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Long-term Stability of Two *Rauwolfia serpentina* Cell Strains

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ABSTRACT

In these latter days there is ever growing demand for medicinal drugs and nutrient supplements of natural origin, in particular derived from plant raw material. At the same time, exhaustion of natural resources has risen the problem of search for new alternative sources of plant raw material. Cell biotechnology may propose the way out of this situation. Development of the techniques for maintenance of the plant cells, tissues and organs under controlled conditions on the artificial nutrient media provides the possibility for production of desirable amounts of ecologically clean plant biomass. In this work we investigated the long-term genetic stability of plant tissue culture, which is prerequisite for its durable use for industrial production of plant based drugs. Genetic analysis of *Rauwolfia serpentina* Benth. cultured tissues from two strains to be sampled with an interval of time more than 10 years was carried out through RAPD-PCR. The genome of cultured plant cells was found to lack of obvious changes during long-term maintenance. The data obtained evidence for significant stability of the tissue culture under growth in standard condition. Used RAPD-markers seem to allow differentiate various strains of cultured *R. serpentina* tissues.

Keywords: *Rauwolfia serpentina*, plant tissue culture, *in vitro*, cell line K-20, cell line K-27, indoline alkaloids production, ajmaline, genetic polymorphism, RAPD-PCR, plant raw material.

INTRODUCTION

In these latter days there is ever growing demand for medicinal drugs and nutrient supplements of natural origin, considerable proportion of which comprises preparations derived from plant raw material. In the developed countries, the total amount of medicinal remedies to be derived from the natural raw material represents over 50% of all the remedies, while in Japan – as much as 90%. (WHO, 2002) At the same time natural resources of plant raw material are far from being inexhaustible, significant part of the valuable medicinal plants is the rare and often endemic species, which are unmanageable for cultivation (Hamilton, 2004). Cell biotechnology may propose the way out of this situation. Development of the techniques for maintenance of the plant cells, tissues and organs under controlled conditions on the artificial nutrient media provides the possibility for production of desirable amounts of ecologically clean plant biomass. However, great genome variability and resulting unstable synthesis of the secondary metabolites may become a substantial obstacle to *in vitro* cell cultures application into industrial production of plant raw material.

Rauwolfia serpentina Benth. a plant of tropical origin, is a source of alkaloids widely used for treatment of cardiovascular diseases (Duke *et al*., 2002; and de Padua *et al.*, 1999). At present *Rauwolfia* reserves are becoming increasingly exhausted while the requirements of pharmaceutical industry of importing countries in raunatine, reserpine, ajmaline etc. cannot be met (Cites, Appendix II; Commonwealth Secretariat, 2001). Therefore the development of new ways for industrial production of alkaloids, in particular cell biotechnology approach, is of great importance. In this study we investigate two *R. serpentina* strains, namely K-20 and K-27, that have been derived from cell line A during 1983-1985 years. On the other hand, the line A was originated from *R. serpentina* callus introduced in culture *in vitro* by R. Butenko in 1964 (Kunakh and Alkhimova, 1989). Strain K-20 was obtained as a result of selection on the medium supplemented with 5-methyltryptophane (Kunakh *et al*., 2001). Strain K-27 has been generated following cell line exposure to ethyleneimine and subsequent selection of most productive clones on the nutrient medium of special composition (Kunakh *et al*., 2006). To ascertain the possibilities for the industrial employment of these strains we perform the evaluation of their genetic stability in conditions of long-term culturing.

MATERIALS AND METHODS

Two highly productive strains of cultured *Rauwolfia serpentina* tissues, K-20 and K-27, sharing a common origin have been studied (Fig. 1). These strains were generated more than 25 years ago and all over the time of maintenance they were distinguished by the invariably stable high level of indoline alkaloid accumulation that provides for their practical value for the industry as a source of raw material for ajmaline production. Genealogy, background, growth conditions, as well as nutrient media composition, productivity, cytological and biochemical peculiarities of these strains are detailed in the works (Kunakh *et al*., 2001; Kunakh, 2005a; Kunakh *et al*., 2006).

The samples of cultured tissues for analysis were collected from both strains in 1990 and 2004 years. In addition to tissues grown in cell culture collection of IMBG NAS of Ukraine there was also examined the variant K-27(Kh) grown in conditions of industrial maintenance at the Kharkiv chemical and

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pharmaceutical corporation "Zdorov'ya" from 1988 up to 1998 followed by the further maintenance in the IMBG NAS cell culture collection.

