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Evolution of cell populations *in vitro*: peculiarities, driving forces, mechanisms and consequences

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This review outlines the major features and distinctions of cell populations, types and directions of selection in such populations. Population-genetic basis for cell adaptation to growth conditions in vitro is elucidated; in particular, peculiarities of genome evolution in the course of cell dedifferentiation and further cell adaptation to growth conditions in passaged culture are evaluated. Main factors of variation and selection in cell populations in vitro, influence of growth conditions on structure of cell populations and some regularities of cultured cells and regenerated plants are considered. Details of creation of stable cell lines-producers of biologically active substances are presented. Views and suppositions of author resulting from analysis of both literature data and own multiyear studies on cell population genetics are set forth. Among others are substantiated such key statements: cell culture in vitro presents dynamically-heterogeneous biological system, clone population, which is developing (evolving) as a result of major driving factors of evolution – variation, heredity, selection and drift of genes (genotypes); interaction between these processes determines the biological characteristics of each particular cell line grown in specific conditions; in adaptation of cells to growth conditions in vitro one can single out three periods: the initial population of isolated cells, the period of strain (cell line) formation and the established strain. The division into periods is determined by the type, direction and intensity of «natural» selection that acts in cell population. The formed (adapted to growth in vitro) strains are genetically heterogeneous, they are characterized by the presence of physiological and genetic homeostasis, which are mostly caused by the action of stabilizing selection; cultured cells of higher plants are able to synthesize practically all classes of secondary (specialized) compounds (alkaloids, steroids, terpenoids, etc.); any somatic cell with living (functionally active) nucleus during its isolation and further cultivation in tissue culture, as a result of the process of «somaclonal» variability occurring according to the N. I. Vavilov's law of homologous series in hereditary variability, can restore in it's descendants, including regenerated plants, the entire genetic polymorphism (or at least a significant part of it) characteristic of the plant's species and may be even it's genus. This provides an opportunity to preserve and restore the natural polymorphism in cultured cells and tissues in vitro.

Keywords: genome evolution, cell populations, selection, growth in vitro.

Introduction. Eukaryotic cell and tissues cultured *in vitro* provide a basis for modern cell and gene technologies. Among these are worth mentioning cell technologies for improvement, conservation and accelerated propagation of unique genotypes, including those using cryoconservation, creation of principally new genotypes (organisms) through cell and gene engineering and cell breeding; obtaining of biologically active compounds including recombinant ones from the biomass of cultured cells and tissues for needs of medicine, cosmetic and food industries, cell therapy methods including technologies based on application of stem cells *etc*. [1–7].

No less extensively cultured cells are also used as model objects and biological systems for studying the most actual problems of current biology: details of occurrence, signal pathways and mechanisms of cell proliferation, cell dedifferentiation including their switch to stem-cell state, totipotency, pluripotency and omnipotency; regeneration of tissues, individual organs and integral organisms *etc*. [2, 8–12].

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The methodology for maintenance and exploring of isolated cells and tissues of higher eukaryotes started to be extensively elaborated in 1930th. Cytogenetic studies of cultured cells approached their peak for mammals and human in 1960th [8–10, 13], while for plants – in late 1960th –early 1970th [2]. As exemplified by numerous species, the cells in conditions *in vitro* are distinguished by significant cytogenetic disturbances. Based on the results generated the idea arose about the high level of hereditary variation of cultured cells, the genetic, first of all chromosomal instability of cell lines and strains.

Major results and key conclusions concerning the experimental data on the features of genetic variation and evolution of cell populations *in vitro* both in plant cultures and mammalian cells (including human) derived both in those times and later on appear to coincide [2, 9–11, 14]. Joint opinion shared by researchers of eukaryotic cultured cells became insight into culture *in vitro* as a new experimentally designed biological system [2, 9, 10]. Switch to the systemic analysis of cultured cell populations proved to be the most productive, as just this approach allowed to reveal major regularities underlying the dynamics of cell populations, their adaptation to conditions of isolated growth, main trends in evolution of both the genome of cultured cells and the cell populations as a biological system.

We consider further details of variability and evolution of cell populations *in vitro*, driving forces, mechanisms and consequences of these events, focusing preferentially on examples of cultured cells of higher plants, object of authors' prime interest. Generalizations provided below are based first of all on experimental data, obtained in the Department of Cell Population Genetics of the Institute of Molecular Biology and Genetics of NAS of Ukraine.

Furthermore, one should take into account that parallels between animal and plant cell cultures may not be unquestionable. For example, even closely related species, cultivars and lines can be distinguished by certain specific features. The same refers to mammalian cells as well (see *e. g*., [2, 9, 10, 13, 14]). *A priori*, cultured eukaryotic cells are comparable only by general traits and key moments of variability and adaptive evolution, just as, for example, they are similar by their cell structure and basic aspects of functioning and metabolism.

We'll start the review focusing on main statements of cell population genetics largely formed as a scientific area in the Department of Cell Population Genetics of the Institute of Molecular Biology and Genetics of NAS of Ukraine.

Major features and distinctions of cell populations. Integration of single individuals into population and their genotypes into gene pool of panmictic population are mediated by the recombination of the genetic material during reproductive process. Populations of cultured cells are lacking such scale exchange by hereditary information. Lack of the classic combinative variation allows referring cell populations to non-Mendelian populations according to Dobzhansky classification [15].

Integrity of non-Mendelian populations is ensured preferentially by non-combinative hereditary variation. In such populations, appearance, change and maintenance of the inherited polymorphism depend largely on two factors, hereditary (non-combinative) variation and selection. Hereditary variability in cell populations is not restricted to mutation one. In the course of evolution, eukaryotic cells, especially cells of multicellular organisms, evolved one more type of hereditary variation, epigenetic one, which in cell populations plays important and sometimes key role. Yu. Vakhtin claims that interaction of hereditary variation and selection can explain the genetic aspects of such processes as cell population adaptation to changes in the environment, their ageing and dying off, transformation of normal somatic cell populations into malignant ones *etc*. [9, 10]. When analyzing the genetic processes in cell populations one should consider interaction of not only mutation variation and selection, but also epigenomic variation and selection as well as joint effect of different forms of variation and epigenetic one on the processes of interplay between mutational variation and selection.

