Field Cultivation and *in vitro* Cultures, Root-Forming Callus Cultures and Adventitious Root Cultures, of *Panax quinquefolium* as a Source of Ginsenosides

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The content of six ginsenosides (Rb1, Rb2, Rc, Rd, Rg1, and Re) was studied in the roots of field-grown plants, as well as in root-forming callus cultures and adventitious root cultures of *Panax quinquefolium* using high-performance liquid chromatography (HPLC). The highest level of saponins was isolated from root hairs (128 mg/g dry weight). The examined *in vitro* culture synthesized all identified saponins, although in smaller amounts than those obtained from field cultivation. Metabolites Rb1 and Re dominated in the ginseng biomass from both field crops and *in vitro* culture.

Key words: American Ginseng, Ginsenosides, *in vitro* Culture, Adventitious Roots

Introduction

American ginseng (*Panax quinquefolium* L.) is one of several species of the genus *Panax* belonging to the Araliaceae family. The roots, as well as leaves, stems, and fruits are known to be a source of bioactive and therapeutic ingredients. Today, approximately 100 of these substances have been isolated. The triterpenoid saponins, known as ginseng saponins or ginsenosides, are the major pharmacologically active compounds in American ginseng. They are known to possess a number of healthy properties, including anticancer, antioxidative, antiangiogenesis, antiproliferative, and apoptosis-inducing properties, in addition to exerting multiple pharmacological effects on the central nervous, cardiovascular, and immune system, respectively. Moreover, they have demonstrated antidiabetic, antifatigue, and adaptogenic effects (Yuan *et al*., 2010).

Ginsenosides are divided into several main groups, depending on their aglycone moieties, which may be protopanaxadiol, protopanaxatriol, oleanane, or ocotillol. Ginsenosides, which are derivatives of protopanaxadiol and protopanaxatriol, are the main constituents of the raw material used in pharmaceutical products and dietary supplements, while the oleanane and ocotillol groups are the minor saponins. Ginsenosides can also be further differentiated on the basis of the nature and number of the sugars, position of hydroxy groups, and side chain modification at C-20 (Qi *et al*., 2010; Yuan *et al.*, 2010).

As a perennial herb, American ginseng is native to eastern North America, and grows in deciduous and mixed forests in the northeast of the United States of America and the Canadian provinces of Quebec and Ontario. As *P. quinquefolium* is a slow-growing plant, and the wild ginseng population is decreasing, ginseng is cultivated in many regions and countries: in Wisconsin, Michigan, North Carolina, and several other states in the USA, in Ontario and British Columbia in Canada (Punja, 2011), and near Lublin in Poland (Kołodziej, 2003; Kochan *et al*., 2008). Field cultivation of ginseng is difficult; seeds have to be stratified, both young and old plants are prone to diseases, and agricultural operations are labour-intensive and expensive. Moreover, field cultivation requires 3–7 years to obtain plants useful for saponin production.

Despite these problems, commercial demand for ginseng and ginsenosides is enormous. *P. quinquefolium* is used primarily in herbal medicines, and also as a dietary health supplement and additive in foods, beverages or cosmetics. Ginseng products are sold as tablets (regular and chewable), capsules, liquid extracts, tinctures, carbonated drinks, powdered root (bulk or packaged), sliced root, chips, soft gels, and teas as well as

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creams or other preparations for external use. These products are mainly produced from *P. ginseng* and *P. quinquefolium* (Yap *et al.*, 2007).

Another alternative source of ginseng biomass and saponins, respectively, is *in vitro* culture, more specifically a cell suspension of *P. ginseng*, developed on a commercial scale in the 1980 s at the Nitto Denko Corporation (Ibaraki, Osama, Japan) (Furuya, 1988; Hikino, 1991; Ushiyama, 1991; Hibbino and Ushiyama, 1998) and later at PhytoLife Ltd. (Tel-Aviv, Israel) (Wu and Zhong, 1999). There are no reports concerning the ginsenoside content of cultures of root-forming callus and adventitious roots of *P. quinquefolium* compared with the content of saponins obtained from the roots of field-grown cultivated plants of this species. Our study presents the contents of total and individual ginsenosides, respectively, in root extracts from field-grown plants in Poland and from *in vitro* cultures, including root-forming callus and adventitious root cultures.

