Molecular and Cell Biotechnologies

ISSN 1993-6842 (on-line); ISSN 0233-7657 (print)
Biopolymers and Cell. 2023. Vol. 39. N 4. P 283–298
http://dx.doi.org/10.7124/bc.000AA7

UDC 582.923.5:581.143.6+547.94+576.53

Comprehensive study of hormone-independent highly productive strain of *Rauvolfia serpentina* tissue culture as a source of indole alkaloids

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Aim. To characterize a new hormone-independent strain K-27M of *R. serpentina* tissue culture. **Methods.** Plant tissue culture, biochemical and cytological analysis, statistical methods. **Results.** The strain is maintained on the specially designed simple-composition hormone-free 10C medium. The maximum biomass yield was at day 69 of subculture (693.5 g/l of live biomass, 43.0 g/l of dry biomass), the weight of cell biomass increased 15–18 times over a subculture period. The maximum content of indole alkaloids was observed from 88 to 108 days of subculture. The dry biomass contained 4.0% of total indole alkaloids, 1.64% of ajmaline-like alkaloids, 0.789% of ajmaline, 0.337% of vomilenine, and 0.006% of each yohimbine and reserpine. The strain is a heterogeneous mixoploid cell population with a modal class of 22 to 33 chromosomes (2n = 22), which characterized by significant cell and nuclear polymorphism. **Conclusions.** The K-27M strain of *R. serpentina* cultured tissue is a prospective producer of indole alkaloids.

K e y w o r d s: plant tissue culture, *Rauvolfia serpentina*, indole alkaloids, cell selection.

Introduction

The Indian snakeroot (*Rauvolfia serpentina* Benth. ex Kurz, 2*n* = 22) is a tropical shrub that synthesizes and accumulates in its roots more than 50 indole alkaloids, which exhibit anti-arrhythmic, hypotensive, sedative, psychotropic, anti-inflammatory or antibacterial activities [1, 2]. This herb is a source of medicinal raw material used especially for prophylaxis and treatment of cardiovascular diseases.

Since *R. serpentina* is a rare medicinal herb, it is relevant to develop a biotechnological approach to obtain plant tissue biomass containing target biologically active compounds (BACs) [3–5]. The *in vitro* tissue culture allows researchers to produce aseptic biomass, enriched in secondary metabolites in environmentally-friendly way, over the whole year, with no harm to biodiversity. Furthermore, optimization of culture conditions for undifferentiated cell growth *in vitro* may notably increase the productivity of tissue culture in terms of target BACs [3, 6–10].

Since late 1970s, the Department of cell population genetics of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine has carried out the experiments to establish highly productive tissue culture strains of different rare medicinal herbs. The physiological, biochemical, cytological, and molecular genetic characteristics of the tissue cultures have been studied, and the optimal culture conditions suitable for the biotechnological production of the target medically important BACs have been developed [3]. In particular, the strains derived from the *R. serpentina* tissue culture established by Butenko R.G. in 1964 have been developed and comprehensively studied. However, at that time, the tissue culture and its derivates (clones, lines and strains) accumulated insignificant quantities of alkaloids. For instance, the highest content of ajmaline in dry biomass was lower than 0.1 % in contrast to 0.3 % in the plant roots [11]. After a series of procedures (mutagen treatments, development of special nutrient media, constant selection of variants with higher productivity) the strain was obtained which accumulated up to 1.1 % of total alkaloids and up to 0.25 % of indole-type alkaloids in dry biomass [12]. Since 1980s, the experiments for establishing new highly productive strains of *R. serpentina* tissue culture were started in the Department, at the beginning in collaboration with the former Leningrad Chemical Pharmaceutical Institute (St. Petersburg State Chemical Pharmaceutical University, Russia), and independently later on.

In the experiments carried out in the Department of cell population genetics, the accumulation of alkaloids in the *R. serpentina* tissue culture occasionally exceeded their content in natural plants several times, reaching 8 % and more in dry biomass, under conditions of culture on the specially developed growth media according to the specially designed protocols [3, 6, 8, 13]. Using various methods of mutagenesis, in particular chemical mutagenesis, and supportive (stabilizing) selection, a number of new suspension cell lines, single-cell clones, and strains of *R. serpentina* tissue culture were established. The abovementioned strains and cell lines in some cases had shown a difference not only in the content of accumulated alkaloids, but also in the alkaloid profile and total productivity [3, 6, 8, 13–15]. Especially productive and technological was the strain K-27, which was created using the treatment of cultured tissues with the chemical mutagen ethylene imine followed by the long-term supporting selection on a specially developed growth regulator-free media. The genealogy of the K-27 strain of *R. serpentina*, the details of its establishment and culturing along with the characteristics and peculiarities were reported earlier [3, 7–9]. Over more than 35 years, this strain was stable in terms of both

productivity, genetic and cytological characteristics, being grown on both liquid and solid media of various composition with or without different organic additives [16, 17, 26, 18–25]. In the optimum culture conditions on 10C growth regulator-free solid agar medium, on the 70th day of subculture cycle, the K-27 strain accumulated 2.4–2.9 % of total alkaloids, 1.2–1.8 % of indole alkaloids, and up to 1 % of ajmaline in dry biomass. Other alkaloids were identified in the biomass of this strain, including vomilenine (up to 0.5 %), reserpine (up to 0.02 %), ajmalicine, serpentine, and 17-O-acetylajmaline. The total yield of indole alkaloids was 0.7–1.0 g per 1 L of nutrient media, with accumulation rate of 12–17 mg/L per day [3]. Using the K-27 *R. serpentina* strain, the Kharkiv Chemical Pharmaceutical Plant has started experimental biotechnological industrial production of ajmaline in late 1980s — early 1990s. However, without state support and funding, this production line was shut down in late 1990s, and no further optimization of technology was undertaken.

