BIOLOGIA PLANTARUM 51 (2): 235-241, 2007

Ginsenoside production, growth and cytogenetic characteristics of sustained *Panax japonicus* var. *repens* cell suspension culture

I.N. SMOLENSKAYA*[1], O.V. RESHETNYAK*, A.V. NOSOV*, S.E. ZORINIANTS*, A.L. CHAIKO**, Y.N. SMIRNOVA* and A.M. NOSOV*

*Department of Cell Biology and Biotechnology, K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya 35, 127276 Moscow, Russia**
*Institute of Food Chemistry and Technology, Ukrainian National Academy of Sciences and Ministry of Agricultural Politics, Osypovskogo 2a, 04123 Kyiv, Ukraine***

Abstract

Cytophysiological and cytogenetic characteristics of cell suspension culture of *Panax japonicus* var. *repens* were studied in relation to the accumulation of ginsenosides (GSs). The minimal time of cell number doubling was 1.3 ± 0.1 d and cell number increased 7 to 8-fold during growth cycle. The cell culture can be considered as aneuploid with about tetraploid (46 - 60 chromosomes) modal class. Upon long-term cultivation, the total content of GSs considerably increased and maximal concentration of GSs was 2.2 %(d.m.). The ratio of seven major GSs only slightly altered both over each and different subcultures. The overall amount of GSs of Rg-group significantly exceeded that of Rb-group. Cell volume and the number of large cellular aggregates with the higher proportion (by 20 %) of parenchymal cells increased late in the subculture. In this time the population contained about 20 % of the cells with doubled amount of nuclear DNA and accompanied with elevation in the GS content. These data prompted us to suggest that biosynthesis of GSs has a link with cell differentiation.

Additional key words: chromosome number, cytodifferentiation, ginseng, *in vitro* culture, nuclear DNA content, triterpenes.

Introduction

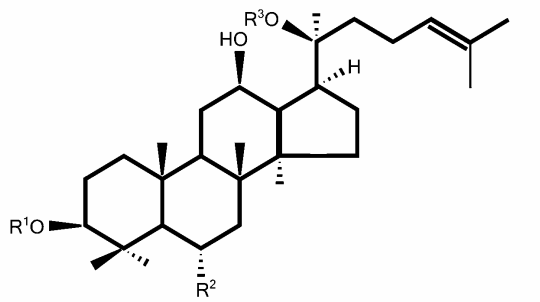
The isoprenoid biosynthetic pathway plays an important role in plant metabolism. Major terpenoid classes contain mono-, sesqui-, di- and triterpenes. Two distinct routes for synthesis of general precursor of terpenoids, isopentenyl diphosphate exist in plants. The acetate/ mevalonate pathway operates in the cytoplasm where sesquiterpenes and triterpenes are produced; 2C-methyl-D-erythritol-4-phosphate pathway occurs in plastids where mono- and diterpenes are formed (Estévez *et al*. 2001). On this basis, it is not surprising that such diterpenoid secondary metabolites (SMs) as sweet steviol-glycosides of *Stevia rebaudiana* were scarcely detected in the non-differentiated cell cultures and were synthesised in the morphogenic callus with the well-developed chloroplasts and a higher amount of these SMs were found in the mature stevia leaves only (Bondarev *et al*. 2001, 2003/04).

A different situation emerges with triterpenes that exhibit a wide range of structural diversity and biological activity. The bulk of plant cell cultures are capable of producing assorted triterpenes at a level of intact plants (Hayashi *et al*. 2001, Flores-Sánchez *et al*. 2002, Suzuki *et al*. 2002).

