CHEMICAL INVESTIGATION OF THE BIOMASS OF A GINSENG CULTURE

V. ATTEMPT TO OBTAIN CELL CULTURES PRODUCING GINSENOSIDES FROM THE ROOTS OF GINSENG OF THE POPULATION OF THE MARITIME TERRITORY

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Callus cultures and radical cultures from the roots of plantation ginseng plants of the Maritime Territory population have been obtained. It has been established that, with respect to the level of ginsenosides that they contain, the new cultures are close to those parts of the root from which they were obtained; the spectrum of the ginsenosides in the culture is somewhat narrower than in the root. Some of the lines obtained are superior to the domestic cultures of ginseng described previously with respect both to the amount and to the variety of ginsenosides.

Cell cultures from ginseng plants were first obtained at the end of the 1960s [1, 2]. In the last two decades in our country industrial technologies for obtaining a ginseng biomass based on the surface and suspension growth of cell cultures have been developed and introduced. At the present time a biomass from ginseng cell cultures is being produced in a number of countries and is being used for medical, nutritional, and perfumery purposes.

Unfortunately, work on the selection of domestic strains of ginseng was carried out for a long time without reliable control of the chemical composition of the cells. Only since 1986 in TIBOKH DVO AN SSSR on the initiative of VNIIBiokhimmashproekt have investigations begun on the chemical composition of ginseng cell cultures and the development of methods of controlling their content of specific groups of substances. At the present time, comparative information has been obtained on the composition of glycosides of triterpenoids of the dammarane series and of oleanolic acid (ginsenosides), lipids, fatty acids, and polysaccharides in ginseng cell cultures and natural ginseng root [3-6]. It has been found that domestic cell cultures differ from the intact root by an extremely low content or the complete absence of ginsenosides [3, 6]. At the same time, Japanese researchers have obtained strains close to the plant with respect to the level of this group of substances [7, 8]. Highly productive strains of ginseng have also been obtained in Sweden [9].

In view of this, in our country work has begun on obtaining new strains of ginseng producing the ginsenosides characteristic for the root. The first success in this direction was achieved in BPI DVO AN SSSR [Institute of Soil Biology, Far Eastern Branch, Russian Academy of Sciences], where ordinary ginseng cultures and cultures transformed by Agrobacterium rhizogenus have been obtained. The most productive cultures proved to be those of transformed calluses of skin origin, the level of ginsenosides in which amounted to 3.32 mg per gram of dry biomass. While considerably exceeding in productivity all domestic strains of ginseng obtained previously, the new cultures nevertheless are inferior to the natural root with respect to the spectrum and amount of ginsenosides and differ with respect to the ratio R_b/R_g of the groups of ginsenosides [10].

The use of preparations based on transforming strains in medicine is apparently encountering difficulties because of the tumorlike growth of the cells of such cultures.

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TABLE 1. Amounts of Ginsenosides in Ginseng Cell Cultures and Ginseng Root (series 2)

Sample No.	Sample of plant raw material	Composition of the nutrient medium	Amount of TGF,* %	Amount of ginsenosides, mg/g of dry biomass									
				R_e	R_{g1}	R_f	NG—R_2	R_{b1}	R_c	R_{b2}	R_d	$\frac{Rb}{Rg}$	Σ
	Ginseng root												
1	Main root	Soil	5,8	1,29	0,79	0,35	0,12	1,35	0,68	0,88	0,15	1,2	5,61
2	Skin	Soil	4,9	1,48	1,3	0,52	0,21	1,29	0,95	1,1	0,16	0,99	7,01
3	Root hairs	Soil	5,8	4,05	0,92	0,67	0,25	4,56	3,58	4,53	2,3	2,5	20,86
4	Callus cultures	MS 2,4-D1K 0.1	3,8	2,27	1,47	Tr.	—	1,26	Tr.	0,13	0,12	0,40	5,25
5		$MS-NH_4NO_3$ 2,4-D1K 0.1	4,8	3,36	1,3	Tr.	—	2,7	Tr.	0,23	0,25	0,68	8,08
6	Ginseng root	MS IBA2K 0.1	2,9	1,0	0,63	Tr.	—	0,91	0,08	0,22	0,03	0,79	2,92
7		MS IBA2K 0.1	7,0	2,4	1,24	Tr.	—	1,4	Tr.	0,5	Tr.	0,72	5,54
8		MS IBA2K 0.1	3,7	3,7	1,24	Tr.	—	2,26	Tr.	0,89	0,38	0,72	8,47
9		$MS-NH_4NO_3$ IBA2K 0.1	4,6	1,48	1,08	Tr.	—	1,3	0,21	0,42	0,21	0,86	4,7
10		$MS-NH_4NO_3$ IBA2K 0, 1	3,9	2,2	1,02	Tr.	—	1,57	0,26	0,5	0,27	0,85	5,82

