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Stability of the genome of highly productive *Rauwolfia serpentina* Benth K-27 cell line at changing maintenance conditions

K. V. Spiridonova, D. M. Adnof, I. O. Andreev, V. A. Kunakh

Institute of Molecular Biology and Genetics, NAS of Ukraine,
150, Zabolotny Str., Kyiv, 03143, Ukraine

i.o.andreev@imbg.org.ua

Genetic analysis of the tissues of long-term passaged strain K-27 of Rauwolfia serpentina, maintained as surface or submerged cultures on the media of different composition for 4 passages, has been carried out via RAPD-PCR. Changes in the maintenance conditions were found to result in minor rearrangements of strain K-27 genome at the molecular level. However, the switch from the surface maintenance to the submerged one appears to affect the genome of cultured tissues more significantly as compared with the changes in the nutrient medium composition. Introduction of the additional subculture on the agarized medium of the intermediary composition preceding the switch to submerged culture seems to allow reducing the level of genome changes.

Keywords: *plant tissue culture, Rauwolfia serpentina, plant genome, RAPD-analysis.*

Introduction. The culture of plant tissues and cells is considered to be a very promising source of ecologically safe raw material, applicable for obtaining biologically active substances of plant origin in conditions of limited natural resources. The technologies for developing pharmacological medications and food additives on the basis of plant tissue cultures are being widely used around the world [1].

Obtaining and selection of highly productive strains and cell lines at the initial stages take place in laboratory conditions, however, upon transfer to large-scale manufacturing, there may arise a problem of laboratory cultivation schedule modification. Accordingly, further use of cell line producers as a source of specific metabolites is possible only under conditions of composition performance and quantity ratio stability maintenance of target products. In view of the genetic control for these characteristics the investigation into genome of the established strains under changing maintenance conditions is of special interest.

The work was performed on *Rauwolfia serpentina Benth*. – a tropical plant, producer of indole alkaloids, widely used in treatment of cardiovascular diseases. The stability of established strain K-27 genome upon modification of the nutrient media composition and maintenance conditions has been studied. The investigated strain is distinguished by its increased productivity of indole alkaloids, antiarrhythmic ajmaline alkaloid, in particular, and it has been cultivated for 25 years in standardized conditions, which provide ajmaline accumulation in the range of 0.9-1.2% in dry biomass upon supporting selection [1, 2]. Current work is a part of complex experiment, aimed at adapting the strain to the conditions of industrial manufacturing, including the optimization of maintenance conditions for *R. serpentina* tissues in

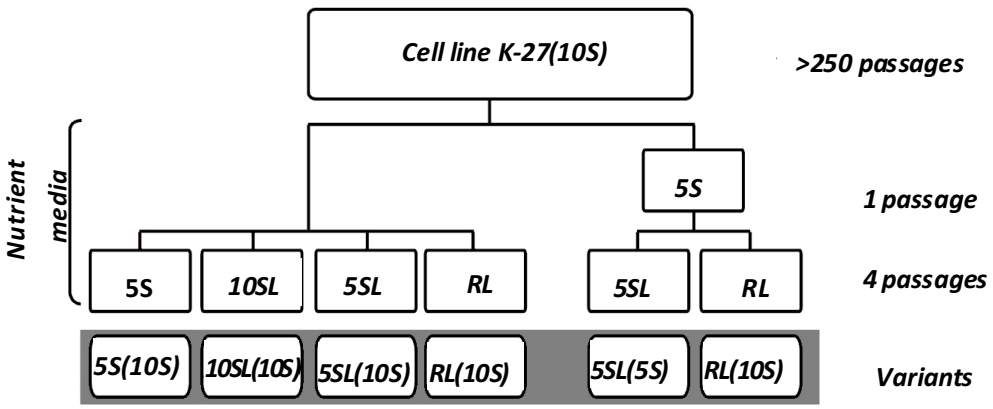

Fig.1 Scheme of experiment on the investigation of the influence of maintenance conditions on genome of hormone-independent cell line K-27(10S) strain K-27 *R. serpentina* with marking of analysed variants of cultivated tissues.

liquid nutrient medium. The investigation results concerning indole alkaloids accumulation and total productivity of K-27 strain under different maintenance conditions (surface and submerged) have been published earlier [3, 4].