True ginsengs, the most famous low-growing and rare medicinal plants in the genus *Panax*, produce at least 30 different tetracyclic dammarane- and pentacyclic oleanane-type triterpene glycosides and they are referred to ginsenosides (GSs). The major active constituents of ginsengs are seven dammarane-type GSs (Fig. 1) and all these are glycosides belonging to two aglycones: 20(S)-protopanaxadiol that yields Rb-group of GSs (Rb_1, Rb_2, Rc, Rd) and 20(S)-protopanaxatriol giving

Received 20 May 2004, *accepted* 3 January 2006.
Abbreviations: HPLC - high-performance liquid chromatography; GS - ginsenoside; PJ - *Panax japonicus* var. *repens*; SM - secondary metabolite.
Acknowledgements: The financial support of the Russian Foundation for Basic Research (Projects 04-04-48139 and 05-04-48564) is much appreciated.
[1] Corresponding author; fax: (+07) 095-9778018, e-mail: ismolenskaya@ippras.ru

In memory of Prof. R.G. Butenko



Ginsenoside	R^1	R^2	R^3
Rb_1	-Glc(1–2)Glc	-H	-Glc(1–6)Glc
Rb_2	-Glc(1–2)Glc	-H	-Ara(pyr)(1–6)Glc
Rc	-Glc(1–2)Glc	-H	-Ara(fur)(1–6)Glc
Rd	-Glc(1–2)Glc	-H	-Glc
Re	-H	-O-Rha(1–2)Glc	-Glc
Rf	-H	-O-Glc(1–2)Glc	-H
Rg_1	-H	-O-Glc	-Glc

Fig. 1. The structure of seven major dammarane-type triterpene glycosides, ginsenosides: Ara(pyr) - arabinopyranose, Ara(fur) - arabinofuranose, Glc - glucopyranose, Rha - rhamnopyranose.

Rg-group of GSs (Re, Rf, Rg_1). Each GS has been shown to have various pharmacological effects, including immuno-modulatory, anti-stress and anti-cancer activities (Shibata *et al*. 1985, Craig 1999, Briskin 2000).

Wild harvesting of ginseng plants can be problematic in terms of biodiversity loss while field cultivation is a slow and laborious with possible variation in the plant quality. Cultivated plant cells and tissues have been explored as more efficient alternatives for both the mass production of ginseng and its bioactive GSs. Research on ginseng cell cultures started in the early 1960s (Butenko 1968) and for different *Panax* species considerable body of information related to GSs content in the plant cells *in vitro* had been accumulated. However, most available data were of a great variety indicating high- (Zhong *et al.* 1996, Liu and Zhong 1998), medium- (Choi *et al.* 1994, Langhansová *et al*. 2005, Wang *et al*. 2005) and low-GS (Nosov *et al.* 2000, Kunakh *et al*. 2003) ginsengs' cell cultures.

One of the interesting endemic ginseng plant with big rhizome is *Panax japonicus* var. *repens* (PJ), known as a variety of *Panax japonicus* C.A. Meyer. It grows under natural conditions in seaboard region of Russia. The major dammarane-type GSs are also synthesised in PJ plants (Shibata *et al*. 1985). The cell suspension culture of PJ had been obtained (Chaiko *et al.* 1999) and so far, more than 120 its subcultures have been performed to produce stable cell population.

The purpose of the present work is to characterise the *in vitro* cell population of PJ in order to clarify two issues: *1*) is PJ cell suspension suitable for biotechnological application in terms of GS productivity and stability? *2*) does the level of triterpene glycoside accumulation is related to growth, cytophysiological and cytogenetic characteristics of the cell culture?

Materials and methods

Plant material: Cell suspension culture of *P. japonicus* var. *repens* obtained in 1998 from the callus originating from the rhizome of the two-year-old plant was used. The cell suspension was grown on the Murashige and Skoog's (1962; MS) medium supplemented with 1 mg dm^{-3} kinetin, 2 mg dm^{-3} α-naphthylacetic acid, 2.5 % (m/v) sucrose, 0.1 mg dm^{-3} thiamine, 0.1 mg dm^{-3} pyridoxine, 0.5 mg dm^{-3} nicotinic acid and 90 mg dm^{-3} mesoinositol. Cultivation was carried out in the dark at 25 ± 1 °C in 500-cm^3 flasks on a shaker (90 - 100 rpm). The ratio of the medium to the initial inoculum was 6:1 (v/v).

