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The indoline alkaloids accumulation by *R. serpentina* cell lines upon surface and submerged maintenance

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Indoline alkaloids accumulation, including ajmaline, in the biomass of R. serpentina cell line culture on the example of hormone independent highly productive strain K-27 at its surface and submerged maintenance in nutrient media with differing mineral composition has been studied. Optimal nutrient media for both ways of maintenance have been identified. The conditions for two-step maintenance in surface, followed by submerged cultures, in compositionally simple nutrient media have been specified. Two-step maintenance increases alkaloids accumulation at the early stages of growth 3-4 times and allows decreasing the time of callus tissue growing from 60-80 days to 20-40 days.

Keywords: *ajmaline, indoline alkaloids, plant tissue culture, R. serpentine, cell lines – alkaloids producents.*

In most known cases the cell cultures of different species of alkaloid plants at long-term maintenance *in vitro* either are not capable of accumulating alkaloids at all, or accumulate them in insignificant quantities [1]. This is advantageous background for cell lines of *Rauwolfia serpentina Benth*, isolated from the callus, obtained by R.G. Butenko from the fragment of a young green stem of a 5-year plant in 1964 in the Institute of Plant Physiology named after K.A. Timiryazev of NAS USSR. These lines are capable of accumulating about 0.2 to 2% indoline alkaloids in dry biomass for more than 40 years already; 70-90% of them is anti-arrhythmic alkaloid ajmaline (scheme 1). In special maintenance conditions the indoline alkaloids content in dry biomass may amount to 20%, and the total productivity in favourable medium sometimes is more than 700 mg of ajmaline from 1 liter [1, 7, 9].

Callus strain K-27 is the most productive of all the known cell cultures of *R. serpentina*. At the maintenance in specially designed agarosed nutrient medium 10C without phytohormones, containing 10% saccharose [8], this strain

Scheme 1. Genealogy and indoline alkaloids accumulation in dry biomass of Rauwolfia serpentina Benth., grown on different nutrition media agar containing (surface growing).

Primary Callus
Obtained by B. G. Butenko on MC medium according to [2], green body fragment of 5 year old plant, 1964

Callus Tissue
MC medium, total alkaloid content 0.1–0.3 %, ajmaline 0.04 % (1964–1968) [3]

Mutagen treatment by nitrogenous yperite in 1968

M1 Mutant Line
MC medium, total alkaloid content 0.2–0.48 %, ajmaline app. 0.2 % [4]

Selection on nutrition medium 5C, without phytohormones according to [5] (1972–1975)

Cell Line A
5C medium without phytohormones, indoline alkaloid content 0.7–0.8 %, ajmaline app. 0.4 %

Mutagen treatment by ethylenimine in 1981 and the adaptation to growth on 10C medium, without phytohormones according to [8] in the course of 1982–1985

K-27 Strain
10C medium, indoline alkaloid content 1.2–1.8 %, ajmaline 0.9–1.2 %

accumulates 1.2-1.8% indoline alkaloids and 0.9-1.2% ajmaline (scheme 1).

Maintenance of this strain in industrial conditions at Kharkiv chemical-pharmaceutical factory in the course of more than 10 years proved the stability of its productivity. However, surface maintenance of callus tissues in agarosed medium on large scales showed low technological effectiveness of raw material obtaining (cell biomass), used as a source of ajmaline. According to the preliminary data, the maintenance of *Rauwolfia* tissue cultures in liquid nutrient medium is more productive.