Materials and Methods. Hormone-independent *R.serpentina* callus strain K-27 whose productivity, genetic, and biochemical details are described in [1, 5], was used. The strain was generated by exposing *A* cell line tissues [2, 6] to mutagen ethyleneimine with subsequent selection according to '*content of alkaloids*' character on specially designed nutrient medium, which provides high level of indole alkaloid accumulation [2]. Strain K-27 has been maintained on agarized medium 10S according to method described in [7] (cell line K-27 (10S)) since 1982. In addition, agarized medium 5S by [8] and the same media lacking agar (liquid media 10SL and 5SL) as well as specially designed medium RL according to [9] were used for submerged maintenance of *R. serpentina* tissue culture. Callus cultures were cultivated in flasks of 250 ml, containing 50 ml of agarized medium, in darkness, at 24-26°C. Liquid cultures were grown in flasks of 250 ml, with 50 ml of medium, on longitudinal shaker, at 60-70 rpm, in dark room, at 24-26°C. The size of explant upon subculturing was 4-5 g of tissue per flask. In the course of experiment (Fig.1) the tissue was transferred onto another medium and cultivated for 4 passages (1 passage = 30 days), then the tissue was used for isolation of DNA.

DNA was isolated by cetavlon according to [10]. Density and DNA preparation performance were determined visually by the fluorescence intensity of DNA-ethidium bromide complexes in UV-light after fractionation in 1% agarose gel.

Genome was analysed using RAPD-PCR. The reaction of amplification was conducted in *Tertsik* thermocycler (*DNA-Technology*, Russia). The reaction mixture of 20 мl contained 1 PCR-buffer with 2 mM of $MgCl_2$ (*Medbioservice*, Ukraine), dNTP density of 0.2 mM each, 1 unit of Taq-polymerase (*Amplisense*, Russia), 0.25 мM primer (*Litech*, Russia), 20 ng of analyzed DNA, on the surface of which mineral oil was layered. The following program was used for PCR: denaturation at 94°C/2 min; 5 cycles of denaturation at 94°C/30 sec, annealing – 36°C/30 sec, elongation – 72°C/1min; 35 cycles of denaturation at 94°C/20 sec, annealing – 36°C/20 sec, elongation – 72°C/40 sec; elongation – 72°C/2.5 min. In the work 25 ten-nucleotide primers were used (Table 1). PCR with each primer was performed in two repeats. Amplification products were separated in 1.7% agarose gel with ethidium bromide in 1 TBE-buffer upon electric field tension of 4 V/cm for 5 hours.

The gels were photographed in UV using "0×2" light filter. In the course of electrophoregram analysis

Table 1. *The list of primers used and the characteristics of products, obtained during PCR with DNA of the investigated variants of K-27 strain of R. serpentina*

№	Primer code	Nucleotide sequence	Number of amplicons	Number of major amplicons
1	A01*	CAGGCCCTTC	15	8
2	A02	TGCCGAGCTG	5	5
3	A03*	AGTCAGCCAC	10	6
4	A04	AATCGGGCTG	7	4
5	A05	AGGGGTCTTG	14	6
6	A07	GAAACGGGTG	11	3
7	A08	GTGACGTAGG	8	5
8	A09	GGGTAACGCC	8	4
9	A11	CAATCGCCGT	3	3
10	A12*	TCGGCGATAG	12	3
11	A13*	CAGCACCCAC	16	6
12	A14	TCTGTGCTGG	7	1
13	A16*	AGCCAGCGAA	12	1
14	A17	GACCGCTTGT	8	4
15	A18	AGGTGACCGT	11	3
16	A19*	CAAACGTCGG	9	3
17	A20	GTTGCGATCC	9	2
18	B01*	GTTTCGCTCC	10	5
19	B02	TGATCCCTGG	5	2
20	B04*	GGACTGGAGT	11	4
21	B05	TGCGCCCTTC	14	4
22	B06	TGCTCTGCCC	11	4
23	B07	GGTGACGCAG	10	6
24	B08	GTCCACACGG	7	3
25	B10	CTGCTGGGAC	9	7
Total			242	103