Growth and cytogenetic parameters of the cell suspension: A number of cells was counted after maceration of the cell suspension with 10 % chromic acid at 60 °C for 15 min. The growth of the culture was characterised in terms of fresh mass and dry mass, which was obtained by drying of cell samples at 100 - 105 °C. To determine viability the cells were stained with 0.1 % (m/v) aqueous solution of eosin selectively penetrating only dead cells. The mitotic index was estimated daily on the carbolic-fuchsin-stained squashed cells, with at least three preparations for each sample and 1000 cells scored in each preparation. The chromosome number was determined by inspecting projection pictures of 100 metaphase plates photographed under a light microscope. The analysis of nuclear DNA content was conducted in the stationary growth phase on squashed cell preparations stained according to the modified Feulgen protocol and DNA content was measured by the two-wave length method, at λ_1 = 500 nm and λ_2 = 550 nm using *Reichert-Jung Univar* cytospectrophotometer (Vienna, Austria) (Zoriniants *et al.* 2003). Near 100 interphase nuclei of suspension cells randomly selected from two preparations were analysed. Mitotic cells of root meristems of *Vigna radiata* (cv. Berken) seedlings were used as a standard of DNA content (4C = 2.1 pg).

Analysis of the GSs by the HPLC: Cell samples (2 - 3 g) were extracted twice (3 h and 1 h) with methanol upon continuous stirring. The extracts were pooled, methanol was evaporated under vacuum at 45 - 50 °C and the dry residue was dissolved in distilled water. The extract was then passed through a *Sep-Pak* cartridge (*Tessek*, Prague, Czech Republic) filled with *Separon*^{TM} *SGX C-18* (60 μm). The cartridge was then washed with distilled water, 20 % (v/v) ethanol and finally 80 % ethanol. The dried residue of the latter fraction was dissolved in the acetonitrile:water (35:65, v/v) mixture, passed through the 0.2-μm pore (*Tessek*) microfilter and used for HPLC.

Separation of GSs was performed on the *Lichrosorb RP-18* (5 μm) column (4 × 250 mm) at a flow rate of 0.5 cm^3 min^1. Acetonitrile:water mixtures (35:65) and (22:78) were used to elute GSs represented by Rf, Rb_1, Rc, Rb_2, Rd and by Rg_1 and Re, respectively. Detection of GSs was carried out at 203 nm and their quantification was performed using individual GSs purchased from *Sigma* (St. Louis, USA). The sensitivity of individual GS detection was 20 μg g^{-1}(d.m.). Identification of aglycones was conducted by the GC-MS method (Butenko *et al.* 1993).

Results

Analysis of PJ cell suspension was carried out over three independent experiments corresponding to the 70, 85 and 100th subcultures; a number of cells and both dry and fresh cell masses were determined throughout the growth cycles (Figs. 2, 3). The growth curve obtained for PJ was typical for the most *in vitro* cell populations and showed (Fig. 2) that the lag-period lasted for 1 d, the exponential phase took 4 - 5 d, and thereafter the process was slowed-down followed by the stationary phase lasted from day 17 to day 19 - 20, and terminated by the degradation phase. Early in the subculture and during the exponential phase the time of cell number doubling was 1.3 ± 0.1 d. It should be noted that only small variations in the growth characteristics of the culture were observed in three independent experiments (Fig. 3). Overall the cell number increased about 7 to 8-fold, and dry and fresh masses increased 8.6-fold and 8.5 to 8.8-fold, respectively. The duration of the exponential phase and the beginning of degradation stage may be slightly changed depending on the amount of the initial inoculum.