DNA from the cultured tissues was isolated by the standard cetavlon method (Draper *et al.*, 1988). The PCR was performed on the thermocycler "Tercyk" ("DNA technology", Russia). 20 ?l reaction volume contained 1×PCR-buffer with 2mM $MgCl_2$, 0.2mM of each dNTP, 1 U Taq polymerase, 0.25μm primer and 20ng analyzed DNA. For DNA amplification the following parameters were used: 94°C – 2 min; then 5 times: 94°C – 30 s, 36°C – 30 s, 72°C – 40 s; then 35 times: 94°C – 20 s, 36°C – 20 s, 72°C – 40 s; and final elongation at 72°C – 2.5min. PCR with each primer was done in duplicate. Amplification products were separated in 1.7% agarose gel in 1×TBE buffer. After visualization of DNA with ethidium bromide the gels were photographed in UV-light.

To quantify the genetic polymorphism the RAPD-PCR amplification patterns were recorded as a binary matrix, in which presence or absence of fragments with the similar size as well as their significant differences by the fluorescence intensity were scored as "1" or "0", respectively. The resulting binary matrix was used as the input file for a computer program PopGene (Yeh *et al.*, 1999) that computes Nei's genetic distances based on the proportion of shared PCR-loci.

The genetic analysis of K-20 and K-27 strains of *Rauwolfia serpentina* cultured tissues to be sampled in 1990 and 2004 (see Fig. 1 for details) was performed through RAPD-PCR. The use of RAPD-markers for identification of genetic variation in cultured tissues was determined by the simplicity and cost-effectiveness of the method. At the same time this type of molecular markers, which amplify different regions randomly distributed in genome, allow better chances for identification of genetic variations in tissue culture *in vitro*. There were used 25 decanucleotide primers with arbitrary sequence, which earlier proved to be successful in identifying the interspecies polymorphism for plants of *Rauwolfia* genus (I. O. Andreev, K. V. Spiridonova, unpublished data) and, hence, may allow to evaluate the variable areas of the genome involved. Primer sequences and characteristics of amplification products are listed in Table (1).

RESULTS AND DISCUSSION

The length of resultant amplification products varied within the range of 200-2000bp. Polymorphism was recognized as differences involving the presence of individual bands of specific size as well as variations in their fluorescence intensity (quantity of amplified DNA) within the spectra of PCR-products (Fig. 2). Upon the analysis of electrophoregrams only clearly discernible and reproducible in repeated reactions fragments were taken into account. Quantitative variations were included only in the case of at least 3-4 times variance in the amplicon fluorescence intensity. Differences were found within the spectra of only 6 primers among the 25 used with most variable spectra being those of amplicons derived from primers A16, A19, B08 (Table 1).

A total of 244 amplified bands were scored and 12 (4.9 %) polymorphic between the studied objects bands was found. Among these, 11 amplicons showed variation between the strains involved and only one of them (0.4 %) (see for spectrum of A16 primer products in Fig. 2) displayed variation between the strain K-27 variants of 1990 and 2004. No difference was found between the variants of strain K-27 maintained separately in standard condition in IMBG collection and at the Kharkiv chemical and pharmaceutical corporation "Zdorov'ya". Strain K-20 failed to exhibit any polymorphism between the variants of 1990 and 2004. Nei's genetic distance between the two strains estimated on the base of RAPD-marker polymorphism was 0.051, and between K-27 variants of 1990 and 2004 – 0.004.

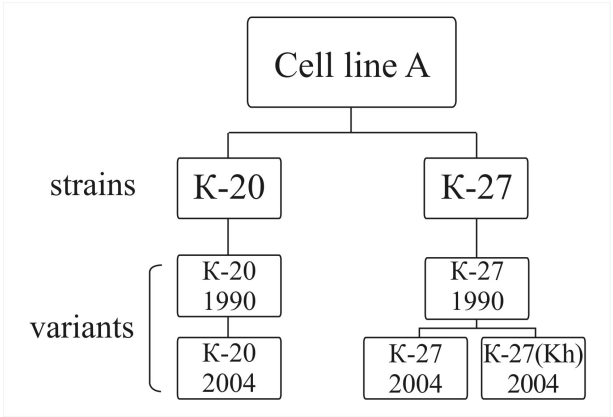

Figure (1): Strains and variants of *R.serpentina* cultured tissue under study.

Lack of the substantial changes within the spectra of RAPD-products from K-20 and K-27 strains taken for analysis with the 14 years interval of time may suggest the high genetic stability of these strains during culturing in standard conditions. At the same time, the polymorphism to be revealed indicates the genetic differences between the strains. Nei's genetic distance between them was 0.051. This value is consistent with the genealogy of the strains studied, which share joint root, i.e. originate from the line A. Strain K-20 was obtained as a result of sequential selection on the media with high content of the antimetabolite 5-methyltriptophane (Kunakh *et al*., 2001). Strain K-27 was generated after exposure of line A to mutagen ethyleneimine followed by the selection by the traits "biomass productivity" and "indoline alkaloid content" on the specially formulated nutrient medium (Kunakh, 2005a; Kunakh *et al*., 2006).