* primers with which the products of amplification reveal the variability between the investigated objects

only clear-cut and reproducible in repeated reactions fragments were registered. For each object the proportion of polymorphic loci in comparison to the original strain K-27 to be cultivated on 10S medium under standard conditions was calculated.

Results and Discussion. The reaction was studied at the level of K-27 (10S) *R. serpentina* cell line genome upon modification of the nutrient medium content and maintenance conditions, in particular upon the transition from surface maintenance of callus tissue to the submerged one. The tissue was transferred from agarized medium 10S to other types of media (5S, RL, 5SL or 10SL), then after 4 passages of cultivation, the analysis of DNA was conducted by RAPD-PCR. The variants of cultivated tissues to be obtained in such a way were marked as S (10S), RL (10S), 5SL (10S), and 10SL (10S). In another case the tissues of K-27 (10S) cell line were cultivated during 1 passage on more simple in terms of composition 5S medium by surface maintenance (30 days), and then the resultant tissue was moved to the liquid medium 5SL or RL and cultivated by submerged maintenance during four passages (variants 5SL (5S) and RL (5S)). The tissue of K-27 strain constantly growing on hormone-independent

Table 2. *Basic differences in composition of nutrient media, used for cultivation of R. serpentina tissues*

Component of medium	10S by [7]	5S by [8]	RL by [9]
Vitamin B_1, mg/l	5	1	1.7
NH_4NO_3, mM	31	6	3.7
KNO_3, mM	3.3	12	12
$MgSO_4$, mM	5.3	2	2
$NH_4H_2PO_4$, mM	1.7	5.2	2.4
$(NH_4)_2HPO_4$, mM	2.27	2.27	1.5
$CoCl_2$, мМ	0.42	0.1	0.1
H_3BO_3, mM	0.185	0.1	0.1
$FeSO_4$, mM	0.363	0.107	0.107
Na_2-EDTA, mg/l	49	37.5	37.5
Sucrose, g/l	100	50	25

Note: 8-9 g/l of agar were added to solid media for surface maintenance.

solid medium 10S was taken as a control. General scheme of the experiment is presented in Fig.1.

The media used in experiment differed from the standard 10S for K-27 strain by content of micro- and macronutrients, sugar, vitamin B_1 and consistency: solid media with agar (5S) and liquid media without agar (10SL, 5SL, and RL). Major differences in content of media are presented in Table 2. Thus, in media 5S and RL sugar content was curtailed 2 and 4 times, respectively. Besides, in 5S and RL media the nitrogen content was reduced almost 2 times, while in 5S medium nitrogen ratio within amides and nitrates was modified towards the increase of the latter.

To investigate the genome of the cultivated tissues RAPD-PCR method was chosen. This method allows covering a significant number of regions, randomly distributed all over genome. Preliminary investigation permitted assorting 10-nucleotide primers with arbitrary sequence which provide clear-cut reproducible amplification spectra, demonstrating interspecific polymorphism of *Rauwolfia* genus (Andreev, Spiridonova, unpublished data). This work deals with 25 primers, whose product spectra for DNA of K-27 cell line is represented by a set of fragments (3-16), on average of 9.9 amplicon per primer (Table 1). Total of 242 clear-cut reproducible amplicons were registered upon electrophoregrams examination. Amplification products varied from 200 to 2000 b.p. to be qualified as major and minor fragments. The sum of major fragments equalled 103, which corresponds to 42.6% of total number of amplicons (Table 1).