However, subsequent experiments using supporting selection were carried out in the Department over the course of years (the details of supporting selection on the basis of inheritability factor are published in a monograph [3]), and a new, modified, stable hormone-independent K-27M strain of *R. serpentina* tissue culture was established. This strain can be potentially used as a biotechnological source of indole alkaloids.

Aim of the study was to investigate comprehensively the main characteristics of the established hormone-independent K-27M strain of *R. serpentina* tissue culture, including the accumulation of wet and dry biomass over subculture cycle, quantitative and qualitative analysis of indole alkaloids accumulated in cell biomass, and cytological characteristics of cultured tissue.

Materials and Methods

Cell biomass of the K-27M strain *R. serpentina* tissue culture derived by the cell selection methods from the K-27 strain, which was maintained *in vitro* for more than 35 years, was studied. The details of establishment and characteristics of the initial K-27 strain were published earlier [3, 16–28]. The newly established K-27M strain was cultured under thermostatic conditions at 27–28 °C in the dark, in 370 mL glass vessels. Each vessel contained 100 mL of the 10C solid agar medium without growth regulators. The composition of the 10C medium was published earlier [13] and patented (patent No.77366) [27]. Noteworthy, the medium has a simple composition and contains only mineral salts, sugar (100 g/L), agar (5 g/L), and B1 vitamin (5 mg/L). The duration of cultivation before harvesting the biomass was 65 to 85 days, the typical subculture cycle duration was 45–50 days, and the mass of tissue used as an explant was 3.5–5 g per vessel or 35–50 g per 1L of medium. To study the biomass growth and alkaloids accumulation over a subculture cycle, the tissue culture was sampled starting from the day of subculturing (day 1 of the new cycle and day 50 of previous cycle). In this study, the biomass was sampled from 5 to 10 vessels over the course of 117-day long subculture period on days 10, 20, 30, 48, 60, 69, 80, 88, 108, and 117. The biomass samples were dried in the infrared dryer (Avangard SVM Service, Ukraine) at 55 °C

and air humidity of 56 % for 24 hours; ambient air was circulated through the dryer for 4 min each 15 min. The content of dry biomass was calculated as a ratio of dry mass to the wet mass of sampled cultured tissue.

Statistics was calculated over 5 to 10 sampled vessels for each day of sampling.

The tissue samples for cytogenetic analysis were taken simultaneously with the abovementioned samples for biochemical study. Callus was fixed for 24 hours in a mixture of 3:1 ethanol:acetic acid. The samples were then stained with 1 % acetoorseine, and squashed preparations were made. To identify the starch grains in the cells, staining with iodine solution was used. Microscopic investigations were carried out using NU-2E Carl Zeiss microscope; microphotographs were taken using Canon 1000D camera.

To quantify the indole alkaloids, the dry biomass was extracted three times with methanol (10 mL per 1 g of biomass) with a drop of ammonia solution using sonication for 30 minutes 4 times. The resultant extracts were combined, and the solvent was removed by rotary evaporation. The extracts were then reconstituted in pure methanol (1 mL per 0.1 g of dry biomass). The content of the alkaloids in dry biomass was determined over the course of 117-day long subculture period. Qualitative and quantitative analysis of alkaloids was performed on the Agilent 1260 Infinity II HPLC-MS system, using C18 column and water-acetonitrile gradients with 0.1 % v/v of formic acid as sensibilizer and pH adjustor. The calibration standard solutions of ajmaline, ajmalicine, vomilenine, reserpine, and yohimbine hydrochloride (0.5–1.0 mg/mL, which corresponded to 0.5–1.0 % mass of this alkaloid in extracted biomass assuming equal injection volumes) were prepared in methanol. Mass chromatograms were taken for $[M+H]^+$ ions of each alkaloid, and alkaloids were quantified by the area of respective chromatographic peaks. For other known (by m value) alkaloids with no standards, the structural similarity to ajmaline was assumed to give similar ionization effectiveness, hence their concentrations were estimated by peak areas relative to the peak of ajmaline standard.

Results and Discussion

Cell biomass productivity. The K-27M strain of *R. serpentina* tissue culture was grown on the simple-composition medium 10C lacking growth regulators, which was specially designed for culturing *R. serpentina in vitro* (for the medium composition see [13, 27]). The growth of callus tissue was stable; visually the tissue looked yellowish, homogenous and dense, with knobby surface; it had bitter taste and characteristic odour. Over the subculture period, the growth medium was largely consumed: from the initial 100 mL of the 10C medium, only 20–25 g remained in a vessel after 70–75 days, with 68–69 g of harvested wet biomass. The authors suggest that such a completeness of the medium consumption indicates a balanced composition and is important for the development of wasteless biotechnological biomass production in industrial scale (Fig. 1).

The growth curve of the strain exhibited a typical S-shape, reaching the stationary phase after 48–50 days (Fig. 2A). The yield of wet callus tissue on the 10th day was 90.7 g per 1 L of medium; thus, the cultured biomass increased 2.5 times compared to the weight of

Fig. 1. General appearance of the K-27M strain of *R. serpentina* tissue culture grown on the 10C medium at different time points after subculture.

initial explant at day 1, and further 5 times on day 20, tenfold on day 30, and 16.7 times on day 48. After day 48, the growth curve exhibited a plateau with a less intense increase and the maximum weight of wet biomass on day 69 (693.5 g/L), which is almost 18 times more than that of initial explant (Fig. 2A). After the day 88 and until the day 117, the yield of wet biomass stabilized within 643.4–666.8 g/L.

