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Characterization of the Variability of Nucleoli in the Cells of *Panax ginseng* Meyer *In Vivo* and *In Vitro*

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Results of karyological study of intact plants and some callus lines of *Panax ginseng* are presented. In the native plants of *P. ginseng* the nucleus with 1 nucleolus (90%) dominate, and nucleus with 2 nucleoli is rare. One nucleolar nucleus also dominate in interphase nuclei of cells of cultivated *P. ginseng* (from 2006), but we also found nucleus with 2 to 3 nucleoli in the same cell lines. Interphase nuclei of *P. ginseng* in long cultivated lines (from 1988) contain 1 to 9 nucleoli, with a predominance of nuclei containing from 3 to 4 nucleoli. It was shown that long-time cells (cultivated since 1988) had cytogenetic changes such as increase level of polyploid and aneuploid cells, increase of nucleoli number into interphase nucleus and decrease of nuclei/nucleoli ratio. These long-time cultivated cells had very low ginsenoside content.

Keywords: *Panax ginseng*, *In vivo*, *In vitro*, Nucleoli, Ginsenoside content

INTRODUCTION

Panax ginseng Meyer (Araliaceae) is a perennial officinal herb. It is a rare relict species and it was listed in the Red Books of USSR and Russian Federation [1,2]. In 2000 by decision of Conference of the Parties of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES) *P. ginseng* (wild plant roots) has been included in annex II the CITES. Introducing of *P. ginseng* into culture *in vitro* and creating collection of its cell cultures is one of the important elements in the strategy which is aimed to retain the gene pool of ginseng. Cell cultures of *P. ginseng* are used for examining of secondary metabolites synthesis and obtaining the alternative source of bioactive substances [3-5]. When we introduced plant cells into the cell culture the latter often lost their genetic stability, and formation of high level polyploidy and aneuploidy could be seen [6]. In literature one can find data about wild *P. ginseng* chromosome numbers that is determined as 2n=24, 44, 48, and karyotype is shortly described as well [7-9]. Data of chromosome cytologic analysis could be used as the evidence to genetic variability of plant cells cultivated *in vitro*; but it is difficult to describe karyotype in detail, because chromosome size of *P. ginseng* is very small (2-5 μm and less). Reliability of data obtained by measuring of length of such chromosomes is quite questionable, because resolution of light microscope is just 0.5 μm. But there are some techniques, which allow to determine the influence of physical and chemical factors on living organisms – by identifying capacity of cell's genome (without determining structural rearrangements of karyotype) [10]. Thus, nucleoli characteristics are successfully used as biotests of high sensitivity. Determination of

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nucleoli number and their size allows to assess gene activity of ribosomal RNA on cytological level [11,12]. In Gorpenchenko's dissertation [13], it has been noted that nuclei with two or more nucleoli can be found in callus cells of *P. ginseng*. In our research work the investigation of nucleolus-forming locus activity in native plants of *P. ginseng* and its cell cultures with different passaging time has been performed using karyometric technique.

MATERIALS AND METHODS

Seedlings were obtained from seeds of cultivated ginseng in the ginseng farm from the Institute of Biology and Soil Science. Callus line 1c was obtained from a leafstalk of a two-month-old plant from a cultivated *P. ginseng* population in 1988 [4]. Callus line no. 8 was obtained from a leafstalk of plant from a cultivated *P. ginseng* population in 2006. Samples were taken after cell and tissue cultures were grown for 18 d. The materials were prepared and analyzed according to generally accepted techniques for fruit plants [14] with some modifications. Cell, tissue cultures (volume 0.5-1 mL) and actively growing root tips were pretreated in 0.2% colchicine solution for 2 to 3 h at room temperature (about 22ºC), fixed in 3:1 ethanol:acetic acid mixture and stained with acetohematoxylin. The slides were prepared using the squash technique. The somatic chromosome number was studied in at least 60 well-prepared metaphase plates. A 50% solution of silver nitrate was used to stain nucleoli [15].

Ginsenoside content

Analytical HPLC of the ginseng samples were performed on a LaChrom (Merck Hitachi, Tokyo, Japan) system (pump L-7100, UV detector L-7400, column oven L-7300, and integrator D-7500). Separations were carried out using Agilent Technologies column Hypersil ODS 5 μm (25 cm×4.0 mm) with a guard column Hypersil ODS. The binary gradient employed water (A) and acetonitrile (B) according to the following profile: 0-17 min, 82%-78% A, 18%-22% B; 17-20 min, 78%-76% A, 22%-24% B; 20-35 min, 76%-65% A, 24%-35% B; 35-40 min, 65%-60% A, 35%-40% B; 40-50 min, 65%-50% A, 35%-50% B; 50-55 min, 50%-30% A, 50-70% B; 55-60 min, 30% A, 70% B. The flow-rate was 1.0 mL/min.