One more way of interrelation between higher eukaryotic cells, but less studied concerning mechanisms, may be release into environment and exchange with products of metabolism as well as genetic material exchange between cells, which provide long established facts (details and ref. see [2, 10, 13, 16, 17].

Lately, the ideas are developing that cell genomes of multicellular organisms are integrated into common information space of organism that presents specific and extremely effective mechanism counteracting to

mutation pressure [18]. It was put forward and substantiated statement that plant presents a system of cell populations to be distinguished by plasticity of the gene pool, which is based on the plasticity of somatic cell genome, whose interaction with cell selection may ensure plant adaptability as integral organism and creates possibility for inheritance (transfer to offspring) of adaptive genome changes acquired through individual development. Most of such genome changes including numerical changes at chromosome level, chromatin, as well as certain DNA sequences appear to be epigenomic since they likely fail to affect a genetic code and in principle may be reversible as is especially clearly seen in the processes of dedifferentiation-redifferentiation [16, 17].

Cell transfer to culture *in vitro* means termination of its existence as one of the structural elements of the integral organism in whose composition they were included earlier. Induction of dedifferentiation (in plants, callus formation) results in changed morphological and functional features to be inherent to regular differentiated cells; some of them start to proliferate while other cells change their reproduction rate. Moreover, cell in conditions *in vitro* may go out of control of correlative factors that direct and regulate activities of various organs, tissues and cells as a unified whole. Conditions and the mode of cell nutrition may also undergo essential changes. These influences, which may exceed reaction norm of cell genome by their force, appear to be stressful and lead to cardinal rearrangements of cell functions and metabolism, significant increase in genome and epigenome variation, change of direction and intensity of cell selection and, eventually, to substantial changes in cell population structure. As a result, populations of cultured cells may differ from original tissues by high level of heterogeneity (polymorphism) and vast genome rearrangements. Magnitude and depth of rearrange ments may, in isolated cases, exceed even interspecies differences to occur in nature (see, *e. g*. [2, 19–28]).

Selection in cell populations. Established (long-term passaged in conditions *in vitro*) strains and cell lines, just as in whole organism, are preferentially subjected to two forms of selection, stabilizing and directional. Cell populations of the intact organisms are dominated by stabilizing selection based on advantages of a norm over all possible departures from it. As a norm for proliferating cell populations in intact organism, for example for plant meristem, appear to be diploid cells showing karyotype and epigenome typical of given tissue. All aberrant forms (cells with chromosome and genome mutations) are less adapted; they either loose capacity for division or divide less extensively and subsequently are displaced from cell population by cells with unchanged genotype (see [2], sections 4.2, 4.3).

Selection in cell populations is based on the differential reproduction of hereditarily distinct cell variants. Differences in proliferation rate involve changes in variant ratio: the cell proportion of rapidly proliferating variant rises while that of variant proliferating more slowly descends. When there occurs simultaneous non-selective death of cells (or their switch from population of meristematic cells to population of specialized ones that further fail to divide, for example, as a result of differentiation), variant proliferating more slowly eventually may disappear, it will be eliminated by selection. If population abundance rises all the time and non-selective death of cells occurs infrequently, elimination of the variant subjected to influence of negative selection will not occur, there will be decreased only its proportion [9].

Rate of displacement of one variant by the other may characterize the intensity of selection. Hereditarily (karyotypicaly) changed variants of cells in the meristem of intact organisms normally are displaced very quickly thus suggesting high intensity of stabilizing selection in cell populations (see [2], section 4.3).

In case of pathological or stress state, organism's internal environment may deviate from norm. This influences in the first place on the cells with regular genotype (and epigenotype); as a result the intensity of stabilizing selection declines, and hereditarily changed variants are displaced from population more slowly. Concurrent with worsening (drastic change) of environment there is increased incidence of emergence of new aberrant forms. On the whole, when environmental demands go beyond the limits of genotypic reaction of the cells, *i. e*. they are caught in really stress environments (*e. g*., upon cell transfer to conditions *in vitro*), destabilizing selection begin to operate in cell population. This form of selection leads to dramatic enhancement of genetic variation as a result of disturbances of organism correlative systems, largely the hormonal system, ari-

sing under stress influences [2], sections 4.4, 8.3, 8.4; [29]. Destabilizing selection may result in considerable increase of genetic diversity in cell populations. Against the background of high genome variation, directional or stabilizing (depending on particular conditions) selection subsequently begins to act as manifestation of changed environmental pressure.

Features and effectiveness of various forms of selection in the course of cultured cell population establishment and in established strains are detailed in the book [2], sections 8.3 and 8.4. Here, we only note that populations of isolated cells compared with populations of mitotically active cells of intact organisms may be genetically much more heterogenous. This heterogeneity is stable (see [2], section 8.1.3) suggesting the prevailing effect of stabilizing selection in such populations. In this case, one should consider that if the trait which selection influenced on is stabilizing, it doesn't mean that the effect of selection on this trait stopped in population: there is changed only the type of selection in population, its effect is targeted to the support of attained structure of population and new average value of the trait. If the intensity of such supporting selection declines, average cell proliferative activity in population drops as well. This selection can not be referred to as stabilizing, if toughly follow the term, because in this case every hereditary variant showing increased proliferative activity would have the elevated selective value. We think, however, that when genetic structure even of very heterogenous population is stable over many cell populations and passages, then it is preferentially subjected to the influence of stabilizing form of selection. Especially clearly it is evident when population maintains the genetic structure against the background of high incidence of new genotypes arising (high level of spontaneous mutations and mitotic irregularities) as it was demonstrated for the first time in our studies [30–33]; (see also [2], section 8.3).

Thus, one of the most important factors of maintenance of the genetic heterogeneity may be phenotypic polymorphism, which ensures existence of population in varying environments, mediating its lability, and the occurrence of pre-adaptations Therefore, one may consider polymorphism as manifestation of evolutionarily formed genetic homeostasis. Natural selection reinforces the existence of polymorphism through control over proportion of the necessary forms [34]. There is direct relationship between selection and hereditary variation. In case of drastic environmental changes, population may adapt to them either using available mutation reserve or at the expense of increased frequency of mutations. The emergence of non-directed spontaneous mutations followed by selection promotes gradual changes in population, with the latter being able to create the impression of being directed. These essentials of population genetics, originally found for higher organisms, currently are considered as common for populations of any kind.