The strains involved are characterized by the increased indoline alkaloid productivity, antiarrhythmic alkaloid ajmaline in particular. Cultured tissues of K-27 strain are capable to accumulate total alkaloids up to 2.4-2.9% per dry biomass, indoline alkaloids – up to 1.2-1.8%; of these the ajmaline – 0.9-1.3% (Kunakh, 2005a). For strain K-20 these indices constitute: total alkaloids up to 2.5-3.3%; indoline alkaloids – up to 1.2-1.6%; ajmaline – 0.9-1.2% per dry tissue mass (Kunakh, 2005a). Reexamining of the above mentioned strains' productivity to be performed in 2004-2005 affirmed that these measures remained invariable following their

Table (1): Characteristics of RAPD-primers and amplification products.

№	Primer	Nucleotide sequence	Amplicon number	Polymorphic amplicon number
1	A01	CAGGCCCTTC	14	1
2	A02	TGCCGAGCTG	5	–
3	A03	AGTCAGCCAC	10	–
4	A04	AATCGGGCTG	7	–
5	A05	AGGGGTCTTG	13	–
6	A07	GAAACGGGTG	8	–
7	A08	GTGACGTAGG	9	–
8	A09	GGGTAACGCC	8	–
9	A11	CAATCGCCGT	10	1
10	A12	TCGGCGATAG	11	–
11	A13	CAGCACCCAC	14	–
12	A14	TCTGTGCTGG	7	–
13	A16	AGCCAGCGAA	15	2
14	A17	GACCGCTTGT	11	–
15	A18	AGGTGACCGT	12	–
16	A19	CAAACGTCGG	7	4
17	A20	GTTGCGATCC	9	–
18	B01	GTTTCGCTCC	10	–
19	B02	TGATCCCTGG	5	–
20	B04	GGACTGGAGT	10	–
21	B05	TGCGCCCTTC	10	–
22	B06	TGCTCTGCCC	11	–
23	B07	GGTGACGCAG	10	–
24	B08	GTCCACACGG	8	3
25	B10	CTGCTGGGAC	10	1
		Total	**244**	**12**

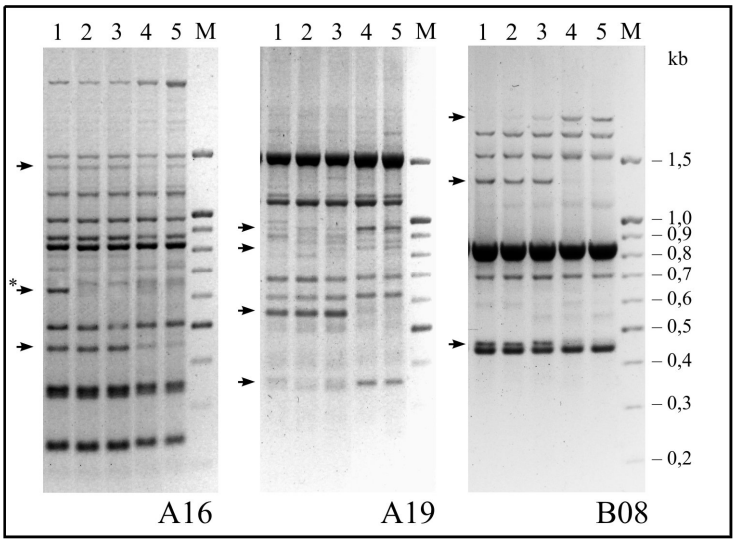

Figure (2): RAPD-polymorphism in *R.serpentina* cultured tissues. Agarose gel electrophoresis of amplified sequences from RAPD-reaction with primers A16 and A19. Template DNA used: (1) strain K-27, 1999 year; (2) and (3) - K-27 and K-27(Kh), 2004; (4) and (5) – strain K-20: 1990 and 2004 years, respectively. M – molecular marker "100 bp + 1.5 kb"; kb – kilobases. Arrows indicate variable amplicons, asterisk indicates amplicon, changed in cultured tissues of K-27 strain 2004. Code names for primers are listed under electrophoregrams

culturing in standard conditions during durable period (Kunakh, 2005a, 2005b; Kunakh *et al*., 2001, 2006).

In conclusion, the results of our studies suggest that consistent maintenance of the established strains of *R. serpentina* tissue culture in standard conditions may insure high genetic stability of cultured tissues over a long period of time. Thus providing one of the substantial prerequisites for employment of plant tissue cultures in industrial production of ecologically clean biomass, which can be used as a raw material for obtaining biologically active substances. Used in the work RAPD-markers allowed to differentiate various strains of *R. serpentina* tissues culture at the molecular and genetic levels.

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