Material and Methods

Materials

For the ginsenoside assay, the following materials of *P. quinquefolium* were used:

- Roots from an experimental field of the Agricultural University of Lublin, Lublin, Poland.
- Root-forming callus cultures. For this, seedlings obtained *in vivo* from the plantation of the Agricultural University of Lublin were used to initiate a callus culture of *P. quinquefolium*. Seeds were supplied by Prof. Bryan F. Zilkey (Dehli-Township, ON, Canada) from specimens confirmed to be *P. quinquefolium*, and germinated on soil. One-month-old seedlings were washed under running water with detergent, disinfected using 1% (v/v) sodium hypochlorite for 10 min, and cut into 1-cm pieces. Aseptic pieces were rinsed 3 times in sterile distilled water. The root-forming callus was maintained on MS (Murashige and Skoog, 1962) basal medium [containing 3% (w/v) sucrose, 0.7% (w/v) Difco Bacto agar (Sigma-Aldrich, Steinheim, Germany), a combination of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/l) and 1-naphthaleneacetic acid (NAA) (1 mg/l), and the cytokinin 6-benzylaminopurine (BAP) (0.2 mg/l)] after 3 weeks of cultivation.
- Adventitious root cultures. The roots developed on the callus described above were cut and transferred to liquid B-5 medium (Gamborg *et al.*, 1968) supplemented with NAA (0.2 mg/l). After every 28 d of culture, adventitious roots were transferred to fresh B-5 medium. Adventitious roots were used in the study after 8 passages.

The roots from field-cultivated plants were divided into main roots, lateral roots, and root hairs, dried on absorbent paper at room temperature, crushed, and ground in a mill, prior to extraction.

The media for *in vitro* culture were adjusted to pH 5.6–5.8 and sterilized in an autoclave at 123 °C and 1 atm for 16 min. Callus cultures were placed in plant culture tubes (19 cm x 2.5 cm, containing 25 ml of medium), and the adventitious root culture was grown in liquid B-5 medium on a rotary shaker (100 rpm). The cultures were incubated at (26 ± 2) °C, in the dark, and subcultured either every 5 weeks (callus) or every 4 weeks (adventitious roots).

All *in vitro* materials were dried as above and then used for ginsenoside extraction and high-performance liquid chromatography (HPLC) analysis.

Preparation of samples

Samples of 1 g dry raw material (weighed to 0.1 g tolerance) were placed in 250-ml flasks. Three extractions were performed in 50 ml of 80% (v/v) methanol for 30 min under reflux. The combined methanol extracts were evaporated to dryness in a vacuum evaporator under reduced pressure at 60 °C. Flasks were kept in a desiccator, and the residues were weighed.

Standard solutions

Ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 were purchased from Roth (Karlsruhe, Germany). A standard stock solution consisting of a mixture of the ginsenosides (10 mg/ml of each ginsenoside) was prepared in methanol of HPLC grade (Baker, Deventer, The Netherlands) and diluted as required.

HPLC analysis of ginsenosides

Dried extracts were dissolved in 2 ml of methanol (HPLC grade) and filtered through 0.2-μm pore diameter Millex®-FG hydrophobic

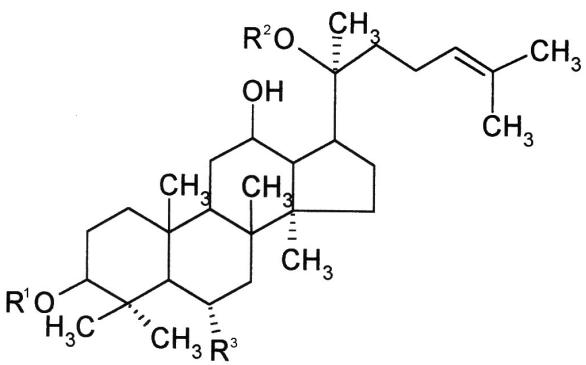
fluoropore filters (PTFE) (Millipore, Bedford, MA, USA). Aliquots of 20 μl were introduced to a liquid chromatography system consisting of a 250–4 LiChroART 100 RP-18 column (Merck, Darmstadt, Germany), a Waters 600 controller pump (Waters, Milford, MA, USA), and a UV-VIS Waters 996 detector connected to a Pentium 60 PC equipped with Millenium software (Waters).