However, populations of cultured cells have some specific features, in particular:

– cell populations differ from populations of multicellular organisms and those of unicellular eukaryotes by the lack of combinative variation, but unlike the former they demonstrate epigenetic variation which is of great value for functioning of isolated cells; such populations are referred to as non-Mendelian with epigenetic variation;

– cell isolation from the whole organism results in disturbances of tissue and organism homeostasis, the common reason for mutations. Therefore, the more living conditions of the cells deviate from optimal, the higher level of hereditary variation and genetic heterogeneity will be, just as is seen in such extreme environments as conditions of isolated growth *in vitro*;

– in the course of generation and establishment of strains able to long-term subculturing, both genomes of individual cells and genetic structure of cell populations become radically changed; individual genome changes may exceed even natural interspecies differences [19–28].

These and other details of populations of cultured cells are reviewed further.

Population-genetic basis of cell adaptation to growth conditions *in vitro*. Plant cells cultured *in vitro* present clone population, in which individual cells play role of the organisms. Original cells of the intact multicellular organisms are not programmed for implementation of these functions. Therefore phenomena to occur in cell populations in the course of their adaptation to conditions of durable cultivation *in vitro* represent the processes of formation of new biological system and are of general biological value. This is a unique model

(but according to experimenter choice being reversible upon morphogenesis and regeneration events) for regressive evolution of biological system, from multicellular to unicellular level. General theory of evolution considers regress (morphophysiologic regress) as a developmental pathway leading to simplified organization, loose of important, even basic morphophysiological features which were characteristic of more or less differentiated progenitors [35].

As a result of dedifferentiation upon introduction of eukaryotic cells and tissues into culture *in vitro* or callus formation in plants, there occurs simplification of organization and structure of the cells, which lost some important and even main morphological and physiological features, inherent to original differentiated cells. Therefore, based on generally recognized evolution terms, one may consider callus formation as regressive way of cell development, like carcinogenesis [8], while accompanying genome changes as regressive ones. Ability to such rearrangements seems to determine possibility for cell dedifferentiation and, eventually, callus formation.

First step for generation of isolated cell culture appears to be induction of dedifferentiation events and subsequent divisions of dedifferentiated cells (proliferation). In many plant species especially in dicotyledons, cells of any level of differentiation and specialization demonstrate potential to dedifferentiation, provided that they carry living nucleus [36]. Upon callus formation by such cells, genome in many cases was found to be rearranged. Both nuclear and extranuclear genome undergo changes.

Genome evolution in the course of cell dedifferentiation. Induction of dedifferentiation events implies genome reprogramming and its return to the state characteristic for proliferating cells, that is genome «rejuveniation» This is evidenced by genome rearrangement diversity, whose level, type and direction vary between different objects. Distinctions in cell genome variation are mediated by plant (species, cultivar, line, form, *etc*.) genotype features, state of genome in the cells of original explant, depth of genome rearrangements as a result of cell differentiation. Especially considerable genome reorganization at every level studied (genomic, chromosomal, molecular) is observed in those plants and tissues, which faced more radical genome rearrangements during ontogenesis. These processes in vitro are substantially affected by particular conditions of callus induction and components of nutrient medium, growth regulators in the first place. Thus, peculiarities of the genome variation occurrence during dedifferentiation are determined by genotype-environment interaction. It is based on the fact that wounding, components of nutrient medium, particular conditions of cell culturing affect expression of genes which are responsible for dedifferentiation (callus formation). The same factors determine activation of certain elements of mutation system.

On the whole, genome rearrangements to be recorded in the course of callus formation *in vitro* represent totality of changes various by the origin that include:

– programmed changes to occur during wounding and induction of dedifferentiation;

– changes and mutations arising in individual development of the original organism and found in case of passing through mitosis *in vitro*;

– changes and mutations arising from influence of conditions for induction of callus formation, which (conditions) in some cases may go beyond the limits of regular reactions of particular genotype and induce genome rearrangements [36, 37].

Mechanisms of genome rearrangements upon induction of cell dedifferentiation (re-differentiation as well) both *in vivo*, and *in vitro* before and during early mitoses of differentiated, especially highly specialized cells, most commonly are based on the processes as follows:

– change in the methylation state of many DNA sequences;

– additional DNA synthesis, which is often rather considerable;

– amplification of individual DNA sequences;

– endoreduplication, other forms of endomitosis;

– change in heterochromatin amount and its distribution pattern within chromosomes;

– extrusion (release of nuclear material outside the cell boundaries);

– loss of a significant amount of nuclear DNA (especially typical for highly polyploid cells), in particular through diminution of chromosome and chromatin;

– cytomixis (exchange of nuclear material between cells);

– changes in B-chromosome number (initially, as a rule, increase in their number, while upon durable passaging, loss of B-chromosomes, especially tissue-specific ones);

– fragmen tation, constriction and budding of nuclei (amitosis);

– anomalies of mitosis and cytokinesis, formation of syncytium, in particular; these commonly result from anomalies of microtubules;

– nuclear fusions in multi-nuclear cells;

– emergence of micronuclei in absence of aberrant anaphases;

– segregation of nuclear material in prophase and metaphase not only in polyploid but also diploid cells that leads to chromosome number reduction;

– emergence followed by gradual disappearance of polytene chromosomes (disappearance may obviously occur gradually, by decrease in thread number in originally polytene chromosomes);

– somatic meiosis and crossing over;

– transposition of mobile genetic elements etc.

To our opinion, it is ability to such genome rearrangements of dedifferentiated cells that underlies the cycle of development inherent to many organisms, in the first place to plants, cycle of development differentiation-dedifferentiation-re-differentiation.

Development of these processes is seen as follows. As initial inducer of dedifferentiation serves trauma, from which variation processes (originally those of epigenomic) are triggered that accompany cell dedifferentiation and early steps of their proliferation, naturally targeted to wound healing. Plant cells change their competence to phytohormones, sucrose and other growth regulators. Use optimal concentrations of these biologically active components during cultivation tissues *in vitro* may not only speed up and enhance, but also distort programmed occurrence of variation processes. In other words, genome variability observed during callus formation presents hypertrophied and somewhat distorted manifestation of the processes naturally occurring during induction of cell dedifferentiation and proliferation, for example, upon wounding [36, 37].