The dry biomass yield also increased rapidly until day 48 of the subculture cycle. Over the next 20 days, these values were the highest and ranged from 41.8 to 43.0 g/L of medium. After day 88, the dry biomass yield decreased slightly and stabilized.

The highest content of dry biomass (dry matter) in the cultured tissue was observed during the first 30 days of subculture cycle (Fig. 2B). Thus, this figure reached 11.7 % on day 10, 11.0 % on day 20, and 10.1 % on day 30. Further cultivation resulted in a 1.9-fold decrease in the percentage of dry mass content (6.3 %) by day 48. After the 48th and up to the 88th day, the dry biomass content was 6.2 %. After day 90 of subculture, the value of this indicator stabilized in the range of 5.3–5.5 %,

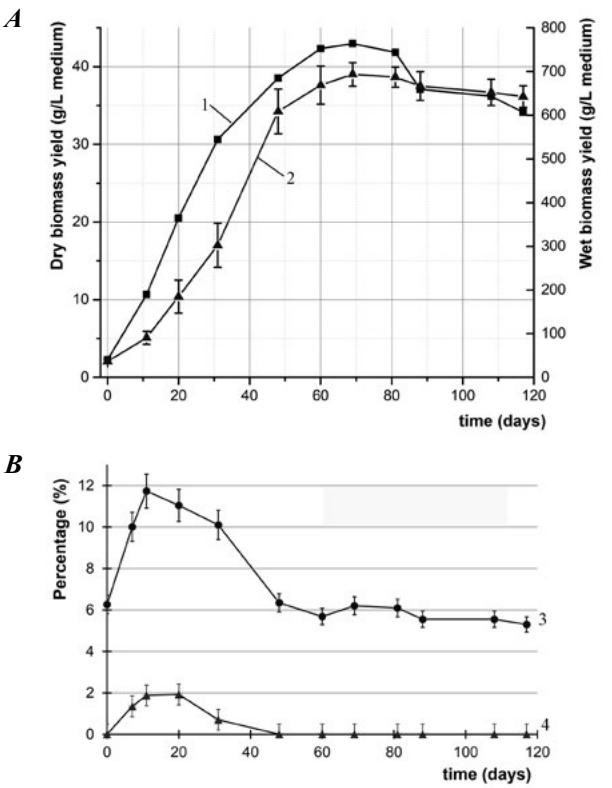

Fig. 2. Changes in dry (1) and wet (2) biomass (*A*); dry biomass content (3), and mitotic index (4) (*B*) of callus tissue of the K-27M strain of *R. serpentina* during a subculture cycle. Error bars indicate 1 SD calculated for 5 to 10 vessels sampled on that day, with each vessel containing 100 mL of medium.

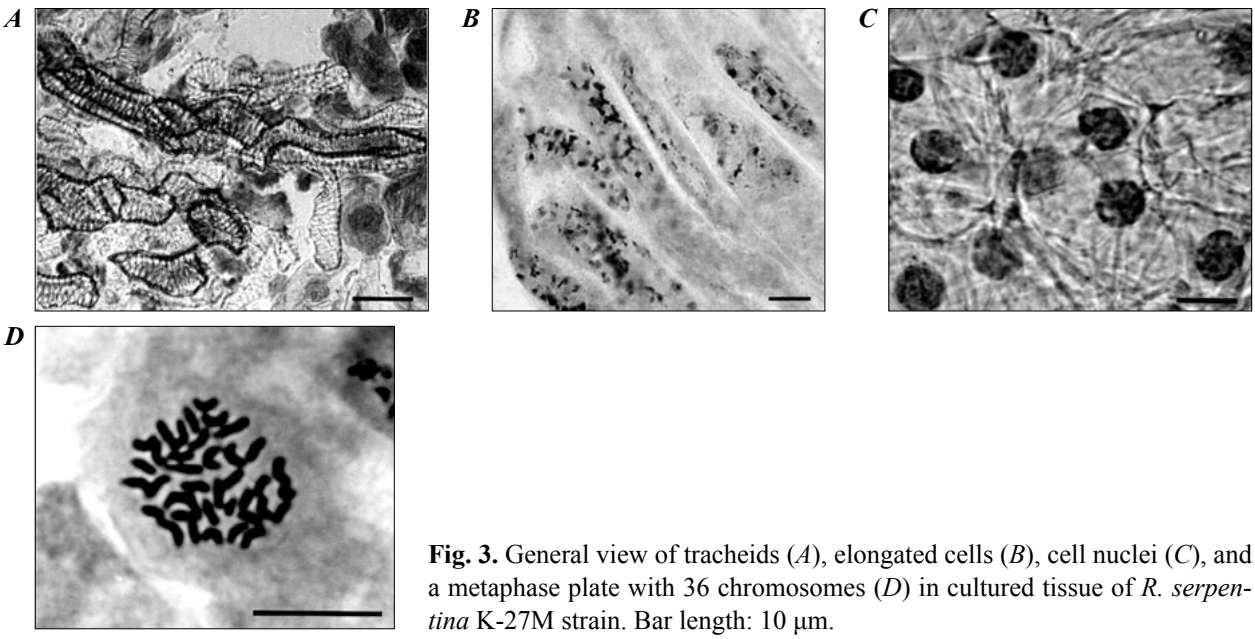

Fig. 3. General view of tracheids (*A*), elongated cells (*B*), cell nuclei (*C*), and a metaphase plate with 36 chromosomes (*D*) in cultured tissue of *R. serpentina* K-27M strain. Bar length: 10 μm.

which is 1.9 times less compared to the beginning of subculture cycle, and 2.2 times less compared to day 10 of subculture (Fig. 2B).