RESULTS AND DISCUSSION

The inherent property of *P. ginseng* is chromosomal variability both for callus culture and wild plants [3,4]. For the latter diploid (2n=24) and tetraploid (2n=48) cytotypes are known [9]. Dividing cells of investigated plants had from 6 to 60 chromosomes, callus cells – from 6 to 150 chromosomes, but cells with 2n=48 chromosomes formed modal class (Table 1 and Fig. 1A).

Studying of nucleoli in interphase nuclei of *P. ginseng* has shown that number of nucleoli in cells of intact plants varies from 1 to 2, in cells of callus no. 8 from 1 to 3, and in cells of old callus from 1 to 9 (Figs. 1B and Fig. 2).

In the first case, average number of nucleoli per cell is 1.14±0.03, in the second – 1.19±0.03, and in the third – 4.3±0.17 respectively. The frequency of nucleoli's occur-

Table 1. Frequency of cells varying in chromosome number in *Panax ginseng*: intact plants, line 1c, and line no. 8

P. ginseng	Cell mixoploidy		
	Minimum	Maximum	>50% modal
Plant native	2n=6	2n=60	24-48
Callus line no. 8	2n=6	2n=90	24-60
Callus line 1c	2n=6	2n=150	36-80

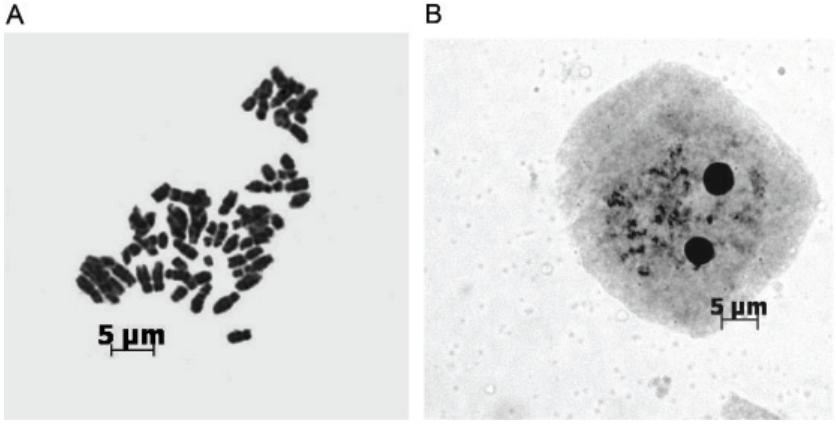

Fig. 1. The cells of intact plants of *Panax ginseng*. (A) Chromosome complement, (B) nucleoli.

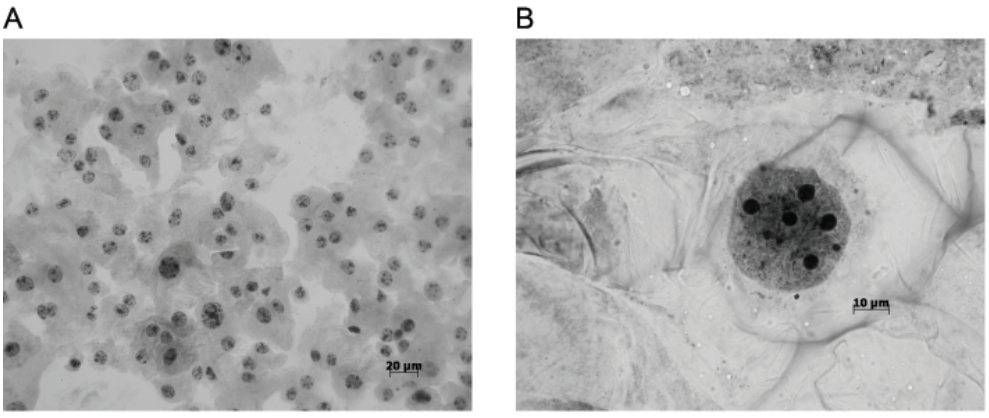

Fig. 2. Cells of the callus line 1c. (A) View of cultivated cells, (B) interphase nuclei with nucleoli.