Two different mixtures of acetonitrile with water were used as eluent, a 30:70 (v/v) acetonitrile to water mixture was used for the determination of Rb1, Rb2, Rc, and Rd (flow rate, 2 ml/min; analysis time, 45 min), and an 18:82 ratio was used for the determination of Rg1 and Re (flow rate, 2.5 ml/min; analysis time, 40 min). Ginsenoside detection was performed at a wavelength of 203 nm, and quantification [mg/g dry weight (dw)] was carried out by comparing retention times and peak areas of standards and samples (Soldati and Sticker, 1980).

Results and Discussion

Ginsenosides Rb1, Rb2, Rc, Rd, Rg1, and Re (Fig. 1) were identified by HPLC in the extracts from both the roots of field-cultivated plants and *in vitro* cultured material of *P. quinquefolium*.

Field cultivation of different species of *Panax* is the traditional and, still main, source of ginseng for commercial purposes, *i.e.* pharmaceuticals,



	Rb1	Rb2	Rc	Rd	Re	Rg1
R^1	Glc-Glc	Glc-Glc	Glc-Glc	Glc-Glc	H	H
R^2	Glc-Glc	Glc-Ara(p)	Glc-Ara(f)	Glc	Glc	Glc
R^3	H	H	H	H	O-Glc-Rha	O-Glc

Fig. 1. Chemical structures of ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1; Glc, glucose; Ara(f), α-L-arabinofuranose; Ara(p), α-L-arabinopyranose; Rha, rhamnose.

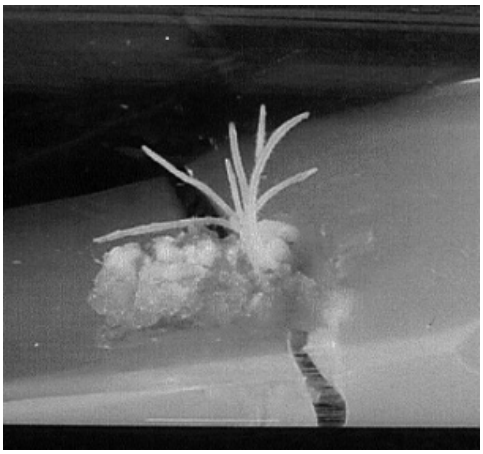


Fig. 2. Root-forming callus of *Panax quinquefolium*.

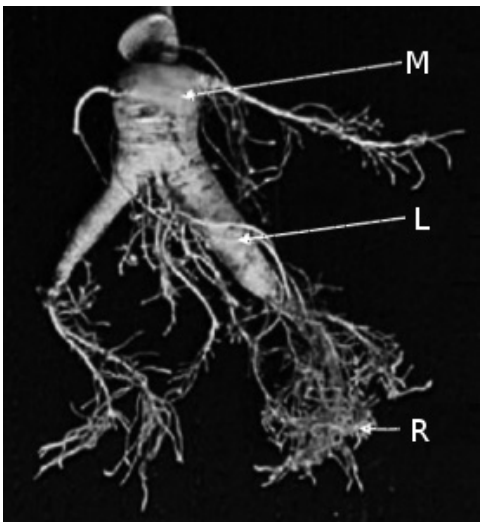


Fig. 3. *Panax quinquefolium* root from field cultivation; M, main root; L, lateral root; R, root hairs.

cosmetics, or functional foods. However, as field cultivation is labour-intensive, relatively expensive, and above all requires a growing period of 3–7 years until harvest, *in vitro* cultures are being investigated as possible alternative sources of ginseng biomass. This study evaluated the production of ginsenosides in root-forming callus (Fig. 2) and adventitious root cultures (Fig. 3). As these cultures are easyly to obtain, and the time for biomass harvest is much shorter than for field-grown plants (only 28 days), they appear to be an attractive research model.

Table I. Ginsenoside content in roots of *Panax quinquefolium*.