Thus, the K-27M strain of *R. serpentina* tissue culture provides the maximum yield of wet biomass (693.5 g/L of medium) and dry biomass (43.0 g/L) on day 69 of subculture. The content of dry biomass in crude tissue culture stabilized after day 48 of subculture and ranged from 5.3 % to 6.2 %. The weight of initial explant tissue for subculture averaged 35–45 g per liter of medium, and the final yield of crude biomass averaged 670–690 g per liter of medium. Thus, the growth index amounted to 15–18, which indicates a high biomass productivity of the newly established K-27M strain of *R. serpentina* tissue culture.

Cytological analysis. The cultured tissue of the K-27M strain of *R. serpentina* consisted of typical callus cells and differentiated cells, mainly tracheids (Fig. 3A). The proportion of tracheid elements was 6–7 % of all cells during a subculture cycle. The callus cells contained many starch grains on days 7, 10, 20, and 30 of growth; they were tightly filled with starch grains. The starch grains were rounded or ellipsoidal in shape and varied in size from 4 to 12 μm. They were formed by the layers of starch, which were usually deposited around the hilum (core) of a starch grain.

The cells and nuclei in callus tissue of *R. serpentina* K-27M strain demonstrated significant polymorphism. Size of cells and nuclei changed during subculture cycle, thus reflecting the growth stages of cultured tissue. With the rise of mitotic activity (Fig. 2B), the cell size decreased, while in the stationary phase of growth, the cell size increased 2–3 times and most cells had an elongated shape. There were round and elongated cells (Fig. 3B, C)

with the nuclei that were round (Fig. 3C), elongated, with apricot-kernel like shape, or spindle-like shape. The cell size ranged from 40 to 60 μm for rounded cells and from 50 to 90 μm in the long axis and 15 to 20 μm in the short axis for elongated cells. Both round and spindle-shaped nuclei were found in round cells, but only spindle-shaped nuclei were observed in elongated cells. The nuclei size was about 10 μm for round nuclei, 12–18 μm in the long axis and 5–7 μm in the short axis for spindle-shaped nuclei. More than 40 % of the nuclei contained from two to six nucleoli. A large number of nuclei were heterochromatized; the number of such nuclei increased during subculture cycle and reached more than 50 % in the second half of the cycle. On day 69 of subculture (at the time of harvesting), the cells were predominantly elongated, with elongated nuclei or nuclei with apricot-kernel like shape. The cells were rather small with small nuclei, 3–4 times smaller than the cells in cultured tissue at the beginning of subculture (approximately 15–20 μm). The cells were tightly adjacent to each other. No tracheids were found. On days 57 and 117 of subculture, many enucleated cells were observed.

The mitotic index was 1.35 % on day 7 of subculture, slightly increased on days 10 and 20 (1.87 % and 1.92 %, respectively). Further during subculture cycle, the mitotic activity decreased and amounted to 0.7 % on the day 30 of the cycle. On days 57 and 117, only single mitoses with highly polyploid chromosome numbers were found.

R. serpentina K-27M strain was found to be mixoploid with a chromosome number ranging from 20 to 70 ($2n$ = 22). The metaphase chromosomes were small in size (1.5–3 μm). The metaphase plate with a hypertriploid number (36 chromosomes) is shown in Fig. 3D. A low level of anaphase aberrations was observed. Among the analyzed anaphases (n = 255), only two cells with aberrations in the form of chromosome lagging were detected, which is less than 1 %. The changes in chromosome number during the subculture cycle are shown in Fig. 4A.

The obtained data indicate that the proportion of diploid ($2n$ = 22) and tetraploid ($4n$ = 44) cells in the pool of proliferatively active cells of the K-27M strain practically did not change during subculture period; they were about 17 % and 4.5 %, respectively (Fig. 4A). Triploid and hypotriploid cells in total accounted for 65 % of the dividing cell population by day 10, then their proportion decreased until day 20, and then remained virtually unchanged during subculture. The percentage of hypotetraploid cells at the beginning of subculture period was 21 %, then their number decreased sharply to 2 % on day 20 and increased slightly (to 12 %) at the end of subculture. The number of hypertetraploid cells at the beginning of subculture period was about 1 % and then increased sharply to 35 % on day 20. On day 30, their proportion decreased to the initial level (Fig. 4A). A significant proportion (69 %) of the highly polyploid cells were the cells with a hexaploid set. Their share in the total population of dividing cells was about 8 % (Fig. 4B).