*TGF — total glycosidic fraction.

TABLE 2. Amounts of Ginsenosides in Ginseng Cell Cultures and Ginseng Root (series 3)

Sample No.	Sample of plant raw material	Composition of the nutrient medium	Amount of TGF,* %	Amount of ginsenosides, mg/g of dry biomass									
				R_e	R_{g1}	R_f	NG—R_2	R_{b1}	R_c	R_{b2}	R_d	$\frac{R_b}{R_g}$	Σ
	Ginsengroot												
1	Main Root	Soil	3,9	0,3	2,83	0,57	0,26	1,16	0,20	0,25	0,18	0,45	5,74
2	Skin	Soil	3,8	1,89	2,03	0,73	0,3	1,76	1,13	1,27	0,2	0,89	9,3
3	Root hairs	Soil	3,7	1,69	1,22	0,7	0,25	1,76	1,26	1,48	0,69	1,34	9,05
4	Lateral root shoot (thick)	Soil	2,4	0,52	1,75	0,36	0,14	0,63	0,18	0,16	0,06	0,39	3,8
5	Lateral root shoot (thin)	Soil	1,7	2,09	3,1	1,39	0,53	3,98	2,2	2,46	1,05	1,36	16,8
6	Callus cultures	MS 2,4-D1K 0.1	3,2	1,77	0,78	Tr.	—	0,98	0,05	0,15	0,14	0,51	3,87
7		$MS-NH_4NO_3$ 2,4-D1K 0.1	3,5	1,24	0,52	Tr.	—	0,7	Tr.	0,08	0,08	0,48	2,62
8	Root culture	MS IBA2K 0.1	19,0	0,5	0,15	Tr.	—	0,57	Tr.	Tr.	Tr.	0,87	1,22
9		$MS-NH_4NO_3$ IBA 2K 0.1	6,9	1,67	0,58	Tr.	—	1,96	0,22	0,51	0,51	1,42	5,45

We have undertaken an attempt to obtain strains of ginseng plant from the Maritime Territory population close to the root with respect to the ginsenosides that they contain without having recourse to the method of transformation. In our work we have based ourselves on the experience of Japanese researchers who have obtained ginsenoside-rich cultures from roots of Korean ginseng [7, 8].

Roots of six-year-old ginseng plants grown in the Far Eastern Zonal Experimental Station and kindly supplied to us by its director A. Zrazhvaya were used as the starting material.

To obtain the callus cultures we used three roots (1, 2, 3) weighing from 60 to 90 g. Cultures were obtained from roots 2 and 3 (series 2 and 3, respectively). The calluses from root 1 proved to be infected.

Segments from the previously sterilized roots were placed in test tubes with media differing with respect to the composition of the growth substances: MS, 2,4-D1K 0, 1; MS, 2,4-D5K1; MS, 2,4-D10K 0, 1; MS NAA2K 1.1. After 10-15 days in the media containing 2,4-D and after 20-25 days in the medium with NAA the appearance of excrescences of cells growing in unorganized fashion was observed on the surface of the segments. The genesis of calluses took place most actively in the medium MS 2,4-D5K1. The callus cells were then removed and were passaged in various media for 30 days. At the end of the third passage structures similar to the root hairs of the plant had begun to form in a number of callus cultures.