Polymorphism among the investigated variants of tissues was observed in spectra of PCR-products of eight primers (Table 3). Electrophoregrams exhibited 10 polymorphic fragments (4%). Most of the differences revealed involved changes in the copy number of individual amplicons, the emergence of two new products as effected by two primers was demonstrated as well. Accordingly, minor fragments were predominantly variable, while only one case of polymorphism of major fragment was documented. Samples of spectra, containing polymorphic amplicons, are presented in Fig.2.

Our findings suggest that in every case changes in maintenance conditions result in variability of PCR-products variability. Analyzed variants of cultivated K-27 tissues differ by the level of polymorphism (Table 3). The lowest level of polymorphism was upon maintenance on 5S and 10CL media (in both cases only one minor fragment was changed) while the highest one was in variants RL (5S) and RL (10S), in which the quantitative changes of six amplicons (including single major one) were observed. Besides, RL (10S) variant showed a new minor

Table 3. *Variability of PCR-products spectra in K-27 R. serpentina tissue culture*

Variant	Primer code								Number of polymorphic amplicons	
	A-01	A-03	A-12	A-13	A-16	A-19	B-01	B-04	abs.	rel., %
5S							1		1	0.4
10SLh(10S)		1					1		2	0.8
5SL(5S)		1			1	1	1		4	1.6
5SL(10S)		1		1	2 ; 1+	1	1		7	2.8
RL(5S)	1	1	*1	1		1	1		6	2.4
RL(10S)	1	1	*1	1		1	1	1+	7	2.8

Note: «+» - the appearance of fragment; - decrease in copy number of fragment; - increase in copy number of fragment; * – changes in major fragments.