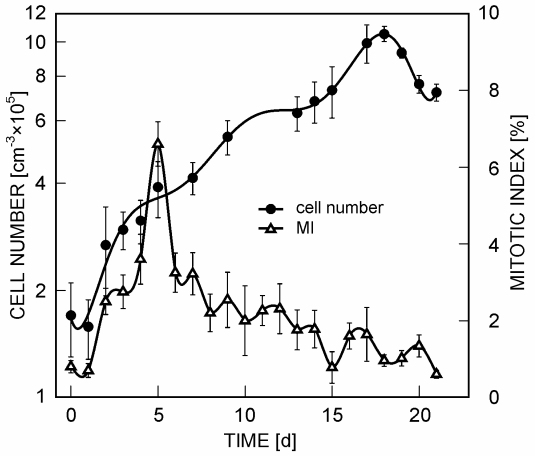


Fig. 2. Dynamics of cell number (semilogarithmic scale) and mitotic index (MI) during the growth cycle of PJ cell suspension. Values are the means ± SD from 3 - 5 cultural flasks of 70th growth cycle. Similar results were obtained for 85 and 100th subcultures.

After replacing the nutrient medium cells began to divide, and the number of mitoses by the fifth day of the subculture reached 7 %. In spite of several variations in the maximum of mitotic index the shape of the curve remained in fact unaltered during different subcultures. In 6 - 8 d of cell cultivation, the mitotic index decreased up to 2 - 3 %, and such value was kept with some fluctuations during subsequent 10 - 12 d (Fig. 2). A cell viability averaged throughout the all experimental periods was 85 ± 0.83 %. After replacing the nutrient medium a percentage of viable cells attained 90 - 92 %, whereas at the end of the growth cycle this index usually declined up to 83 - 85 % (data not shown).

Number of cells in an aggregates varied throughout the subculture: at the first day single cells and small cellular aggregates (from 1 to 10 cells) represented about

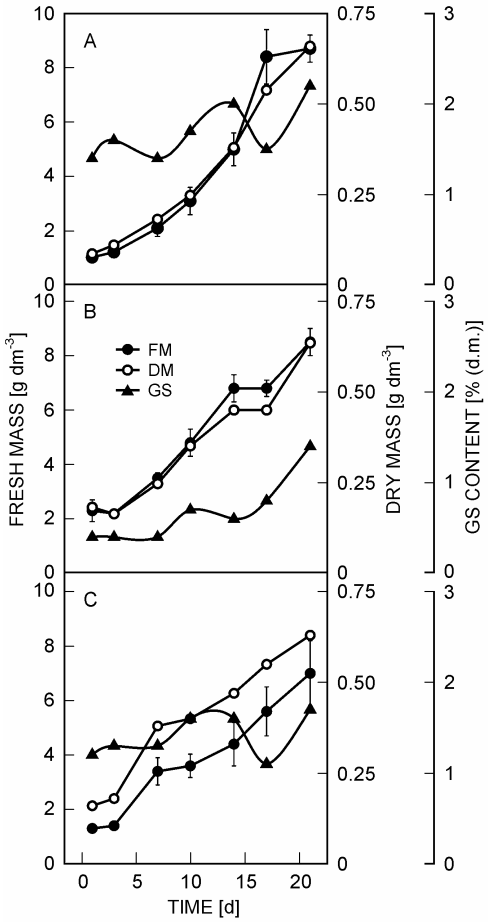


Fig. 3. Dynamics of fresh mass (FM), dry mass (DM) and GS content (GS) in 70 (*A*), 85 (*B*) and 100th (*C*) growth cycles of PJ cell suspension. Data are the means ± SD from 3 cultural flasks for mass parameters and the values from mixed, averaged samples for GS content.

Table 1. The content of cell aggregates [%] in PJ cell suspension with different number of cells in each aggregate. Values are means ± SD of 3 replications from 3 cultural flasks of 70th growth cycle.