Cell adaptation to growth conditions in passaged culture. Adaptation of cells to conditions of long-term growth in passaged culture presents complex and multi-step process. As stated above, it needs radical rearrangement of both function and metabolism of the original cells of multicellular organism and cell population structure. Cell adaptation to conditions of durable growth *in vitro* seems to occur similarly to other biological communities based on interaction of processes of variation and selection.

Variation at the early steps of culturing results from physiological adaptation. Subsequently, during subculturing there occur processes of genetic adaptation manifested as change in the genetic structure of cell populations. In the process of cell population adaptation to growth conditions *in vitro* we singled out three periods: period of primary population of isolated cells, period of formation and that of established strain [33, 38].

The cells found in the period of primary population (primary callus and first 2–3 passages) are characterized by comparatively insignificant genetic differences from mitotically active cells of the original plant (original explant or wound callus), relative stability of most features as a result of prevailing effect of stabilizing selection. Reorganizations of genome in this period are totality of the changes various by origin (see above).

Within the period of formation, dramatic one in the course of cell adaptation to growth conditions in passaged culture, there occurs ultimate disappearance of organism's integrating mechanisms, cells are preferentially subjected to the effect of destabilizing selection. The period of formation covers two to eight, sometimes to 12th passages beginning from cell introduction into culture *in vitro*; at this time there occurs substantial rearrangements of physiological processes in cells and structure of cell populations as a whole. It is this period that is distinguished by most radical changes as a result of which there occurs adaptation of cell associations, as biological system, to changed relative to primary callus (zero passage) environmental conditions. Specifically, in most cases period of formation exhibits such phenomena:

– morphology and growth rate of cell strains (callus tissues and suspension cultures) is changed;

– parameters of proliferation, *i. e.* division and growth rates, tend to decrease;

– rhythms of physiological processes, in particular circadian rhythms of mitotic activity, are disturbed;

– mitotic regime and distribution of cells by duration of cell cycle and its individual phases are changing,

cell populations display increased heterogeneity by both duration of cell cycle as a whole and mitosis, in particular;

– the level of genome changes is increasing and their spectrum is expanding, in the first place proportion of cells with changed chromosome number rises;

– level and spectrum of chromosome aberrations are changing; among these changes most essential role plays the cycle breakage-fusion-bridge;

– frequency of mitosis irregularities increases preferentially due to impaired spindle (microtubules?);

– expression of genetic information which accompanies further changes in methylation of various DNA sequences is changing;

– level of heterogeneity for majority of cytological, biochemical and molecular-biological markers is rising;

– simultaneously, there is positive selection of cells adapted to conditions *in vitro* and elimination of non-adapted ones etc.

Against the background of high level and broad spectrum of variability and heterogeneity, the directional selection results in genetic adaptation of cell populations. However, some lines cease to grow and die, apparently due to the absence of adaptive changes. For related cell strains practically by all characteristics, there are observed possible types of evolution: divergence, convergence, parallelism [2, 28, 38–43].

Main factors of variation and selection in cell populations *in vitro*. It is known that hormonal system is in forefront when organisms respond to stress factors and processes of adaptation, affecting gene expression, transcriptional and translational processes (resulting in apoptosis as well). However, hormones are part of the total mutagenic and anti-mutagenic systems of plants, which determine the level of natural (spontaneous) mutations. Analysis of the results of our experiments and literature data allowed us to assume that the main reason for the high genomic variability of cultured plant cells is a hormonal imbalance [2], section 8; [44]. Let's consider this statement and the consequences ensuing from it, in detail.

In intact organisms hormonal status and competence of proliferating tissues is aimed at creating optimal conditions for cell growth and division. Normally, hormones and appropriate cell competence cause stabilizing selection, as a result of which cells with genomic disorders arising from various reasons are removed from the population of dividing cells. In differentiating tissues, hormones and competent cells are different – depending on future functions not only morphological and biochemical changes occur in cells under the influence of phytohormones, but also take place programmed changes in their genome, aiming ultimately at performing a specific function (see [2], section 4).

In a culture *in vitro* situation is different. Here one of the main tasks is getting the cells that divide and grow (proliferate) rapidly from the differentiated (specialized) tissues. For this, relevant conditions are empirically specified: in a nutrient culture medium not only the necessary macro-and micronutrients and energy source (sugars) are included, but also added growth stimulators in different amounts and proportions. Under these conditions, initiation of dedifferentiation and active proliferation of cells, and the primary callus formation occur. Induction of dedifferentiation and callus formation involves reprogramming of the genome and its return to a state characteristic of proliferating cells, *i. e*., genome «rejuvenation». This is manifested in a variety of genomic alterations, whose level, type, and direction vary between different objects. These changes are mainly programmed (see [2], section 7.2). Therefore, we believe that for plants, which are naturally capable of forming wound callus (*i. e.* where callus formation is evolutionarily fixed and genetically conditioned process (see [2], section 5), variability in primary callus results mainly from physiological adaptation of cells on the basis of epigenomic changes. When applying optimal influences for proliferation, including hormonal ones, living conditions of cells in primary callus *in vitro* are comparable to the conditions *in vivo* in whole (but injured) organism. These conditions are maintained *in vitro* for some time, usually during the first two, at most four passages. As a result, primary callus and cells in the first few passages represent a system that is comparable in general with meristem of intact plants or wound callus. Hormonal balance of this system ensures its relative homogeneity, stability, precise rhythm of mitotic activity (which is governed, by the way, with phytohormones), and dominance of stabilizing selection.

In the process of further subculturing, there occurs selection of cells that divide most intensely and/or areas of isolated tissues, which grow most extensively,

i. e. cells with altered hormonal balance and/or altered reaction (competence) to hormones are selected. Furthermore, there gradually disappear integrating mechanisms of plant organism, cells switch to the living conditions that are outside the norm of reaction of their genotype, i. e. fall into stressful conditions. As a result, destabilizing selection begins to act in population of isolated cells.