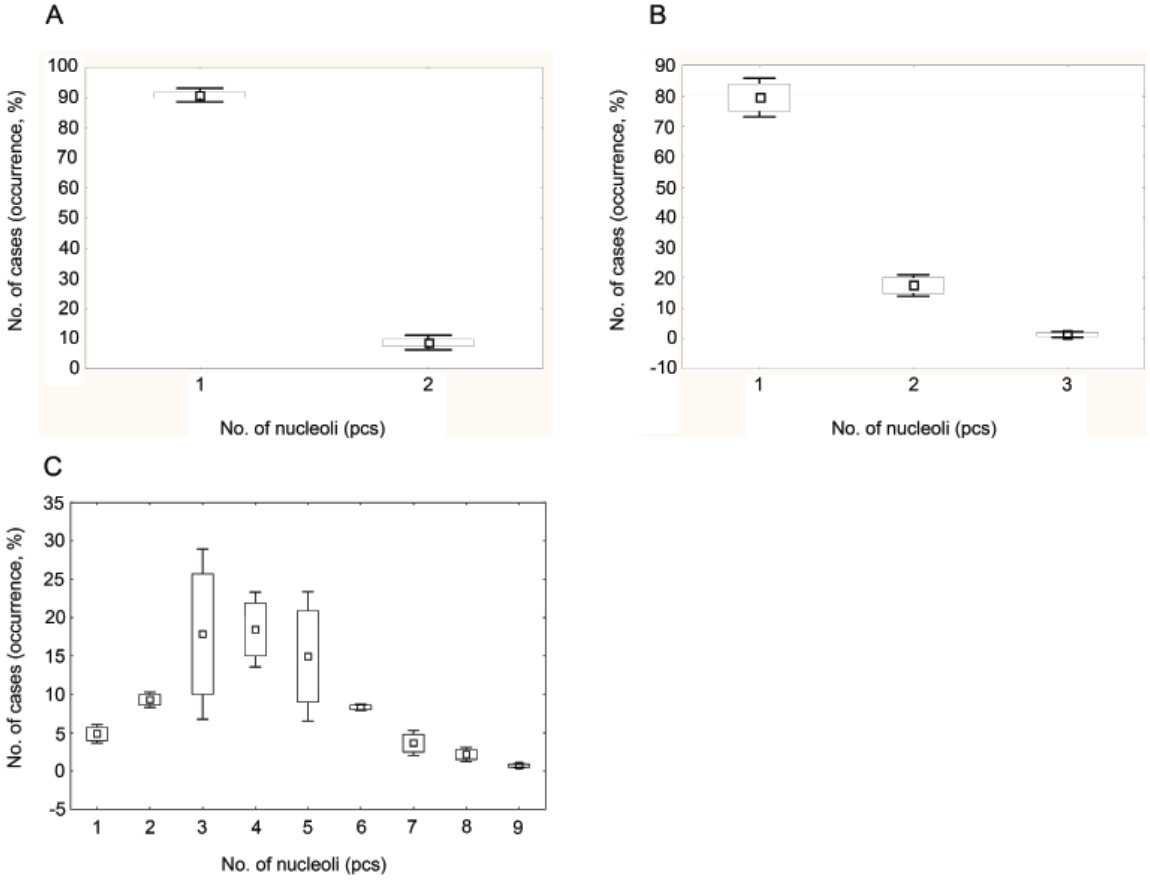

Fig. 3. The number of nucleoli in interphase nuclei of *Panax ginseng*. (A) In the cells of native plants, (B) in the cells of callus lines no. 8, (C) in the cells of callus lines 1c. The square symbols in each box indicate the mean value, the bottom and top parts of the box the standard error and the bottom and top parts of the bars the standard deviation. pcs, pieces.

rence in interphase nuclei of *P. ginseng* is shown on Fig. 3.

In native plants interphase nuclei with 1 nucleolus occur more frequently (90%), and nuclei with 2 nucleoli – much less frequently (10%). In the cells of cultivated lines (from 2006) there are 80% (of total amount of nuclei analyzed) of interphase nuclei with 1 nucleolus, and much less nuclei with 2 to 3 nucleoli (18% and 2% respectively). Cell cultures passaged for a long time (since 1988) have more interphase nuclei with 3, 4, and 5 nucleoli (60% of all cells).

Table 2. Parameters of the nucleus and nucleolus in *Panax ginseng*: intact plants, callus lines

P. ginseng (no. of studied nucleus)	Nucleus area (μm^2)	Area nucleoli (μm^2) (sum of the areas of all nucleolus)	Nuclei/nucleoli ratio
Intact plants (663)	882.77±85.04	68.01±10.49	12.98±0.54
Callus line 1c (252)	961.63±60.63	100.27±3.76	9.59±1.01

Nucleoli formation is determined by some chromosome locus activity – so called nucleoli organizers commonly situated in the zone of secondary chromosomal constrictions, where genes controlling synthesis of rRNA and formation of ribosomes (triggers for synthesis of protein) are placed [11]. Number of nucleoli formed in mitosis telophase allows to estimate the number of nucleolar chromosomes. Maximal nucleoli number in interphase nuclei allows to determine the number of nucleoli-forming chromosomes. Interphase nuclei of native *P. ginseng* have from 1 to 2 nucleoli, that allow to suggest the presence of one pair of nucleolar chromosomes. Nuclei of callus cells of *P. ginseng* (2006) have from 1 to 3 nucleoli, and nuclei of old ones – from 1 to 9 nucleoli, so we can suggest activity of 1 to 2 and 4 to 5 pairs of nucleolar chromosomes, respectively.