Cultivation [d]	1-10	10-20	20-50	>50
1	22.0 ± 3.0	20.2 ± 1.6	22.8 ± 1.6	34.9 ± 1.3
4	12.2 ± 1.4	16.0 ± 1.2	30.5 ± 0.6	40.3 ± 1.5
11	9.0 ± 0.8	19.0 ± 2.0	22.0 ± 1.1	50.0 ± 3.2
21	8.4 ± 0.6	14.0 ± 3.2	29.0 ± 1.6	50.0 ± 2.5

22 %, while the large cellular aggregates containing more than 50 cells achieved 35 %. At the end of the growth cycle the amount of the large aggregates increased up to 50 % (Table 1). Similar results were obtained for different growth cycles.

The cultured cells of PJ differed widely in shape and size. A major part of the population was the meristematic cells of size below 100 μm as well as parenchymal cells with sizes varying from 100 to 200 μm. Later in the subculture the elongated cells which were more than 200 μm in length constituted less than 10 % of the cell population, while parenchymal cells reached 65 % (Table 2). Averaged cell volume derived from division of the fresh mass by the cell number declined after the first day of cultivation. Marked increase in cell volume was observed in 11 - 19 d, and this was most prominent at the end of the growth cycle (Table 2). The resemblance of different cell types distribution and changes in cell volume were observed in three independent experiments.

Table 2. Partitioning of different cell types [%] and changes in cell volume [$\mu m^3 \times 10^3$] in PJ cell suspension. Means ± SD of 3 replications from 3 cultural flasks of 100th growth cycle.

Cultivation [d]	Meristematic cells (<100 μm)	Parenchymal cells (100 - 200 μm)	Elongated cells (>200 μm)	Cell volume
1				6.68 ± 1.12
3	53.6 ± 2.1	46.2 ± 1.7	0.0 - 2.0	4.16 ± 0.74
5				4.60 ± 1.17
9				4.86 ± 0.68
11	49.8 ± 1.0	48.5 ± 0.4	1.8 ± 0.4	7.62 ± 1.35
17	35.2 ± 1.5	59.4 ± 3.0	5.4 ± 0.8	8.57 ± 1.27
21	24.5 ± 1.9	65.0 ± 4.0	10.5 ± 2.1	12.11 ± 1.34

Chromosome counts on the metaphase plates showed that *in vitro* cells of PJ were characterised by noticeable aneuploidy (Fig. 4*A*). The modal class of the chromosome number ranging between 46 and 60 represented 55 % of the cells. The cell partitioning by chromosome number remained practically unaltered throughout the all examined subcultures.

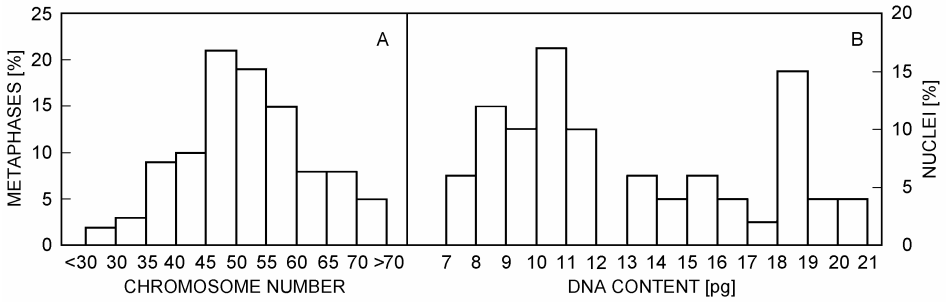


Fig. 4. Distribution of the chromosome number (*A*) and nuclear DNA content (*B*) in the cell suspension. Figure represents the values from 100th growth cycle. Similar results were obtained for 70th and 85th subcultures.

In the stationary growth phase, the amount of nuclear DNA in suspension-cultured cells was found to vary considerably, and near 50 % of cells contained about 8 - 12 pg of DNA, whereas maximal DNA level corresponded to 21 pg (Fig. 4*B*). In addition, it can be seen that the suspension contained 20 % of cells with doubled amount of DNA (18 – 20 pg) relative to the cells constituting the greater part of the population.