In case of cell culture initiation, effectiveness of destabilizing selection depends not only on the conditions of their cultivation, but also on the plant species, its genome, features of primary explants, *i. e*. the state of the genome in the original cells. In other words, the effectiveness of destabilizing selection is determined by genotype-environment interaction where the critical role belongs to exogenous hormones and competence of the cells (primarily competence to hormones). Cells of wild species with simple genomes are characterized by a low level and a narrow range of genome variability, karyotypic in particular. The cells of most cultivated species are characterized by much broader range of variability *in vitro*. This is, apparently, caused not only by polyploid state of most plant genomes, but also by prolonged exposure to destabilizing selection in the process of domestication and subsequent breeding. As a result, selected forms appear to be more labile, respond more noticeably to changes in growing conditions (including the hormonal influences): during the introduction into the culture *in vitro*, they demonstrate a wider range of variability, especially karyotypic one. In many such plant species the results of destabilizing selection are seen already in primary callus (see [2], section 7.2).

Against the background of high genomic variability, directional selection begins to prevail (as a manifestation of changed environment pressure) in cell populations that forms the population of cells capable of unlimited growth in specific conditions of isolated culture. The main mechanisms of variation underlying the evolution of genomic structure of the cell population with crucial role of selection, are:

– changes in the number and morphology of chromosomes associated with disturbances and deviations from the normal course of mitosis, breakage-fusion-bridge cycles and other processes (see previous section);

– somatic crossing over; changes in gene expression, including their repression and derepression, resulted mainly from changes in DNA methylation;

– amplification and deletion (reduction) of DNA repeats;

– transpositions;

– gene mutations.

Because the changes in chromosome number and increased rearrangements of chromosome structure, changes in the number and distribution of C-heterochromatin are inherent to the culture of isolated cells and tissues of intact plants, while other deviations occur more rarely within the period of formation, it is reasonable to assume that the first and most probable and most effective mechanism of emergence of new genotypes and increase of genetic variability *in vitro* is the occurrence of cells with different sets of chromosomes, stabilization of their number and ratio at a certain level, the selection of a certain frequency of structural chromosome rearrangements that lead to their morphological changes. Moreover, at first, it is usually seen polyploidization of certain part of cell population mainly due to endomitosis, with further increase in genetic heterogeneity of the population by the number and morphology of chromosomes as a result of deviations from the normal course of mitosis and breakage-fusion-bridge cycles. And only further on those changes (rearrangements) mainly occur, which form new cellular variants with differences at the level of DNA sequences.

The results confirming just this course of events are in almost all published papers dealing with the studies on the genome variability in dynamics of subculturing (passaging) of cell cultures of various plant species (see, *e. g*., [19–28, 45–51]).

After the 8–10th passage the strains are usually stabilized by many characters studied. Such stable heterogeneity is observed against the background of high (sometimes over 50 %) level of chromosomal mutations and mitosis disorders that lead to the emergence of new genomes, i. e. against the background of permanently high mutation pressure [2], section 8.3.2; [52]. It should be noted that one of the ways for regulating the level of mutation is a change in the efficiency of elimination of cells with break age-fusion-bridge cycle. The effectiveness of elimination of such cells, and, hence, also the level of cells with chromosome aberrations can be regulated by exogenous phytohormones [30, 53]. By changing availability and ratio of phytohormones in a nutrient medium one can also affect the ploidy level of cultured

cells. Mechanisms of such regulation are discussed in publications (see [2], section 8.3.2; [31, 54]).

It should be emphasized that cultivated for a long time (tens of passages and more) cell cultures with active disorganized growth represent heterogeneous populations of cells with reorganized genomes. Populations with prevalence of the cells with the original genome (typical of cells/tissues from source explants), usually did not grow in passaged culture, they stopped growing and died in the early stages of subculturing (within the period of strain formation). Some experiments demonstrated a direct correlation between callus ability to long-term growth *in vitro* and proportion of the cells with reorganized genome (details and ref. see [2]. Thus, the adaptation of cells to conditions of continued growth *in vitro* is determined by reorganization of original cell genome regardless of its state in the original explant and even in primary callus.

Hence, the accumulated results of numerous experiments at all levels of exploring, from the cellular to the biochemical and molecular genetic ones, suggest that within the period of established strain most of cell populations are characterized by relative stability of characteristics, which became established during the period of formation, the presence of physiological and genetic homeostasis caused by the overwhelming influence of stabilizing selection (direction and strength of which differ significantly from those in the intact organism and wound callus).

Genomic reorganizations in the established strains exhibit nonrandom, canalized character thus indicating that mechanisms of adaptation and evolution are shared to some extent by the plant genomes both in nature and in culture *in vitro*.

Similar processes and periods (stages) are known for animal cells in culture during the formation of permanent cell lines, tumor growth, as well as in the normal ontogeny [9, 10, 13, 14, 55]. Such processes and phenomena are inherent to prokaryotes during the sharp changes in environment (see, *e. g*., [56]). This indicates the universality of cell population adaptive mechanisms, regardless of the degree of evolutionary development of organism.

Effect of growth conditions on the genetical structure of cell populations. The influence of some culture media components and conditions for induction of dedifferentiation and callus formation (temperature, illumination, mineral composition of the medium and content of various organic additives, sugars, vitamins, amino acids and the like, ratio among different types of growth regulators, especially phytohormones) on level and spectrum of genomic changes during callus formation were detailed in [2], sections 7.2, 9; [31, 57]. The composition of the nutrient medium and other external factors largely determine the direction of genome changes during cell adaptation to growth *in vitro* and therefore may be used as regulators of cell variability not only in case of induction of callus formation, but also during generation of passaged cultures and established cell strains. Obtaining and further passaging of cell cultures in different conditions can lead to the formation of strains that differ in many ways, namely by the:

– level and type of chromosome aberrations;

– range of variability by the chromosome number and their modal class;

– cell distribution by nuclear DNA content;

– ratio of various fractions of repeated DNA sequences;

– structural and functional state of DNA, level of DNA methylation, amount and distribution of heterochromatin along the chromosome *etc*.