Nuclei/nucleoli ratio is good parameter, reflecting activity of system of protein synthesis in the cell [16]. This ratio shows differences in the rate of protein biosynthesis, i.e., decreasing of niclei/nucleoli ratio means increasing of nucleolus volume in the nucleus, and that implies intensification of biosynthesis in the cell, and vice versa. Data on value of nuclei/nucleoli ratio are given in Table 2.

On the basis of obtained data we can conclude that synthesis of rRNA and some stages of ribosomes formation and, therefore, protein synthesis is much more intensive in the cells of that lines of *P. ginseng*, which were cultivated for a long time due to increased quantity of nucleoli organizers. Probably, extra-activation of nucleoli-forming zones (that are inactive in native plants) occurs during long passaging. Greater amount of nucleoli in interphase nuclei seems to be connected with greater ploidy level (thus, in culture 1c part of mixoploid cells 2n=91, 100, 150 is very high).

It is known from literature, that some species are able to maintain stability of chromosomes morphology if callus is cultivated for a long time [17]. And it was also shown for callus cells of *P. ginseng*, that they have the same modal class of chromosome number as in intact plants, but callus lines had high aneuploidy, and at the same time low DNA variability, determined by RAPD [4].

Present data clearly shows that unlike native plants of *P. ginseng* (which karyotype has one pair of chromosomes with secondary constrictions), callus lines have up to 4 to 5 pairs of such chromosomes. Kozyrenko et al. [4] have shown that culture 1c is characterized by mixoploid cells with maximal chromosome number 2n=72 (5% of all cells analysed), and in 8 years research we found mixoploids 2n=91, 100, 150 in callus cells of culture 1c and their part in culture is 25%, this factor probably influences the number of nucleoli in interphase nuclei.

Total number of nucleoli per nucleus is increasing when polyploidy (and also aneuploidy) takes place, but increasing of chromosome complements does not lead automatically to duplication of total nucleoli number [11,12,18]. Examples of polyploidy in cell cultures are given in articles of Kunakh et al. [5] for *P. ginseng* and of Dorofeev et al. [19] for *Atragene speciosa* Weinm. Authors suggest that change of flavonoid and saponin levels (type of compounds which includes glycosides) is the result of genetic variability of culture *in vitro*, because of changes in ploidy [5,19].

It should be noted that in the case of *P. ginseng* polyploidy led to growth enhancement but was not accompanied by increased accumulation of triterpene glycosides, and in the case of *A. speciosa* on the contrary polyploidy promoted almost double increasing of saponines content. Maximal accumulation of glycosides is inherent to cell cultures of *P. ginseng*, which cytogenetic characteristics are close to that of intact plants [5].

Quantitative analysis of ginsenosides from ginseng cell cultures, induced from leafstalk of seedling and intact leafstalk of seedling were examined (Table 3). Content of ginsenosides from long-term cells cultivated since 2006 was noticeably higher than from long-time cells cultivated since 1988, but it was less ginsenoside content from intact leafstalk of seedling. Rg/Rb ratio indicated that contents of protopanaxatriol glycosides were dominated in all patterns.

It was shown that long-time cells (cultivated since 1988) had cytogenetic changes such as increase level of polyploid and aneuploid cells, increase of nucleoli number into interphase nucleus and decrease of nuclei/nucleoli ratio. These long-time cultivated cells had very low ginsenoside content. Long-term cells cultivated since 2006 had cytogenetic characteristic like intact plant.

Thereby, it is possible that nucleoli test will signal about processes of biosynthesis in cultivated cells of

Table 3. Content of ginsenosides

Panax ginseng	Content of ginsenosides (mg/g)	$Rg^{1)}/Rb^{2)}$
Leafstalk of seedling	5-10	2.7-14
Callus line no. 8 (2006)	1.35-1.66	1.05-8.9
Callus line 1c (1988)	0.24	11

[1)]Rb=Rb1+Rb2+Rc+Rd+F2; [2)]Rg=Re+Rg1+F1

P. ginseng, and results of the given test will allow assessing of similarity of cultivated cells to the cells of native plants.

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