Thus, the proliferatively active portion of the cell population of *R. serpentina* K-27M strain was represented mainly by hypotriploid cells with a chromosome number of 25–30, as well as by di- and triploid cells (Fig. 4B). The data obtained indicate that *R. serpentina*

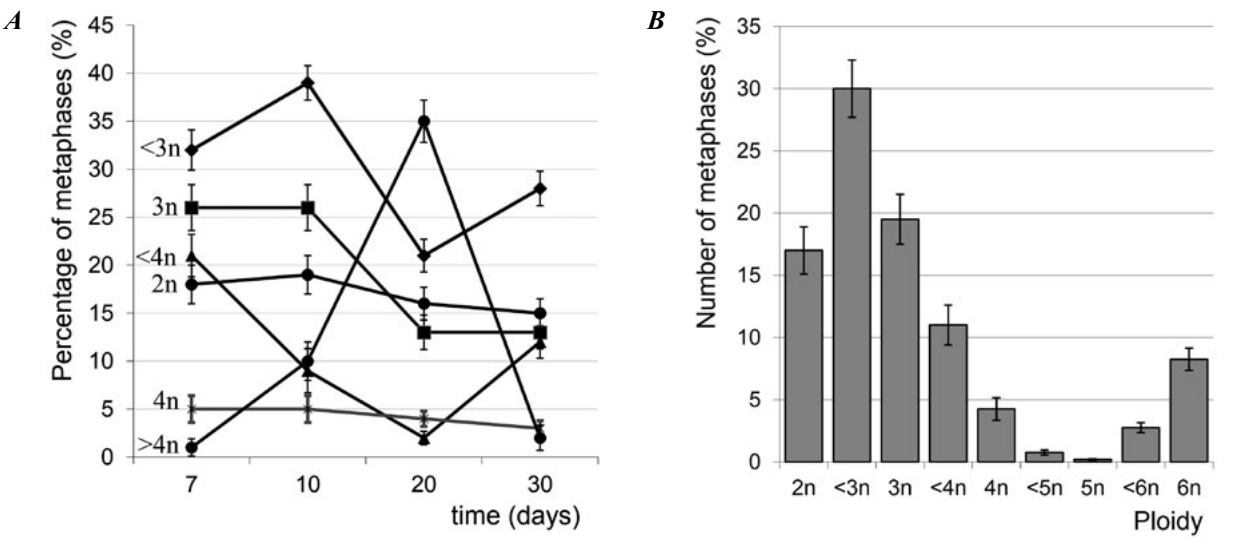

Fig. 4. Changes in the proportion of cells with different ploidy levels during the subculture cycle (*A*) and distribution of cells according to their ploidy level in the proliferatively active pool (averaged data over a subculture period) (*B*) in cultured tissue of *R. serpentina* K-27M strain.

K-27M strain is a mixoploid cell population with the chromosome numbers ranging from 20 to 70 and a modal class consisted of the cells with chromosome numbers ranged from 22 to 33. The strain exhibited a very low level of anaphase aberrations, less than 1 %, compared to other described tissue cultures, which is comparable to the spontaneous level of aberrations in normal plants in nature. The culture growth during the subculture period was accompanied with an increase in the cell and nucleus size. The highest percentage of polyploid cells in the proliferatively active pool was observed on day 20 of subculture simultaneously with the most intense increase in biomass. After day 30 of subculture, the mitotic activity stopped and the size of cells decreased.

An interesting, previously unknown phenomenon was the accumulation of a large number of starch grains in the *R. serpentina* cultured tissue cells at initial stages of callus growth. It can be assumed that starch is further used as a source of energy for the biosynthesis and accumulation of alkaloids (see below).

Content of indole alkaloids. The HPLC studies revealed a sharp decrease in the content of indole alkaloids in the biomass at the beginning of subculture period, with the lowest values during the most intensive growth of cell biomass. In particular, on day 20 after subculture, the content of ajmaline was 2.8 times less, vomilenine — 4.0 times less, and ajmalicine — 2.8 times less compared to the explant tissue used for subculture (Fig. 5). Yohimbine and reserpine were found in residual amounts. In contrast, the content of dry matter in wet biomass increased by 1.8 times compared to the explant tissue; and the cells contained a large amount of starch grains.

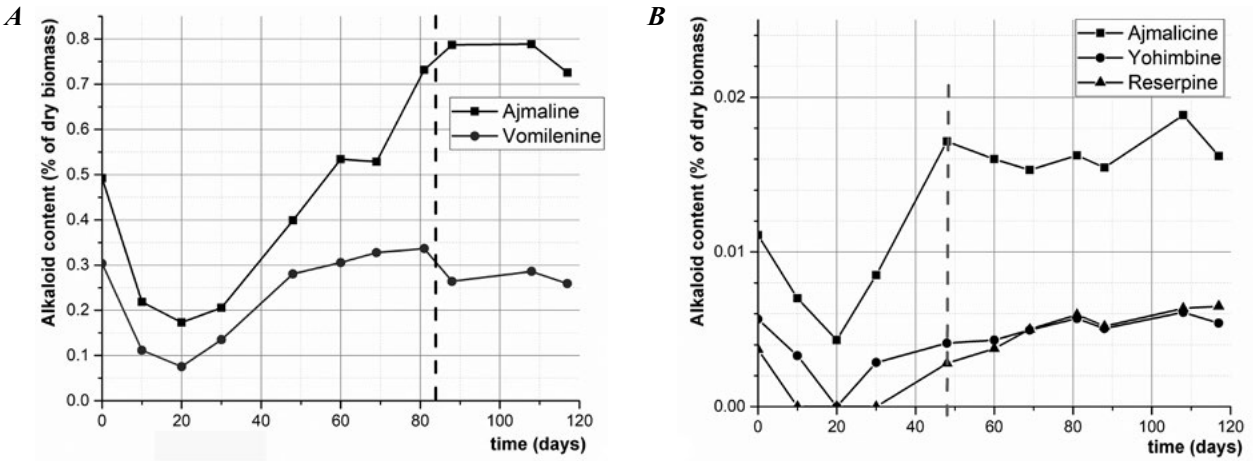

Fig. 5. Dynamics of accumulation of ajmaline and vomilenine (*A*); ajmalicine, yohimbine, and reserpine (*B*) during a subculture period of *R. serpentina* K-27M strain.