In qualitative terms, at least seven major GSs of both Rb- and Rg-group inherent in PJ plant were synthesised in the cell suspension and were detected in all samples tested. According to the quantitative analysis, the total content of GSs over the 70th and 100th subcultures varied from 1.2 % [11.7 mg g^{-1}(d.m.)] to 2.2 % [21.9 mg g^{-1}(d.m.)], whereas it was in the range of 0.4 - 1.4 % of dry mass over the 85th growth cycle (Fig. 3). Fluctuations of the GS accumulation were evident especially in the 70th and 100th subcultures. In general, a tendency of increasing of the GS amount took place throughout each of studied experiment (Fig. 3). Nevertheless, the proportions of individual GSs were affected slightly both over individual growth cycle and those separated by 90-week-long interval. The overall amount of GSs of Rg-group [9.8 - 16.5 mg g^{-1}(d.m.)] significantly exceeded the net content of GSs of Rb-group [1.7 - 4.5 mg g^{-1}(d.m.)] and the ratio of Rg_1 to Rb_1 (2.25 ± 0.56 and 2.76 ± 1.17 for 70th and 100th subcultures, respectively) was rather stable (Fig. 5).

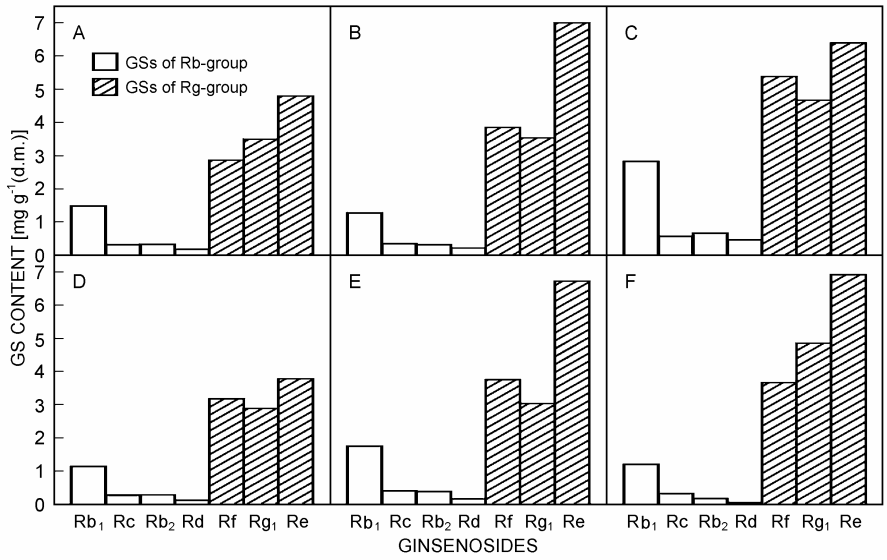


Fig. 5. The content of seven individual GSs in PJ cell suspension. Data are the means of mixed, averaged samples from 3 cultural flasks at the 1st (*A*, *D*), 10th (*B*, *E*) and 21st (*C*, *F*) days of 70th (*A*, *B*, *C*) and 100th (*D*, *E*, *F*) subcultures separated by 90-week-long interval.

Discussion

The suspension-cultured cells of PJ have been highly adapted to the *in vitro* conditions. At the end of the first year of cell population development, its growth indices in terms of dry and fresh masses were in the range of 4.7 - 5.7 (Chaiko *et al.* 1999). For the 100th subculture, these indices increased more than 1.5 times. On the average, the suspension culture had a sufficiently high and stable viability and characterised by a short lag-phase and displayed the maximum mitotic index 7 % which is typical for many actively proliferating cell populations *in vitro*.

From a biotechnological standpoint economically profitable cell cultures should be advantageous for production of biomass and biologically active substances and should be stable in a level and spectrum of SMs of interest. Firstly, bearing in mind above requirements, we compare the growth curve of PJ *in vitro* cell population with that of another cell cultures of some *Panax* species (Liu and Zhong 1998, Kunakh *et al*. 2003, Wang *et al*. 2005). From this comparison it is apparent that growth indices of PJ are at least two-fold higher, suggesting that PJ cell culture has substantial potential for biomass production.