Root formation was observed in the presence both of 2,4-D and of IBA, but predominantly in the latter case. The selection of morphologically homogeneous fragments permitted characteristic root and callus cultures to be obtained. However, as a rule, after several passages both types became mixed if selection with respect to morphological characteristics ceased.

Analysis of the chemical composition of the biomass was carried out by the HPLC method 12 months after the beginning of callus formation in the cultures of series 2 and seven months after in the cultures of series 3. The amounts of ginsenosides in the initial roots were determined simultaneously.

Individual sections of the roots (root hairs, skin, main root, lateral shoots) contained the same set of triterpenoid glycosides of the dammarane series but differed substantially with respect to the level of these compounds (Tables 1 and 3). We found the highest content of ginsenosides in lateral shoots of the root, the skin, and the root hairs and the lowest in the main root and a thick offshoot from it, which agreed with published information on Korean and Japanese ginseng [11, 12].

It is interesting that in the two ginseng roots analyzed (2 and 3) the maximum amount of ginsenosides was characteristic for different parts: in root 3, a thick lateral root (16.8 mg/g) and in root 2 the root hairs (20.86 mg/g dry weight). It must also be mentioned that the ratio of the ginsenosides of the R_b group [glycosides of (20S)-protopanaxadiol] to the R_g group [glycosides of (20S)-protopanaxotriol] varied considerably in different sections of the root and nonidentically in different roots (Tables 1 and 2). Thus, for the main root 3 a R_b/R_g ratio of 0.45 was characteristic, and for root 2 a ratio of 1.2. Other parts of the root differ; the ratio R_b/R_g ranges from 0.39 to 2.5.

All the cell cultures that we obtained from roots contained glycosides of triterpenoids of the dammarane series. The majority of cell lines that we analyzed contained the ginsenosides R_e, R_{g1}, R_{b1}, R_c, R_{b2}, and R_d that are characteristic for the root (see Tables 1 and 2). The presence of ginsenoside R_f could not be regarded as reliably established (samples 4-10, Table 1, and samples 6-9, Table 2). Ginsenoside NG-R_2 was not recorded in the callus tissues. The ratio of the groups of ginsenosides, R_b/R_g, varied considerably in different cell lines but fell within the range of values characteristic for different sections of the root. At the same time, the ratio of the ginsenosides within the R_b and R_g groups differed considerably from that in the intact root.

With respect to the amounts of ginsenosides that they contained, the cell cultures were closest to that part of the root from which they were obtained, i.e., in both cases, to the main root. We did not succeed in obtaining calluses from other parts of the root because of the difficulty of their sterilization. It is not excluded that the solution to this problem will open up possibilities for obtaining more productive strains.

The cell lines of both series varied considerably in their amounts of ginsenosides. Two lines of series 2 (Table 1, samples 5 and 8) substantially exceeded the main root with respect to the level of ginsenosides, three lines (4, 7, and 10) practically coincided,

and two (6 and 9) were inferior. In the case of series 3, one line coincided with the main root (Table 2, sample 9) and three (6, 7, and 8) were inferior. The differences in the amounts of ginsenosides did not reveal a correlation with the composition of the medium or the morphological features of the cultures. They also appeared on growth under identical conditions in cultures of one and the same morphological type – for example, lines 6, 7, 8, 9, and 10 (see Table 1). The reason for this may be genetic, physiological, or other, differences in the segments that had been obtained from the main root and had given rise to the cell lines, or the phenomenon of fluctuations in the characteristics from passage to passage that we have described previously for the case of the ginseng strain BIO-2 [13].