The change of growth conditions in many cases leads to a change in the ratio of cells with different genomes (changes in gene pools of populations). Strains with different duration of cultivation *in vitro* react differently to changes in culture conditions. Not-formed strains found in the stage of primary population or within the period of formation following the changes in culturing conditions give rise to culture that is usually different from the original population by genetic and other parameters. Changes in culturing conditions for strains that had been formed (such as those subcultured over a year) lead in many cases to changes in the genetic structure of cell populations that could be revealed best of all via chromosomal analysis. After 2–4 passages (sometimes later) in changed conditions there occurs either stabilization of the population at new level of genetic heterogeneity or approximation (return) to the original genetic structure of populations. That is, the established strains (as opposed to those found within the period of formation) are characterized by the presence of not only physiological, but also genetic homeostasis caused by the

prevailing action of stabilizing selection (see [2], sections 8.3, 8.4; [58, 59]).

Formed strains in a stable environment, even during decades of cultivation on an industrial scale in factories, rarely change genetic structure. Change in culturing conditions of mixoploid strains leads to increase in proportion of diploid cells while in diploid strains there is rising of heterogeneity by chromosome number (range of variability), sometimes occurs a change in the modal class. As further cultivation is going on in changed conditions, population may frequently restore the original ratio of the cells with various chromosome number [2], sections 8, 9; [54].

Some variability patterns of cultured cells and plant regeneration. The accumulated results indicate the presence of certain patterns of genomic variation in cultured cells: the changes that have occurred in cultured cells, in nature caused both intraspecific and even interspecies variability. It is found both at chromosomal level and at level of various DNA sequences, rDNA sequences, in particular. It is inferred the nonrandom nature of the genome changes in cultured cells of studied plant species, namely, their similarity to the changes that occur naturally in the process of speciation, a certain unity of mechanisms of adaptation and evolution of plant genome in nature and in culture *in vitro* [16, 17, 19–28].

The analysis of biochemical changes also suggests that in some cases the variability *in vitro* may go beyond species limits, that is cells with altered genome may acquire characters typical of members of other genera of this family. For example, a tropical medicinal plant *Rauwolfia serpentina* in nature is distinguished by the accumulation of several dozen alkaloids, mainly reserpine. The cultured cells are also able to accumulate valuable alkaloids (cell selection allows to obtaining strains-superproducers of some alkaloids – see [2, 3, 46, 61]), but there is virtually no reserpine, and about 90 % constitutes ajmaline and in some cases vomilenin. That is, in terms of this biochemical trait cultured cells of *R. serpentina* resemble cells of the intact organisms of other species of the genus, *R. canescens* or *R. vomitoria*, depending on the spectrum and number of synthesized alkaloids [46, 61]. It is also known that cultured cells of different species of poppy, including *Papaver bracteatum* and *P. somniferum*, usually do not accumulate morphine alkaloids. In their biomass dominated sanguinarine and its derivatives in amounts and ratios close to those of the cells of intact plants of another species of the family *Papaveraceae*, *Macleaya*. There are many similar examples [22, 23, 62, 63].

The observed feature, canalizing of genomic changes in the adaptation of cells to growth conditions *in vitro*, to some extent makes it possible to predict these changes and provides targeted search for somaclonal variants just as it is used in work with intact plants on the basis of N. I. Vavilov law of homologous series in hereditary variability. Experimentally it has been proven in work with corn: after cell selection, plants-regenerants were derived with new features that were previously found only in rare cases in individual genotypes (lines), in particular, somaclonal variants showing high inherited ability to regenerate whole plants were obtained [64, 65].

However, not all genomic changes that occur in populations of cultured cells are found at the level of plant regeneration. Regeneration of plants in long-term cultivated strains with substantially rearranged genome is induced with low-frequency; majority of the regenerated plants is abnormal and usually many of them die in the early stages of ontogeny. The obtained viable regenerants usually have normal karyotype, they are diploid, rarely tetraploid; the frequency of plants with large genome rearrangements among them is low. It was found by us in the case of many plant species (for details and references, see [2], section 7.3).

Based on these results, we concluded that in genetically heterogeneous populations, predominantly diploid, rarely – tetraploid cells without visible chromosomal aberrations demonstrate ability to regenerate [66–69]. An exception to this rule is some polyploid and hybrid origin species, among which the regenerant forms with changed number and morphology of chromosomes are much more likely to occur. This feature of cultured cells led to almost collapse of aspirations and hopes that were pinned on the culture of isolated cells and tissues as an inexhaustible source of new plant forms with previously unknown characteristics valuable for genetics and plant breeding. Today, you can count on one hand somaclonal variants with fundamentally new features (evidence and references, see [2, 4, 6, 7, 11, 12, 22, 23, 68–71].

Thus, genome rearrangements that are found in cultured cells and regenerated plants follow N. I. Vavilov law of homologous series in hereditary variability. Fur-

thermore, range of variability among cultured cells can sometimes go beyond the genus limits, and among regenerated plants range of somaclonal variability only rarely goes beyond the limits of particular plant species. Most commonly, variability among regenerated plants derived from one genotype lies within population variability of original plant.

Establishment of stable cell lines, producers of biologically active substances. The above discussed dynamic stability of genetic structure of formed cell strains (stability of their gene pool) is underlying the creation and industrial application of cell biotechnologies for obtaining valuable plant raw material for medicine, food and cosmetic industries and so on (see details [2, 3, 5, 46]). A number of such cell lines and strains developed in the Department of Cell Population Genetics of the Institute of Molecular Biology and Genetics of NAS of Ukraine possess stability of productivity and genetic structure for dozens of years of cultivation both in laboratory and industrial conditions. Stable performance of strains-producers is largely due to the use of supporting selection and special compositions of nutrient media and other cultivation conditions developed by us. This concerns first of all the most productive in the world strains of *R. serpentina*, which for nearly 40 years invariably accumulate 1.8–2.2 % ajmaline, and highly productive, able to growth in industrial conditions strains of *Panax ginseng*, *Rhodiola rosea*, *Arnebia euchroma*, *Ungernia victoris*, *Echium plantagineum*, some species of *Papaver*, *Gentiana* and others [2, 5, 61–63, 72–76].