After the 48th day of subculture, the growth of the tissue slowed down, and the cell resources were mainly directed to the synthesis of secondary metabolites (Fig. 5). The maximum contents of all studied alkaloids were observed after the 80th day of subculture (Figs. 5, 6). On days 88–108 of the subculture period, the amount of ajmaline was 0.787–0.789 % of dry weight, which is 1.6 times more than at the starting point and 4.5 times more than on day 20 of subculture. The content of vomilenine reached the maximum on day 80 (0.337 % of dry weight) (Fig. 5A). The content of ajmalicine from day 40 to day 117 ranged from 0.016 % to 0.017 % and was highest on day 110 (0.019 %). Yohimbine and reserpine were accumulated in cultured tissue in small amounts. From day 80, their contents were stable within the range of 0.005–0.006 % (Fig. 5B).

Thus, during the subculture period, the cultured cells of *R. serpentina* K-27M strain actively undergo mitosis, grow intensively, and accumulate biomass and starch for the first 45–50 days. In the following days, the growth of the culture slowed down, the cells were intensively differentiated, the mitotic activity and the number of starch grains in differentiated cells decreased, and cell resources were mainly directed to the synthesis and accumulation of indole alkaloids. The maximum contents of the studied compounds were observed on days 88–110 of subculture period. In particular, the total content of indole alkaloids was estimated to be 4 % of dry biomass on day 88 of subculture, which is one and a half times higher than that of the original strain K-27 (2.4–2.9 %). The amount of ajmaline-like alkaloids was estimated at 1.64 % of dry weight, which is close to the figures of the original strain K-27 (1.2–1.8 %). No significant differences were found between two strains in the profile and content of other alkaloids identified in the biomass.

The presence of a large proportion (about 83 %) of polyploid cells in the cultured tissue of the K-27M strain is noteworthy, along with