The second important aspect is the level of GS biosynthesis. It is necessary to stress that there are two ways to quantify the ginsengs' saponins: determination of individual GSs concentrations, and that of total saponin. During the 1990's in some publications mention was made of ginsengs' cell cultures with large amount of total ginseng saponin (Zhong *et al.* 1996, Liu and Zhong 1998). It is reasonable to suggest that values of ginsenoside-type saponin content about 7 - 11 % were more than 2.5-fold overestimated because of nonselective vanillin reagent and incorrect multiplication factor were used for ginseng saponin detection and calculation, respectively. Hence, it would be more properly to compare the data where quantification of individual GSs was performed. On reviewing the recent literature data it may be stated that the level of total GSs may vary with cell or tissue strain and cultivation condition, for example: 30-year-old strain of *P. quinquefolium* (Nosov *et al.* 2000), 13-year-old cell suspension of *P. ginseng* (Kunakh *et al*. 2003), non-organogenic callus of *P. ginseng* (Choi *et al.* 1994), root-forming callus of *P. ginseng* (Bonfill *et al*. 2002), high-density cell culture of *P. notoginseng* after methyl jasmonate elicitation and sucrose feeding (Wang *et al*. 2005) accumulated up to 0.1, 0.23, 2.0, 5.4 and 2.3 %(d.m.) of GSs, respectively. In our case the maximal concentration of total GSs in the cell suspension of PJ was 2.2 % (d.m.) and recently it has been attained more than 3 % (our unpublished data). Notice that the value of this parameter increased more than 20 times as compared to that found since the initial cell culture was obtained (Chaiko *et al.* 1999). From the aforesaid, it might be assumed that PJ cell suspension has a reasonably high the net content of GSs.

The third biotechnological point, as applied to ginsengs' cell cultures, is stability in the level and spectrum of GSs. It is appropriate at this moment to remind that the biological activity of ginseng is largely determined by the proportion of individual GSs rather than their absolute content. Overall, the two most important GSs are Rb_1, a depressant of the central nervous system, and Rg_1, a weak stimulant of the central

nervous system (Shibata *et al*. 1985). The analysis of the GS spectrum showed that at least seven major GSs inherent in PJ plant were synthesised in the cell suspension and their proportions were rather stable both over individual growth cycle and different ones separated by prolonged interval (Fig. 5) as compared with unstable GS composition of other ginsengs' cells *in vitro* (Nosov *et al*. 2000, Bonfill *et al*. 2002). The cell culture of PJ has the high ratio of Rg_1 to Rb_1 and GSs of Rg-group are prevalent. In *in vitro* cultures of *P. ginseng* (Choi *et al.* 1994, Bonfill *et al*. 2002) and *P. notoginseng* without methyl jasmonate elicitation (Wang *et al*. 2005) the prevalence of the GSs of Rg-group was found as well. It is notable that in the roots of the *P. ginseng* the reciprocal ratio of the GSs of the two groups had been revealed (Bulgakov *et al*. 1991, Choi *et al*. 1994).

Therefore, it might be assumed that PJ cell suspension is suitable for biotechnological application in terms of GS productivity and its stability.

The genome of cultured plant cells is known to be capable of undergoing considerable changes (Bayliss 1980). A certain cytogenetic status of *in vitro* cells is generated, and it depends on the plant species, epigenetic features of the explant and chosen cultivation conditions. The PJ plant is characterised by three chromosome numbers including diploid ($2n = 2x = 24$) and tetraploid ($2n = 4x = 44, 48$) ones (Fedorov 1969). Based on the chromosome numbers in the suspension-cultured cells of PJ (Fig. 4*A*) it will be obvious that a tendency to the polyploidisation is inherent in this cell culture, which can be considered as aneuploid with about tetraploid (46 - 60 chromosomes) modal class. Similarly, for *P. ginseng* cell cultures the maintenance of about tetraploid modal class along with a high level of aneuploidy was observed (Kozyrenko *et al.* 2001, Kunakh *et al*. 2003). Although the greater GS production was revealed in the cell cultures with cytogenetic characteristic close to intact plants (Kunakh *et al*. 2003), it would be misleading to suggest a close relation between ploidy level and GS production since PJ cell suspension in hand and one of *P. ginseng* (Kunakh *et al*. 2003) have about tetraploid modal classes but differ ten-fold in GS accumulation.