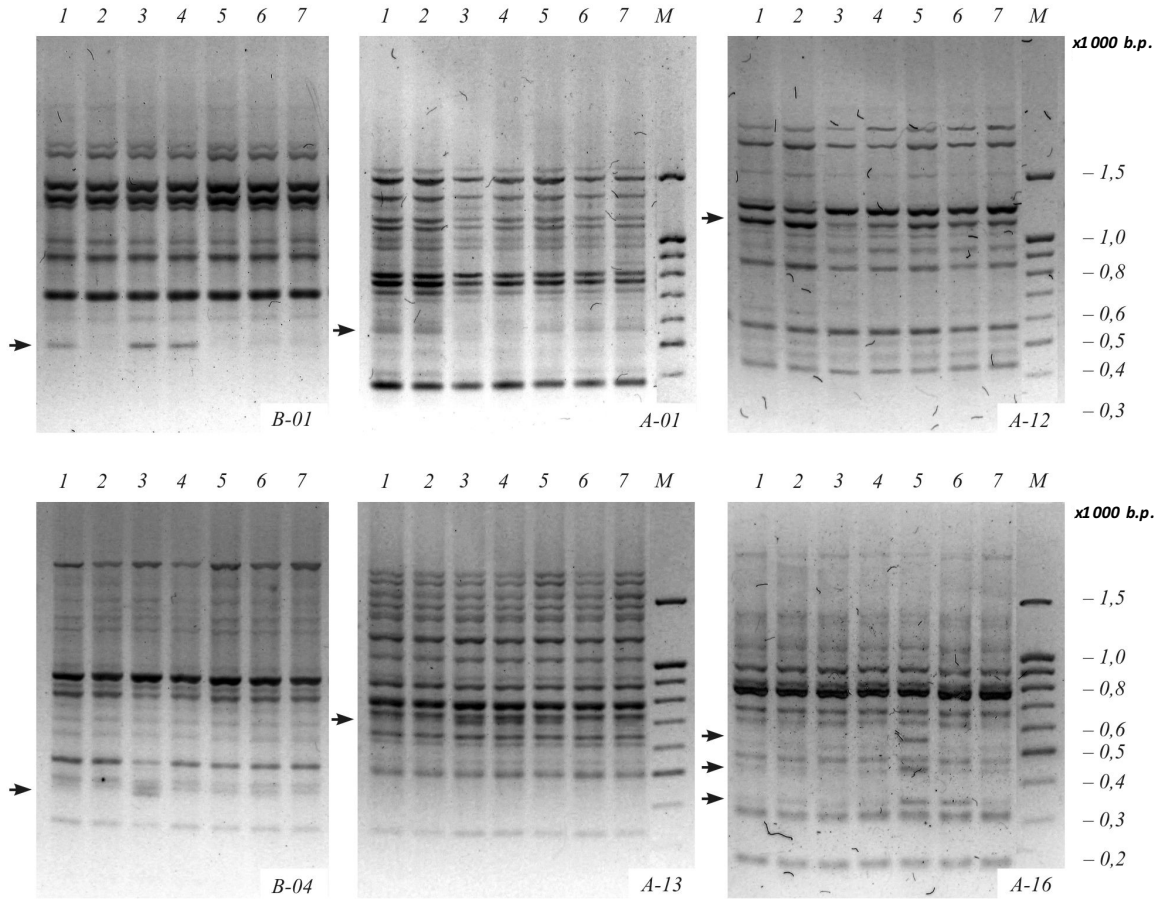

Fig.2 Variability of RAPD-spectra of variants of *R.serpentina* cultivated tissues of strain K-27, obtained as a result of changes of nutrition medium composition and maintenance conditions. *1* – control: cell line K-27(10S); *2 – 7* – callus tissue of K-27(10S) line after 4-passage maintenance at different conditions: 5S(10S), RL(10S), RL(5S), 5SL(10S), 5SL(5S), and 10L(10S). *M* – marker of DNA fragment lengths "100 b.p. + 1,500 b.p.". Arrows indicate variable amplicons; the name of primer is indicated in right bottom corner of electrophoregram.

fragment lacking in the spectrum of PCR-products of the original l strain and in RL (5S) variant. Variants 5SL (5S) and 5SL (S) are characterized by somewhat lower level of differences, versus the variants cultivated on RL medium, *i.e.* their RAPD-spectra exhibited four and seven polymorphic amplicons, respectively.

Apart from differences in the level of variability, induced by transfer to the media of various compositions, preliminary cultivation for one passage on 5S medium was found to result in decreased number of changes upon subsequent submerged maintenance (Table 3). Thus, the introduction of intermediary passage on 5S medium upon the transition from 10S to 5SL medium caused the decrease in number of polymorphic fragments from seven to four, while upon transfer from 10S to RL the number of polymorphic fragments declined from seven to six.

It is noteworthy that the majority of polymorphic fragments in several variants of K-27 strain display uniform quantitative changes. The number of some variable amplicons, namely, those obtained with primers A-03, A-13, and A-19, varied similarly in variants, cultivated on 5SL and RL media. One of the polymorphic amplicons, obtained with primer B-01, proved to be variable in all investigated variants of K-27 strain, however, upon the cultivation on RL medium, its number rose, while in the rest of the cases dropped conversely. Other polymorphic amplicons appeared to be specific and capable of distinguishing the variants, cultivated on 5SL and RL media.

