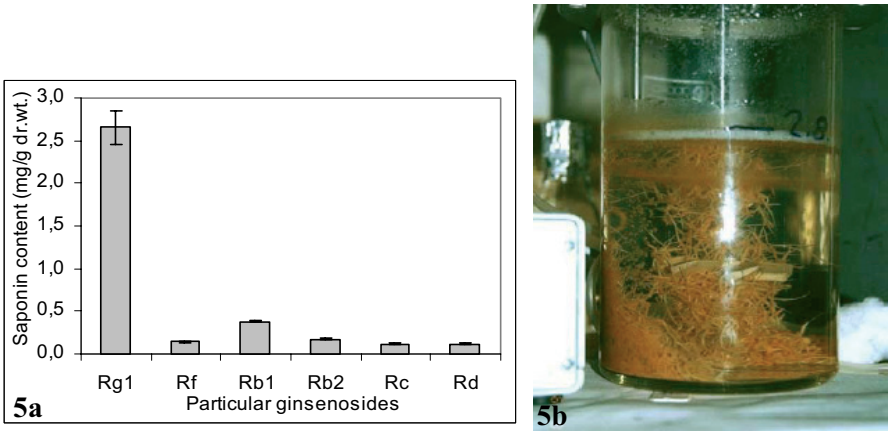

Figure 5: Results from (5a) the "Applikon" Bioreactor (Applikon, Netherlands) with total volume of 3-litre (5b)

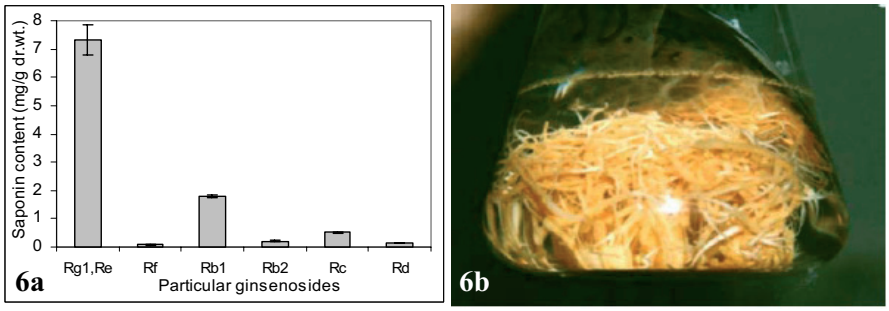

Figure 6: Results from (6a) 250 ml Erlenmeyer flasks on rotary shaker (125 rpm) (6b)

2.3 Detection of ginsenosides by HPLC

Adventitious roots were homogenized and extracted with methanol (7 ml g^{-1} fresh weight) for 5 days at room temperature. The sample was then filtered, and evaporated to dryness under vacuum. The residue of extract was re-dissolved in distilled water and partitioned with dietyl-ether, and twice in n-BuOH saturated with water. The n-BuOH layer was concentrated *in vacuo* to obtain the crude saponin fraction (Sanada, 1974). The n-BuOH soluble fraction was analyzed by HPLC for detection and quantification of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1.

HPLC analyses were performed using a system consisting of two high pressure pumps (DeltaChrom, SDS 020 a SDS 030) with a mixer (SunChrom GmbH) and PDA detector (JASCO, MD 1510); a stainless steel column (250 x 4 mm) packed by reverse phase Si-C18, 7 µm (Biospher); flow-rate 1 ml min^{-1}. The injection volume was set up at 20 µl in the autosampler (TSP, AS300). Eluents: (A) 15 % acetonitrile and water, (B) 100 % acetonitrile; Gradient elution profile: 0 – 40 min, 0 – 35 % B; 40 to 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 comparison of retention time and UV spectra with authentic ginsenosides purchased from Carl Roth GmbH and Co., Germany. Ginsenoside content was expressed in mg g^{-1} of dry weight. Presence of ginsenosides was additionally confirmed by LC-MS.

3. Results and discussion

We have monitored ginsenoside production in callus cultures previously (Langhansova et al., 2002). We found callus cultures to have a high proliferation rate and also high yielding. However, the production of saponins varied significantly during the year. The content ranged between 0 – 2 % dry weight and the saponin content in callus as well as in cell suspension cultures was limited to two major ginsenosides, Rb_1 or Rg_1. The full range of ginsenosides distribution analogous to roots of native plants was only detected in adventitious roots. Hence, in this study we have concentrated on adventitious root production *in vitro.*

The proportion of particular ginsenosides compared with native material is almost identical after cultivation in the rocking TIS of our own construction (Figure 1a). This suggests that the rocking TIS is the optimal way of producing these adventitious roots *in vitro*. The range in protopanaxadiol (ginsenosides: Rb_1, Rb_2, Rc and Rd) and protopanaxatriol (ginsenosides: Re and Rg_1) groups were similar also in RITA® TIS

(Figure 4a) and in Erlenmeyer flasks (Figure 6a). In roots cultivated in the simple airlift bioreactor (Figure 2a) and in Applikon bioreactor (Figure 5a), we observed inhibition of the protopanaxadiol group while in ½-litre "Mafe" bioreactor the biosynthesis of the protopanaxadiol group was significantly stimulated (Figure 3a).