During study on the biosynthesis of secondary metabolites in plant cell culture it has been accumulated a wealth of information, which indicates the existence of such regularities:

– cultured cells are able to synthesize almost all classes of secondary compounds of (specialized) metabolism (phenols, glycosides, alkaloids, including steroids, flavonoids, terpenoids, *etc*.);

– primary cell cultures often contain an insignificant, if any, amount of compounds of specialized metabolism, but it can be significantly improved by optimizing the composition of the culture medium and specifying growing conditions, using methods of cell selection, artificial mutagenesis etc.;

– synthesis of some specific substances (dimer, indole and morphine alkaloids, cardenolides and some others) in dedifferentiated cultured cells hardly occurs, with a clear trend: the more complex the structure of substance and the more specific stages of its synthesis (after the «branching» from the primary metabolism), the less probable the synthesis of this compound in culture of dedifferentiated cells; in many cases, the synthesis of secondary compounds begins only in the case of occurrence of differentiated (morphogenic) structures in the cell culture;

– synthesis of secondary compounds usually improves in case of slowing or stopping the growth of cell cultures;

– stability of secondary compounds synthesis varies among different classes of substances and different cell cultures: synthesis of steroid glycosides is usually stable, while the synthesis of many types of alkaloids – unstable (except, for example, indoline alkaloids in cell lines of *Rauwolfia serpentina* derived by us);

– metabolism of secondary compounds in plant cell culture is often characterized by both regressive changes in ontogenetic and phylogenetic plane, that is specialized turnover in culture shows indications typical of phylogenetically archaic groups of plants or juvenile stages of intact plants (for details and references, see [2, 3, 5]).

Given these regularities, the cell strains were created by obtaining cell populations of corresponding (adequate) genotype (gene pool) which are capable of highly efficient synthesis of desired compounds and full realization of this capacity.

The technology of creation of highly productive strains and specifying optimal conditions for their cultivation involves the following steps:

– matching of donor-plant species: different species have different ability to synthesis of the target substances in cultured cells, for example, different poppy species in culture *in vitro* demonstrate varying potential ability to synthesize target alkaloids;

– matching of high-performance donor-plant (original genotype), and also sometimes its particular organ or tissue for generation of cell culture;

– manipulations with cell culture, including obtaining of mutants, somaclones and other approaches of cell selection, aimed at generation of genetically modified high performance strains (cell populations with altered gene pool);

– specifying composition of culture medium, conditions and methods of cultivation, optimal for a stable realization of genetically predetermined capacity for synthesis of target compounds;

– impact on cell growth (proliferation) in tissue culture aimed at stopping or slowing down cell growth that may switch cell metabolism towards the synthesis of substances of specialized turnover: for example, for this purpose were successfully used inhibitors of transcription and translation;

– search for signals by which plants control the synthesis of secondary metabolites in cells (elicitors, nonspecific stressors, *etc*.) and use of them to increase the yield of the target product in cell cultures;

– obtaining of organogenic cultures such as root cultures, which in many cases facilitates cultivation conditions and increases the content of target secondary metabolites;

– obtaining transgenic crops (both cell and tissue cultures and whole plants) aimed at synthesizing the target product, for example of animal origin, vaccines, specific human proteins, etc. (molecular farming) (for details and references see [2, 3, 5].

In our Department mathematical models were developed that allow more purposefully create and characterize new cell lines and strains-producers [77–80].

Plasticity of genome of somatic cells and adaptability of the plants. Summary. Analysis of the results from long-term study of dynamics of the cell populations genetic structure, the role and characteristics of selection in the adaptation of cells to growth conditions *in vitro*, variability and features of evolution during long term growth in passaged culture for 25–30 years or longer allows the following generalization:

– cell culture *in vitro* presents a dynamic biological system, clonal population, which is developing (evolving) as a result of action of major driving factors of evolution – variation, heredity, selection and drift of genes (genotypes); interaction between these processes determines the biological characteristics of each particular cell line grown in specific conditions;

– adaptation of cells to conditions of prolonged cultivation *in vitro* is complex and multistage; at different stages of formation of culture *in vitro* (dedifferentiation of cells and their subsequent proliferation, the first passages *in vitro*, long-term subculturing), there are different types and levels of variability observed, there are different types of natural selection, the destabilizing, directional or preferably stabilizing operated;

– induction of cell dedifferentiation and subsequent cell proliferation involves reprogramming of genome, «rejuvenation» of his state, switch of the cell genome from «specialized» state to that of characteristic for stem cells;

– the process of adaptation of cells to *in vitro* growth conditions can be divided into three periods: the primary population of isolated cells, the formation of strain, the established strain; the division into periods is determined by the type, direction and intensity of «natural» selection that acts in cell population;

– cell populations of established (adapted to growth *in vitro*) strains are characterized by physiological and genetic homeostasis, which are caused mostly by the action of stabilizing selection;

– the established strains are genetically heterogeneous cell populations; range of variability of some features may for some species (or their individual genotypes?) exceed the interspecies variability in nature;

– much of genome reorganizations in cultured cells is canalized: variability observed in culture *in vitro*, is often similar to the natural variability of plants of related species; DNA sequences that undergo changes are mainly characterized by natural interspecies polymorphism within the genus; individual (rearranged in culture *in vitro*) sequences resemble sequences characteristic of genome of the intact plants of closely related species in nature, the dominance in genetically heterogeneous populations of canalized changes may indicate the adaptability of namely such genomic changes;

– genetic polymorphism of cultured cells derived from the same plant (genotype), may reflect (restore) all or at least a significant part of both intrapopulation and interpopulation diversity inherent to this plant species;

– similarity of genomic changes that occur in the course of adaptation to the conditions of cell growth *in vitro* and genomic variability in nature, including those in the course of speciation, suggests the possibility of applying N. I. Vavilov law of homologous series in hereditary variability to cell culture, this allows to predict the features of genomic variability *in vitro*;

– similarity of genomic evolution, chromosomal in particular, in cell cultures and genome rearrangements

that underlie speciation is certainly important for understanding some regularities of the evolutionary process and offers the possibility to simulate it at a cellular level *in vitro*;

– much of the genomic changes that occur and found in cultured cells can not be revealed in plants-regenerants: cells with significant genome rearrangements can not regenerate viable plants, this reduces reality of expectations for somaclonal variants with properties previously unknown to breeders, this concerns primarily plant species with simple (not polyploid) genomes;

– elucidation of features and identifying the key factors and driving forces of genomic variability of cell populations *in vitro* allows to some extent regulate not only the genetic structure of cell populations, but also the function of their genome, including the biosynthesis of secondary metabolites; because of that it were created highly productive cell lines and strains of rare and especially valuable medicinal plants, an alternative source of environmentally friendly raw material for pharmaceutical production.