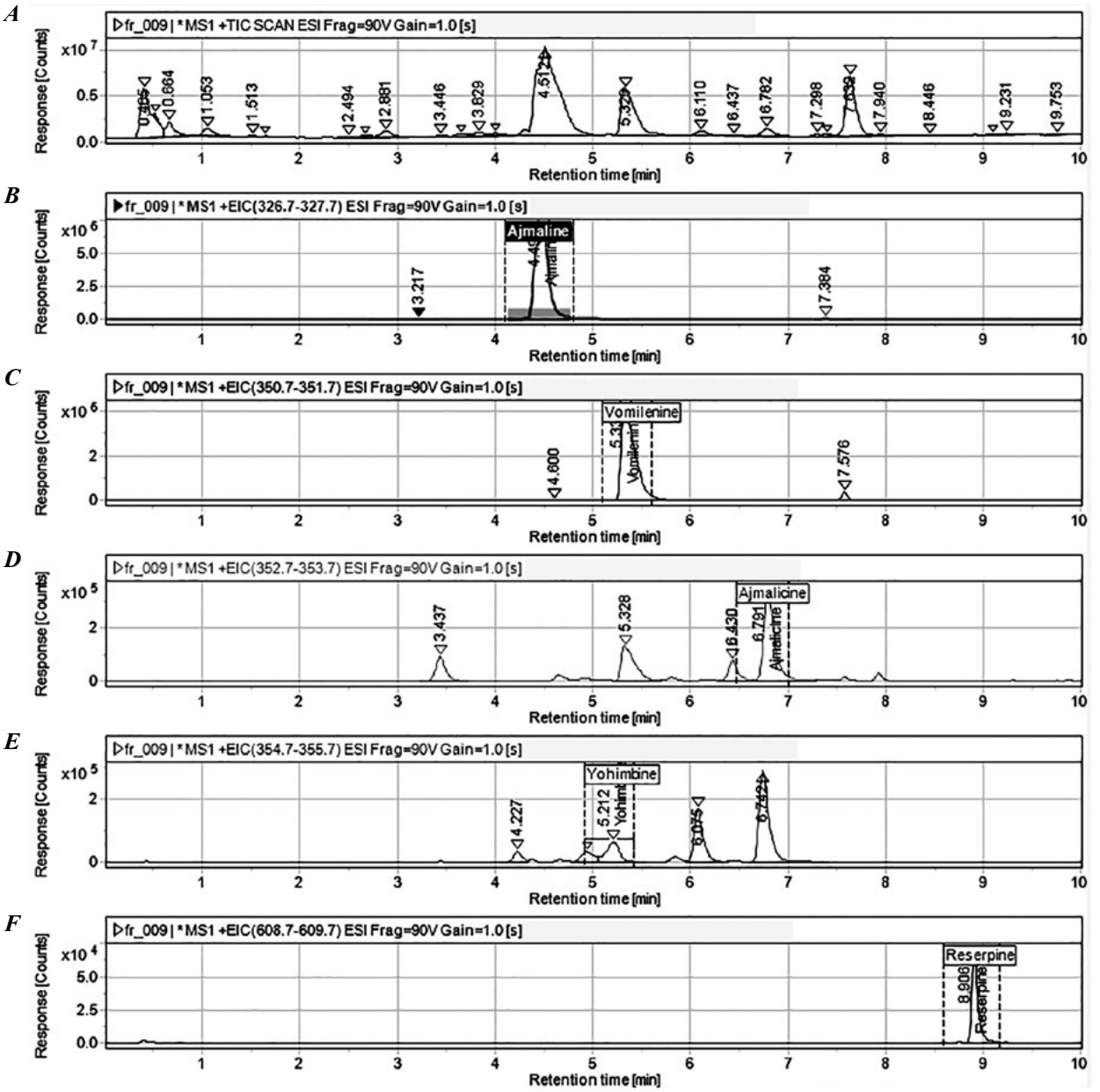

Fig. 6. Chromatogram of indole alkaloids from the cell biomass of *R. serpentina* K-27M strain harvested on day 88 of subculture. *A* — total ion current chromatogram (TIC) of cell biomass extract; *B* — extracted ion chromatogram (EIC) for ajmaline ion ($[M+H]^+$, m/z = 327); *C* — EIC for vomilenine ion ($[M+H]^+$, m/z = 351); *D* — EIC for ajmalicine ion ($[M+H]^+$, m/z = 353); *E* — EIC for yohimbine ion ($[M+H]^+$, m/z = 355); *F* — EIC for reserpine ion ($[M+H]^+$, m/z = 609).

the changes in the number of cells with different chromosome numbers during subculture period. It should be noted that similar phenomena were characteristic of all studied derivates (derived cell lines) originated from the primary callus culture established in 1964 by Butenko R. G., including the strain K-27. In particular, in the K-27 strain, during the first five days, mainly diploid cells were mitotically active, which accounted for 70 % of dividing cells. Then, the number of dividing diploid cells decreased sharply and after 25–27 days of subculture they were not observed. The frequency of triploid cells in mitosis on days 11–17 was 40 %. Tetraploid and polyploid cells entered mitosis later. Their share was 12-20 % during the first five days, 50 % on day 11, and more than 80 % after day 27 of subculture. By the end of subculture period, only tetraploid and high-ploidy cells were dividing (see for review [3]).

Comparison of these data with the dynamics of biomass accumulation and alkaloid synthesis may indicate a relationship between the productivity of *R. serpentina* tissue culture and the presence of polyploid cells in the population. We have previously shown that the amount of DNA in cultured cells increases during a subculture cycle when they switch to alkaloid biosynthesis [29]. Obviously, in mixoploid cell populations of *R. serpentina* culture *in vitro*, there may be a dependence of strain productivity, in particular indoline alkaloid biosynthesis, on the ploidy of cells that formed the modal class [30]. However, other researchers found a negative correlation between the chromosome number and the accumulation of secondary metabolites in cultured *R. serpentina* cells [31].

Interestingly, in nature, both induced and spontaneous tetraploid forms of *R. serpentina* accumulate more indole alkaloids than diploid plants [32, 33]. On the other hand, the contrary data on the amount of nuclear DNA, chromosomes structure, and reserpine content were reported in the study of five populations of *R. serpentina*, *R. vomitoria,* and *R. canescens*. The authors suggest that the genetic control of reserpine content in *Rauvolfia* species does not necessarily depend on the number of chromosome sets, but varies at the intraspecific and interspecific levels [34]. It should be noted that the phenomena of increasing the content of nuclear DNA and the number of individual repeats in cells and cell polyploidization during differentiation in the course of plant ontogeny have long been proven. High level of polyploidization is especially typical of highly differentiated cells and storage tissues (see for review [3, 9]). Nevertheless, the issue of the relationship between the ploidy level of cultured cells of different plant species and the amount of accumulated BAC, and the overall productivity of cultured tissue obviously requires further study.

Since the 1960s, a large number of studies on *R. serpentina* tissue and cell culture have been carried out by other researchers to establish the productive *in vitro* culture strains that would accumulate indole alkaloids used in medicine (see for review [3, 5, 8, 10]). For example, different concentrations and ratios of growth regulators [5, 35], $AlCl_3$ and $CdCl_2$ [36, 37], sucrose [38], thiamine-HCl, pyridoxine-HCl, nicotinic acid, *etc.* [5] were added to the culture medium to increase productivity. An increase in the biomass growth and accumulation of indole alkaloids in *R. serpentina* tissue

culture was shown with the addition of beet sugar to the culture medium: the dry biomass yield after 40 days of growth was 24.4 ± ± 2.11 g/L, which is 17.3 % higher than in the control. The content of ajmaline on day 60 of subculture was 0.507 % of dry weight, which is 61.98 % higher compared to the control. In addition, after genetic transformation PCR analysis of the *rol* genes confirmed the genetic stability of the clone when cultured *in vitro* for six years [38].

In our experiments, the dry biomass yield of the K-27M strain grown on the growth regulator-free, simple-composition medium 10C amounted to 38.6 g/L on day 40 of subculture, which was almost twice as high as reported in [38]. The content of ajmaline was 0.534 % of dry weight on day 60 of subculture and reached 0.789 % on days 88–108, which is 1.56 times higher than the best values reported in the literature. This indicates the highest productivity of the K-27M strain of *R. serpentina* compared with all known *R. serpentina* tissue cultures. Taking into account that the growth regulator-free nutrient medium containing simple cheap reagents is used to culture the K-27M strain, economic profitability and technological simplicity of the established conditions for maintaining *R. serpentina* tissue culture, which contribute to the production of a large amount of raw materials with a high stable content of indole alkaloids, the K-27M strain may be of economic interest to the biotechnology industry as a promising source of indole alkaloids, in particular ajmaline.