Attention is attracted by the fact that the cell lines of series 2 on the whole were superior to those of series 3 with respect to the total amount of ginsenosides. The differences in the productivity of the lines of the two series may depend on the season in which callus formation was initiated, the number of lines obtained, and the duration of passaging. In the latter case, it must be recognized that in the course of the first year of passaging the biosynthetic capacities of the cultures rose, since the lines of series 2 were obtained six months earlier than those of series 3. All these questions require further investigation.

EXPERIMENTAL

To obtain sterile segments from the roots we used the technique described by Furuya [8]. To initiate callus formation and during the passaging of the cell cultures we used the Murashiga–Skoog (MS) medium [14] and a modification from which ammonium nitrogen had been excluded ($MS–NH_4NO_3$) [11]. The following growth substances were used: 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthylacetic acid (NAA), indol-3-ylbutyric acid (IBA), and kinetin (K). The concentrations of the substances in the abbreviated designations of the media are expressed in mg/liter.

The production of cell cultures from root 2 began in November, 1988, and those from root 3 in April, 1989. Cell cultures were grown in glass vessels with a volume of 50-100 ml in the dark at 26°C. The biomass for chemical analysis was dried in a thermostat at 50°C to the air-dry state.

Determination of the Ginsenosides. To determine the ginsenosides by the HPLC method we used a Milikhrom microcolumn chromatograph with a 2 × 64 mm steel column filled with the sorbent Spherisorb ODS (5 μm). Detection was carried out at 204 nm.

For the good separation of the peaks of ginsenosides R_{g1} and R_e we used successive elution of samples first in the isocratic regime (20% solution of acetonitrile) and then with a gradient acetonitrile–water mixture: (20:80, v/v) → (50:50, v/v). The quantitative determination of the ginsenosides was carried out by the internal-standard method that we have proposed previously.

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ALKALOIDS OF Rauwolfia SPECIES GROWING IN VIETNAM

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Reserpine, ajmaline, serpentine, ajmalicine, isoreserpiline, and other alkaloids have been isolated from the roots of Rauwolfia cambodiana Pierre ex Pitard, R. verticillata Baill. and R. serpentina Benth. R. vomitoria Afz. was the richest in alkaloids.

Plants of the devilpepper genus Rauwolfia L., family Apocynaceae are widely known as producers of cardiovascular drugs. An investigation of some of Rauwolfia species growing in tropical regions of the terrestrial globe has shown that they contain valuable alkaloids possessing hypotensive and antiarrhythmic action [1].

The object of our study were the following plants growing in Vietnam: Rauwolfia verticillata Baill., R. cambodiana Pierre ex Pitard [2], R. serpentina Benth. [3], and R. vomitaria Afz. [4]. The quantitative determination of the total alkaloids in the bark of the roots of the above-mentioned species showed that they were rich in alkaloids (Table 1). A particularly high level of alkaloids was found in the bark of the roots of R. vomitoria.

A number of alkaloids were isolated from the root bark of Rauwolfia species from the Vietnam flora, and of these, in all the species studied, ajmaline and reserpine were identified; serpentine was detected in R. verticillata and in R. serpentina. The results, which are given in Table 1, confirm the fact that Vietnam species of Rauwolfia can serve as an industrial source of the hypotensive drug reserpine, the antiarrhythmic drug ajmaline, and preparations based on the total alkaloids.

The structures of all the compounds isolated were shown by physicochemical methods, qualitative reactions, and comparison with authentic samples. The composition of R. vomitoria was investigated in more detail. From the bark of the roots of this plant we isolated 16 alkaloids, of which 8 were identified.

The results obtained show that with respect to the composition of the main alkaloids and their amount, the Rauwolfia species from the Vietnam flora are not inferior to plants growing in other regions of Southeast Asia and Africa.

EXPERIMENTAL

UV spectra were taken on a SF-26 instrument (USSR). IR spectra were obtained on a Specord 75-1R instrument (Germany) for solutions of the substances in paraffin oil. Melting points were determined on a Boëtius stage.

The quantitative determination of the total alkaloids was made by a gravimetric method [5], and that of individual alkaloids by an extraction-photometric method [6]. For thin-

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