Total saponin content in the root of nature Panax ginseng is commonly in the range of 1 - 3 % dry weight (Bruneton, 1995). The highest content of 28.5 mg g^{-1} dry weight (2.85 %) of ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1) in adventitious roots was achieved in RITA® TIS (Figure 4a). However, in order to reach effective production we have to combine two factors,; both the saponin content and the biomass growth. The biomass growth in RITA® TIS was high and comparable to cultivation in the maintenance system of 500 ml Erlenmeyer flasks. High growth was achieved also in roots cultivated in the Applikon 3-litre bioreactor and in the simple airlift reactor. Growth values in the rocking TIS and in the "Mafe" bioreactor were considerably lower (Figure 7). We considered these systems except RITA® TIS not suitable for ginseng adventitious roots biomass production because of the distribution of the different saponins and because the growth rate was much too low to be economical.

In conclusion, here we found root culture in the RITA® TIS optimal for ginsenoside production. It is possible to use Erlenmeyer flasks system as a proliferating step since this also gives a high growth rate, but sadly not the right profile of the saponins. Our next investigation will lead toward developing a better production system which should be based on establishment of

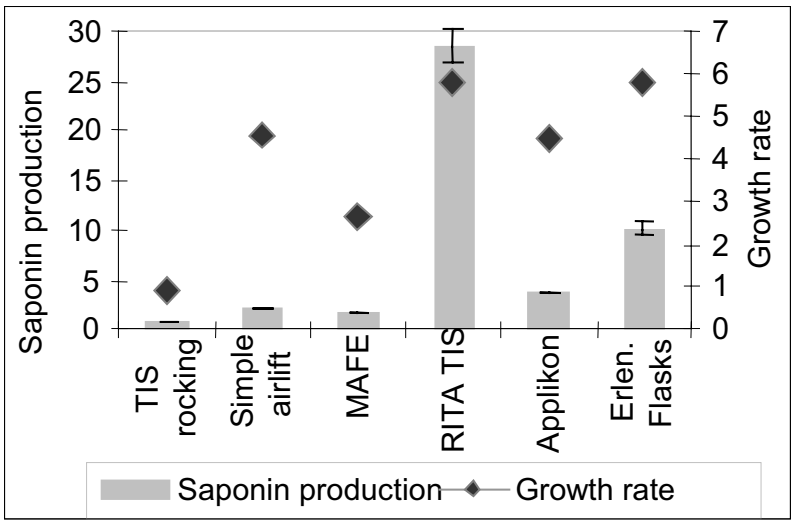

Figure 7: Total production of saponins and biomass growth in different bioreactor systems.

media composition in order to increase a biomass growth using the Erlenmeyer flask system in a proliferation step and to enhance ginsenoside production using RITA® TIS in following production step.

Acknowledgement

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References

Bruneton J (1995) Pharmacognosy, Phytochemistry, Medicinal Plants (p. 915) Lavoisier Publishing

Choi SM, Son SH, Yun SR, Kwon OW, Seon JH & Paek KY (2000) Pilot-scale culture of adventitious roots of ginseng in a bioreactor system. Plant Cell, Tiss. Org. Cult. 62 (3): 187-193

Inomata S, Yokoyama M, Gozu Y, Shimizu T & Yanagi M (1993) Growth pattern and ginsenoside production of Agrobacterium - transformed Panax ginseng roots. Plant Cell Rep. 12: 681-686

Langhansová L, Nepovím A, Maršík P & Vaněk T (2002) Saponin production from *in vitro* cell and root cultures of *Panax ginseng* C. A. Meyer. The 10th IAPTC&B Congres. Plant Biotechnology: 2002 and Beyond. (82-A) Orlando, USA

Langhansova L, Konradova H & Vanek T (2003a) Polyethylene glycol and abscisic acid improves maturation and regeneration of *Panax ginseng* somatic embryos. Plant Cell Rep. (in press)

Langhansova L, Vanek T & Marsik P (2003b) Production of saponins from *Panax ginseng* suspension and adventitious roots cultures and its „scale up" to laboratory 3-l bioreactor. Planta Med. (submitted)

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497

Nepovim A & Vanek T 1998. *In vitro* propagation of *Stevia rebaudiana* plants using multiple shoot culture. Planta Med. 64 (8): 683-780

Newall CA, Anderson LA & Phillipson JD (1996) Herbal Medicines. A Guide for Health–care Professionals (p. 296) The Pharmaceutical Press. London

Petersen TG & Palmqvist B (1990) Utilizing column selectivity in developing a high - performance liquid chromatographic method for ginsenoside assay. Jour. of Chromatography 504: 139-149

Pietta P & Rava PM (1986) Improved high - performance liquid chromatographic method for the analysis of ginsenosides in *Panax ginseng* extracts and products. Jour. of Chromatography 356: 212-219

Sanada S (1974) Studies on the saponins of ginseng. I Structures of ginsenoside - Ro,- Rb_1, - Rb_2, - Rc and - Rd. Chem. Pharm. Bull. (22) 2: 421-428

Schenk RU & Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204

Soldati F & Sticher O (1980) HPLC Separation and quantitative determination of ginsenosides from *Panax ginseng*, *Panax quinquefolium* and from ginseng Drug Preparations. Planta Medica 38: 348-357