It should also be added that the cell biomass of the original strain K-27 also accumulates more than 20 indole alkaloids [3, 39], which have a vasodilator effect and exhibit an α-adrenergic blocking effect [40], as well as a sedative effect [41]. The original strain K-27 has been confirmed to be stable in terms of productivity for more than 35 years; it did not change after 15 years from the last analysis: the total alkaloid content in dry biomass was 2.8 %, the content of ajmaline and ajmaline-like alkaloids was 1.6 %. The content of ajmaline and its derivates was the largest, 0.69 % of dry weight, those of yohimbine and reserpine were slightly lower (0.02 % and 0.009 %, respectively) [39]. The biomass of the K-27M strain contained 0.787–0.789 % of ajmaline and 0.337 % of vomilenine, while the content of yohimbine and reserpine was 0.006 %, which is slightly lower than in the K-27 strain. The total alkaloid content in the K-27M strain dry biomass was estimated at 4.0 %, the content of ajmaline and ajmaline-like alkaloids at 1.64 %, while for the natural raw materials (roots of 5–7 year old plants) it ranges from 0.8 to 1.3 %.

Thus, in the Department of cell population genetics of the Institute of molecular biology and genetics of the NAS of Ukraine, we established the genetically stable, hormone-independent, highly productive strains K-27 and K-27M of *R. serpentina* tissue culture, developed a method to culture these strains on a simple composition medium, and conducted their comprehensive study. The obtained strains of the *R. serpentina* tissue culture have no analogues in the world in terms of productivity and accumulation of indole alkaloids. The total content of indole alkaloids in the established *R. serpentina* strains is significantly higher compared to other tissue cultures and reaches 4 % in the biomass of the K-27M strain.

Conclusions

The newly established hormone-independent K-27M strain of *R. serpentina* tissue culture was comprehensively characterized over the subculture cycle of 117 days under conditions of maintenance on the simple-composition agar media 10C lacking growth regulators. The changes in the accumulation of wet and dry biomass, dry biomass and alkaloids content, as well as cytological and cytogenetic characteristics of the strain were studied during the subculture period.

The biomass yield of the *R. serpentina* tissue culture K-27M strain was found to reach the maximum at day 69 of subculture and amounted to 693.5 g per L of medium for wet biomass and 43.0 g/L for dry biomass. The dry biomass content in tissues ranged from 5.32 to 6.20 % w/w during the stationary stage of growth. Over a subculture period, the weight of cell biomass increased 15–18 times (from 35–45 g/L of the initial explant to 670–690 g/L of biomass at the 70–75 days of subculture cycle).

The strain exhibited significant polymorphism of cells and their nuclei; their dimensions and shape changed at different stages of cultured tissue growth. Most of the cells had elongated shape, the round-shaped cells also occurred. The shape of cell nuclei varied widely: the elongated, apricot-kernel like, and spindle-shaped nuclei were observed in the cultured tissue.

The K-27M strain of *R. serpentina* tissue culture was a heterogeneous mixoploid cell population with $2n = 22$, in which the cells of different ploidy had different time of onset of mitosis. Proliferative pool consisted of cells with 20 to 70 chromosomes. The frequency of anaphase abnormalities was low (<1 %). Modal class consisted of di-, hypotri-, and triploid cells with chromosome number ranged from 22 to 33, which accounted for more than 65 % of all mitotic cells. Among the highly polyploid cells, a significant proportion were the cells with a hexaploid set (8 % of the total population of dividing cells). Their share increased up to 35 % on day 20 of subculture at the start of intensive accumulation of indole alkaloids.

Maximal accumulation of indole alkaloids was observed on the 88th to 108th days of the subculture cycle. In particular, the content of ajmaline was 0.789 %, vomilenine — 0.337 %, yohimbine and reserpine — 0.006 % of dry biomass weight. Total alkaloid content was 4.0 % of dry biomass weight, including 1.64 % of alkaloids structurally related to ajmaline.

Therefore, the K-27M strain of *R. serpentina* callus cultured on a simple-composition growth regulator-free medium is a prospective producer of indole alkaloids.

Funding

The study was partially supported by the National Research Foundation of Ukraine 2020.01/0258 “Development of the scientific grounds for the biotechnological production of novel hypotensive and antiarrhythmic alkaloids from *Rauwolfia serpentina* Benth.” and by a grant from the Simons Foundation, USA (Award #1030279, I.O. Andreev, I. I. Konvalyuk).

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Комплексне дослідження гормоннезалежного високопродуктивного штаму культури тканин *Rauvolfia serpentina* — джерела індольних алкалоїдів

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Мета. Характеристика нового гормононезалежного штаму К-27М культури тканин *R. serpentina.* **Методи.** Культура тканин, біохімічний та цитологічний аналіз, методи статистики. **Результати.** Штам вирощується на спеціально розробленому безгормональному, простому за складом середовищі 10С. Максимальний вихід біомаси був на 69-й день росту (693,5 г/л живої біомаси, 43,0 г/л сухої біомаси), кількість біомаси зростала за пасаж у 15–18 разів. Максимальне накопичення індольних алкалоїдів виявлено на 88–108-й день росту культури. Їх вміст становив 4,0 % у перерахунку на суху біомасу, аймаліноподібних алкалоїдів 1,64%, аймаліну 0,789 %, воміленіну 0,337 %, йохімбіну та резерпіну 0,006 %. Штам є гетерогенною міксоплоїдною клітинною популяцією з модальним класом від 22 до 33 хромосом (2n = 22), яка характеризується значним поліморфізмом клітин та ядер. **Висновки.** Штам К-27М *R. serpentina* є перспективним продуцентом індольних алкалоїдів.

Ключові слова: культура тканин рослин, *Rauvolfia serpentina*, індольні алкалоїди, клітинна селекція.

Received 07.09.2023