The results obtained indicate fluctuations in the GS content with a tendency of increasing of the GS amount over the growth cycle of PJ cell suspension. These changes more likely were related to a partial cell synchronisation which was represented by the changes in proportions of different cell types during the subculture.

In the context of cell suspension unimodality based on chromosome numbers, an important point is the presence of the second peak in the DNA content late in the subculture, when the mitotic activity was minimal, suggesting that the population contained about 20 % of the cells with doubled (endoreduplicated) amount of nuclear DNA, which remained in the G_2 phase of the cell cycle or transited into the stage of the proliferative rest (R_2). It is necessary to stress that the number of the large cellular aggregates increased considerably late in the subculture; in this time average cell volume raised significantly and the suspension contained the higher portion of parenchymal cells (Tables 1, 2). It is safe to assume that such cells possessed doubled amount of DNA and were under a cytodifferentiation. This cell status was accompanied with marked increase in the GS content.

There are a number of arguments to support the assumption that a cytodifferentiation is required for elevation of GS biosynthesis: *1*) It is known that in *P. ginseng* and *P. quinquefolium* plants (Kubo *et al.* 1980, Smith *et al.* 1996) most of GSs are localised in the outer part of the roots consisting of parenchymal cells. *2*) In plants, known physiological function of triterpene saponins is defense molecules against pathogenes (Dixon 2001). The biosynthesis of such type SMs is induced by jasmonates, key signal compounds in the elicitation process (Gundlach *et al*. 1992). Treatment with methyl jasmonate increased GS biosynthesis in cell culture of *P. notoginseng* (Wang *et al*. 2005), expression of genes related to triterpene biosynthesis as well as accumulation of triterpenes in cell suspension of *Medicago truncatula* whereas sterol composition remained unaffected (Suzuki *et al*. 2002). Jasmonic acid had no effect on the coumarin production in embryogenic cell line of *Angelica archangelica* (Eeva *et al*. 2003). In addition, upregulation of genes related to triterpene biosynthesis occurred after methyl jasmonate treatment in ginseng adventitious roots but this was not the case for gene related to sterol formation (Lee *et al*. 2004). Note that jasmonates effectively prevented mitosis (blocked cells in G_2 phase) when applied during DNA synthesis in tobacco cells (Świątek *et al*. 2002) and inhibited cell growth in *P. notoginseng* culture (Wang *et al*. 2005). All the above indicate that jasmonates do not enhanced biosynthesis of ubiquitous and non-defensive small molecules (phytosterols and coumarins) but do increased various triterpenes and GSs formation since cell cycle arrest and certain cell differentiation are required for these processes. *3*) The highest GS content was found in root-forming calluses of *P. ginseng* (Bonfill *et al*. 2002) when obvious cytodifferentiation occurred. *4*) Biosynthesis profile of GS (Kunakh *et al*. 2003) and different triterpenes (Hayashi *et al*. 2001, Flores-Sánchez *et al*. 2002) is similar. Maximal triterpene accumulation coincided with the ceased of the sterol biosynthesis and was observed at the end of the growth cycles.

Therefore, there is extended evidence that bio-synthesis of GSs has close link with cell differentiation manifested in parenchymal cell accumulation in PJ cell culture. Thus, the suspension-cultured cells of PJ may be used as a model system for studies of the factors favouring the synthesis of GSs, as well as an alternative source for production of biologically active compounds.

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