Plant material	Ginsenosides (mg/g dw ± SE)					
	Rb1	Rb2	Rc	Rd	Rg1	Re
Main roots	30.68 ± 0.46	0.49 ± 0.12	8.93 ± 1.98	4.52 ± 1.08	2.33 ± 0.56	28.10 ± 2.89
Lateral roots	41.86 ± 1.78	0.99 ± 0.31	17.58 ± 2.79	9.58 ± 0.55	2.86 ± 0.67	22.55 ± 3.66
Root hairs	35.44 ± 1.95	2.16 ± 0.42	22.44 ± 1.96	13.45 ± 1.66	3.05 ± 0.15	51.76 ± 4.34
Root-forming callus	2.06 ± 1.17	0.79 ± 0.08	0.51 ± 0.05	0.96 ± 0.11	0.99 ± 0.02	1.75 ± 0.58
Adventitious roots	3.03 ± 0.45	0.93 ± 0.06	0.24 ± 0.03	1.10 ± 0.16	1.22 ± 0.15	1.78 ± 0.27

Table I shows the ginsenoside content in different parts of 3-year-old roots of field-cultivated *P. quinquefolium*. The total content of the six examined ginsenosides was highest in the root hairs (128 mg/g dw) and lowest in the main roots. Previous studies (Qu *et al*., 2009; Shi *et al.*, 2007) yielded similar results for 5-year-old roots of *P. quinquefolium* and *P. ginseng*, but the roots examined in those studies had a lower content of ginsenosides, *i.e.* about 70 and 86 mg/g dw, respectively. However, Yamaguchi *et al*. (1988) reported that lateral roots were the most abundant source of saponins in *P. ginseng.* Our results reveal that metabolites belonging to protopanaxadiol derivatives represent 60–70% of all examined saponins from various parts of the roots of field-cultivated plants.

Regardless of the root parts used in the study, metabolite Rb1 and saponin Re were dominant among the protopanaxadiol and protopanaxatriol derivatives (Fig. 4), respectively. In previous studies, Rb1 and Re were described as the main ginsenosides in ginseng roots (Chung *et al.*, 2012; Qu *et al.*, 2009; Wang *et al*., 2005). Contents of Rb2, Rc, Rd, and Rg1 were seen to increase from root hairs to lateral roots to the main roots, where they were highest. The level of the metabolite Rb2 was the lowest, while that of the ginsenoside Rg1 was slightly higher (Table I).

In contrast to the results of this study, Qu *et al*. (2009) noted a significant increase in the content of metabolite Rg1 in root hairs as compared to the main roots. Chung *et al*. (2012) reported the absence of ginsenoside Rc from the roots of the *P. ginseng* variety "Jankyung" growing in three locations in Korea. These results are also in contrast to the observations of Shi *et al*. (2007) and Corthourt *et al.* (1999). These differences might be due to a multiplicity of factors related to genetic or enviromental determinants (temperature, soil conditions, light intensity, root microflora), the ginseng cultivar, and processing conditions (Chung *et al*., 2012; Du *et al*., 2004).

Root-forming callus and adventitious root cultures of *P. quinquefolium* synthesized all examined metabolites; their total amount was lower than in the roots of 3-year-old, field-cultivated plants (Table I). However, it should be empha-