The data obtained indicate that the change in maintenance conditions, in particular, mineral composition, sugar content, and type of the medium (solid or liquid) is accompanied by genome rearrangements of the formed K-27 strain from cultivated *R. serpentina* tissues at the molecular level, which are detected as polymorphism of PCR-fragments, obtained with random primers. Based on the evaluation of the number of variable PCR-products in K-27 variants, cultivated in different conditions, one may emphasize the following details of the genome rearrangements involved. Even upon transfer of the cultivated tissues into the liquid medium without changing its composition and subsequent submerged maintenance during four passages, some slight changes in spectra of RAPD-fragments were observed. At the same time, during surface maintenance on solid medium 5S, the level of variability was much lower than in the case of submerged maintenance in liquid 5SL medium. During submerged maintenance in liquid nutrient media 5SL and RL, the level of differences from the original strain proved comparable, despite significant differences in the composition of these media in sugar content and mineral composition (nitrogen ratio, as NH_4^+– and NO_3^-–groups). On the whole, it seems reasonable that the transition from surface to submerged maintenance may exert more powerful stress effect on genome of the cultivated tissues than the change in nutrient medium composition. It seems likely that it results primarily from the direct contact of cells with components of nutrient medium in conditions of submerged culture, whereas upon cultivation on solid medium only a subset of cells is in contact with it. Constant agitation and liquid medium aeration degree may contribute to stress effect as well.

Secondly, though the level of changes in conditions of submerged maintenance of K-27 tissues (10S) in 5S and RL media was shown to be comparable, the introduction of additional passage on 5S medium preceding maintenance in liquid medium decreased genome variability significantly only in variant to be passaged on 5SL. Thus, preliminary cultivation on solid intermediary medium in terms of content of some components, sucrose, in particular (Table 2) allows loosening the stress effect resultant from simultaneous changes in composition and consistency of the medium. This result is of great value since the introduction of intermediary passage on 5S medium during transition to submerged maintenance in liquid medium was shown to allow increasing the yield of ajmaline versus the direct transfer from 10S medium [3, 4].

Polymorphism of PCR-fragment spectra among K-27 strain variants to be cultivated in different conditions, displays one more interesting detail – in most cases, it is revealed as the changes in quantitative representation of minor fragments. On the one hand, it may be explained by increased lability of genome loci, which provide minor PCR-fragments upon amplification. But the following suggestion seems more probable. Strain K-27 presents a heterogeneous cell population [1, 5] and it was demonstrated that

modification of maintenance conditions, in particular, the transition to poorer in terms of composition or liquid media may result in alterations in genetic structure of cell populations, including the changes in relative proportion of cells with different levels of ploidy [1]. In that case, discovered polymorphism of PCR-products may reflect the changes in the quantitative ratio of cells, bearing different variants of RAPD-loci within the genome. Then the results obtained may be interpreted as not only the reflections of genome rearrangements induced by changes in maintenance conditions, but also as a consequence of selective pressure redirection. If it were possible, the data obtained would indicate a sufficiently high level of genetic stability in long-term passaged K-27 tissue of *Rauwolfia serpentina* upon changes in maintenance conditions.

Conclusions. The changes in maintenance of long-term passaged highly productive cell strain K-27 of *R. serpentina* tissue do not result in significant genome rearrangements at the molecular level, with the change in maintenance conditions from surface to submerged being of greater effect on genome of cultivated tissues versus the changes in nutrient medium composition. The introduction of intermediary passage on the solid medium of modified composition, preceding the transition to submerged culture, allows decreasing the level of genome changes.

Е. В. Спиридонова, Д. М. Адноф, И. О. Андреев, В. А. Кунах

Стабильность генома высокопродуктивной клеточной линии **К-27** *Rauwolfia serpentina* Benth. при изменении условий выращивания

Резюме

Методом RAPD-ПЦР проведен генетический анализ тканей длительно пассируемого штамма К-27 R. serpentina, выращиваемых в условиях поверхностной и глубинной культуры на средах различного состава в течение четырех пассажей. Установлено, что изменение условий выращивания не приводит к существенным перестройкам генома штамма К-27 на молекулярном уровне. При этом смена условий выращивания с поверхностного на глубинное оказывает более сильное воздействие на геном культивируемых тканей по сравнению с изменением состава питательной среды. Введение дополнительного пассажа на твердой среде промежуточного состава, предшествующего переходу к глубинной культуре, позволяет снизить уровень происходящих изменений генома.

Ключевые слова: культура тканей растений, Rauwolfia serpentina, геном растений, RAPD-анализ.

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