The above generalized data allow the following assumptions:

any somatic cell with a living (functionally active) nucleus during its isolation and further growing in culture *in vitro* as a result of dedifferentiation and «somaclonal» variability (the latter acts within the law of homologous series in hereditary variability by N. I. Vavilov) may restore in their descendants, including regenerated plants, genetic polymorphism (or at least a significant part of it) characteristic of the species and maybe even genus of plants. This feature of somatic cells opens up new horizons both in cell biology and evolutionary theory, and in various areas of applied research, including for the conservation and restoration of natural polymorphism, by cultivating cells and tissues in conditions *in vitro*.

В. А. Кунах

Еволюція клітинних популяцій *in vitro*: особливості, рушійні сили, механізми та наслідки

Резюме

Розглянуто основні ознаки та відмінності клітинних популяцій, типи і напрями дії добору у таких популяціях. Висвітлено популяційно-генетичні основи адаптації клітин до умов росту in vitro, зокрема, проаналізовано особливості еволюції геному в процесі дедиференціювання клітин та подальшої адаптації їх до умов росту в пересадній культурі. Обговорено головні чинники мінливості та добору в клітинних популяціях in vitro, вплив умов вирощування на структуру клітинних популяцій та деякі закономірності мінливості культивованих клітин і рослин-регенерантів. Наведено особливості створення стабільних клітинних ліній – продуцентів біологічно активних речовин. Викладено погляди і припущення автора, сформовані в результаті аналізу як літературних даних, так і багаторічних власних досліджень з генетики клітинних популяцій. Серед низки інших обгрунтовано такі ключові положення: 1) культура клітин in vitro є динамічно-гетерогенною біологічною системою – клоновою популяцією, яка розвивається (еволюціонує) в результаті дії основних рушійних чинників еволюції – мінливості, спадковості, добору і дрейфу генів (генотипів); взаємодія цих процесів зумовлює біологічні особливості кожної конкретної клітинної лінії, що вирощується за конкретних умов. 2) У процесі адаптації клітин до умов росту in vitro виявляються три періоди: первинної популяції ізольованих клітин, становлення штаму (клітинної лінії), сформованого штаму; поділ на періоди визначається типом, напрямом та жорсткістю «природного» добору, що діє в клітинній популяції; сформовані (адаптовані до росту in vitro) штами є генетично гетерогенними, для них характерна наявність фізіологічного і генетичного гомеостазу, що визначається здебільшого дією стабілізуючого добору. 3) Культивовані клітини вищих рослин здатні до синтезу практично усіх класів сполук вторинного (спеціалізованого) обміну (алкалоїди, стероїди, терпеноїди та ін.). Будь-яка соматична клітина з живим (функціонально активним) ядром при її ізолюванні та подальшому вирощуванні за умов культури тканин внаслідок процесів «сомаклональної» мінливості, що відбуваються в рамках закону гомологічних рядів у спадковій мінливості М. І. Вавилова, може відновити у своїх нащадках, у тому числі серед рослин-регенерантів, увесь генетичний поліморфізм (або, принаймні, значну його частину), властивий даному виду, та, ймовірно, навіть і роду рослин. Це відкриває можливість збереження і відновлення природного поліморфізму в культурі клітин і тканин in vitro.

Ключові слова: еволюція геному, клітинні популяції, добір, ріст in vitro.

В. А. Кунах

Эволюция клеточных популяций *in vitro*: особенности, движущие силы, механизмы и последствия

Резюме

Рассмотрены основные признаки и отличия клеточных популяций, типы и направления действия отбора в таких популяциях. Освещены популяционно-генетические основы адаптации клеток к условиям роста in vitro, в частности, проанализированы особенности эволюции генома в процесссе дедифференцирования клеток и последующей адаптации их к условиям роста в пересадной культуре. Обсуждены главные факторы изменчивости и отбора в клеточных популяциях in vitro, влияние условий выращивания на структуру клеточных популяций и некоторые закономерности изменчивости культивированных клеток и растений-регенерантов. Приведены особенности создания стабильных клеточных линий – продуцентов биологически активных веществ. Изложены взгляды и предположения автора, сформированные в результате анализа как литературных данных, так и многолетних собственных исследований по генетике клеточных популяций. Среди прочего обоснованы следующие ключевые положения: 1) культура клеток in vitro является динамично-гетерогенной биологической системой – клоновой популяцией, развивающейся (эволюционирующей) в результате действия основных движу-

щих факторов эволюции – изменчивости, наследственности, отбора и дрейфа генов (генотипов); взаимодействие этих процессов обусловливает биологические особенности каждой конкретной клеточной линии, выращиваемой в конкретных условиях. 2) В процессе адаптации клеток к условиям роста in vitro выявляются три периода: первичной популяции изолированных клеток, становления штамма (клеточной линии), сформированного штамма; разделение на периоды определяется типом, направлением и жесткостью «природного» отбора, действующего в клеточной популяции; сформированные (адаптированные к росту in vitro) штаммы являются генетически гетерогенными, для них характерно наличие физиологического и генетического гомеостаза, что обусловлено в основном действием стабилизирующего отбора; 3) Культивированные клетки высших растений способны к синтезу практически всех классов соединений вторичного (специализированного) обмена (алкалоиды, стероиды, терпеноиды и др.). Любая соматическая клетка с живым (функционально активным) ядром при ее изолировании и последующем выращивании в условиях культуры тканей вследствие процессов «сомаклональной» изменчивости, происходящих в рамках закона гомологичных рядов в наследственной изменчивости Н. И. Вавилова, может восстановить в своих потомках, в том числе среди растений-регенерантов, весь генетический полиморфизм (или, в крайнем случае, значительную его часть), присущий данному виду, и, вероятно, даже и роду растений, что открывает возможность сохранения и восстановления природного полиморфизма в культуре клеток и тканей in vitro.

Ключевые слова: эволюция генома, клеточные популяции, отбор, рост in vitro.

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