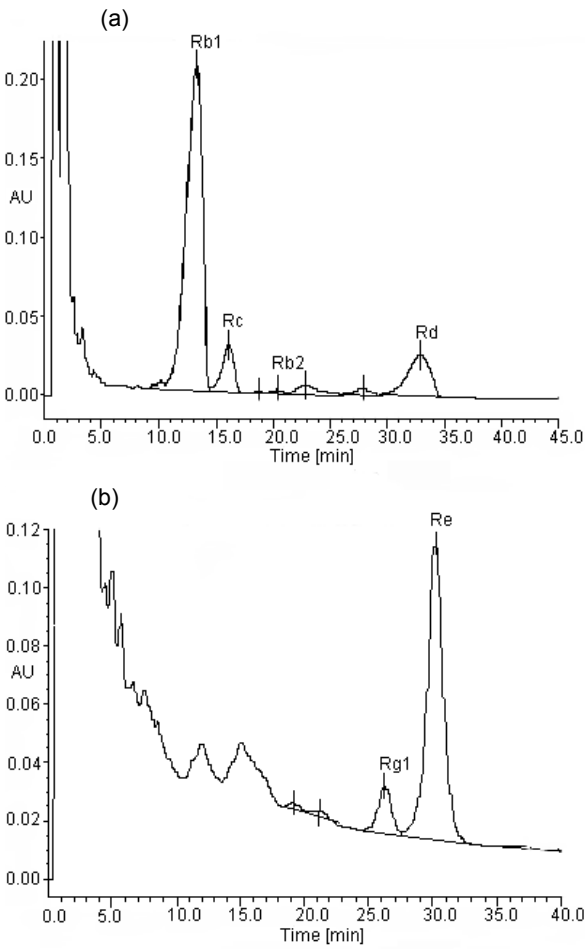


Fig. 4. Representative HPLC chromatograms of *Panax quinquefolium* root extract: (a) protopanaxadiols; (b) protopanaxatriols.

sized that the cultures grew only for 28 days. Studies by Choi *et al.* (2000) on 6-year-old roots of field-grown plants of *P. ginseng* showed that the total content of the examined saponins in some lines reached levels (7.23 mg/g dw) comparable to those in root-forming callus and adventitious root cultures of *P. quinquefolium*.

The results of this study indicate that protopanaxadiol derivatives predominate, regardless of whether the ginseng biomass was from field cultivation or *in vitro* cultures. In both *in vitro* cultures, metabolite Rb1 accumulated in greater amounts relative to other saponins. The content of ginsenoside Rc was the lowest of all saponins in the *in vitro* cultures, which was in contrast to the roots of field crops.

The profile of the individual ginsenosides isolated from the root-forming callus of another species of ginseng, *P. ginseng* (Bonfill *et al.*, 2002), is different from that of the *P. quinquefolium* culture. The callus of *P. ginseng* contains a much greater amount of protopanaxatriol derivatives, and among them Rg1 predominates. There are no previous reports on the ginsenoside content of *P. quinquefolium* root-forming callus culture.

Likewise, data in the literature related to the ginsenoside content of adventitious root cultures of *P. quinquefolium* is also very limited. While Ali *et al.* (2005) showed the content of total saponins to be comparable to our results, this present study is the first to present an analysis of the individual ginsenosides in *P. quinquefolium* adventitious root cultures.

A number of studies (Yu *et al.*, 2002; Kim *et al.*, 2003, 2004, 2005; Langhansova *et al.*, 2005; Ali *et al.*, 2008; Bae *et al.*, 2006; Jeong *et al.*, 2006, 2009; Paek *et al.*, 2009) have been published concerning the synthesis of ginseng saponins in adventitious root cultures of another species, *P. ginseng.* Some authors (Ali *et al.*, 2008; Bae *et al.*, 2006; Jeong *et al.*, 2006) reported that root suspension cultures of *P. ginseng* synthesize a lower level of total saponins than cultures of *P. quinquefolium.*

Kim *et al.* (2003, 2004) and Paek *et al.* (2009) demonstrated that lines of adventitious root cultures of *P. ginseng* accumulate all examined ginsenosides to a level similar to that of the cultures in this study. Ratios of protopanaxadiol and protopanaxatriol derivatives were similar, too, and metabolite Rb1 was dominant in the Rb group, but in contrast to our results, metabolite Rg1 dominated among the protopanaxatriol derivatives. However, other authors (Yu *et al.*, 2002; Langhansova *et al.*, 2005) reported that some of the adventitious root culture lines of *P. ginseng* synthesize more saponins (about 11 and 17 mg/g dw saponins, respectively) than the adventitious root cultures of *P. quinquefolium* presented in this paper.

Conclusion

The results presented in this paper confirm that both the roots of field-grown plants and *in vitro* cultures are sources of ginseng saponins. The amounts of ginsenosides in the *in vitro* cultures are lower than those in the roots grown by traditional cultivation, however, comparable biomass is obtained *in vitro* within a much shorter time.

Future industrial applications of ginsenosides obtained from *in vitro P. quinquefolium* cultures require further yield improvement. Hence, the introduction of *in vitro* cultures to the commercial preparation of ginsenosides requires further research, particularly in areas related to the genetics of the ginsenoside biosynthesis pathway, the optimization of *in vitro* culture conditions, and process scale-up.

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