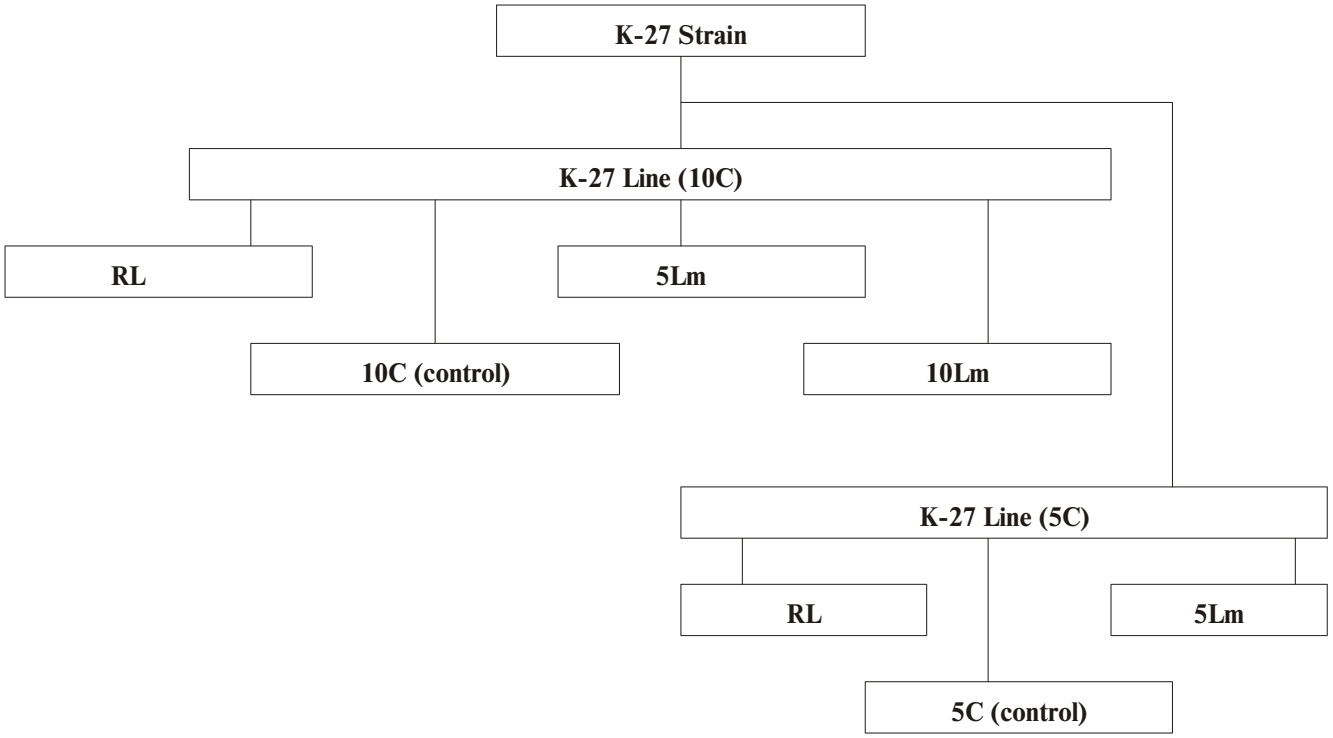
Previously the highly-productive strains of suspension cultures of *R. serpentina* were obtained [1, 10]. However, they proved to be of little use for mass-scale production due to their increased sensitivity to the regime of maintenance and need for special equipment.

The alternative may be the maintenance of callus tissues of highly-productive strains in liquid medium in the form of submerged culture at constant stirring. However, the peculiarities of maintenance and productivity of callus tissues of *R. serpentina* were studied only on the example of the cell line A, comparatively low productive [11]. Callus tissues of more productive strains, including strain K-27, in the liquid medium grow badly or do not grow at all and perish in a short period of time [1].

The present work shows the results of studying the accumulation of indoline alkaloids and total productivity of highly-productive strain K-27 at it maintenance in different conditions (surface and submerged) in different types of nutrient media, specially designed for the tissue cultures of *R. serpentina*.

Materials and methods. The object of investigation was hormone-independent callus strain K-27 *R. serpentina*, the productivity, genetic and biochemical properties of which are described in the works [1, 12]. Since 1982 the strain has been grown on agarosed medium 10C according to [8] (cell line K-27 (10C)), and since 2000 it has been grown on agarosed medium 5C according to [5] (cell line K-27 (5C)).

Scheme 2. The study on growing conditions on biosynthesis level of indoline alkaloids in hormone-independent cell lines of K-27 Rauwolfia serpentina strains.



The same media were used in the experiment without agar (liquid media) – 10Cl and 5Cl, together with the specially designed liquid nutrient medium Rl according to [13] for submerged maintenance of tissue cultures of *R. serpentina* (scheme 2). Cultural media are considerably different in their mineral composition (the quantity and ratio of macro- and microelements), as well as in the saccharose content (10, 5, and 3% correspondingly). Growth regulators were absent in all the media, except for thiamine was in its content out of all the vitamins.

Tissues were grown in the vials of 250ml volume, containing 50ml of media. The size of the explant under transferring was 4-5g of tissue per vial. Liquid cultures were maintained on the shakers with the cycle of oscillation 60-70 r.p.m. The maintenance was conducted at the temperature of 25-27°C without illumination.

Callus tissues were investigated for 90-130 days without transplantation, while the usual duration of the passage before the transplantation for the investigated strain was 35-40 days, and 60-80 days till the harvest output. In the course of every passage every 10 days 3-5 vials of each variant with the medium growth rate were taken for the analysis. The amount of fresh and dry biomass was measured and the content of indoline alkaloids in the dry biomass was determined by photometric method [14]. In some cases individual alkaloids were defined by microchromatography way [15]. The measurements were repeated 3-5 times.

Results and discussion. At usual maintenance on the 50-60Th day of growth the cell line K-27 (10C) on agarosed medium 10C accumulated about 55g of dry biomass out of 1 l of nutrient medium (Table 1; Figure 1, a), while the line K-27 (5C) on agarosed medium 5C about 40g per l (Table 1; Figure 2, a). Further, the output of dry biomass decreased in the course of the passage.

The accumulation curve for indoline alkaloids had a more complicated character. As a rule, in the course of the passage two rises of alkaloids content in dry biomass were found. For example, for line K-27 (10C) one peak of alkaloids content was observed at the time of maximum output of dry biomass (about 1% on the 60th day of the maintenance), and the second one – after 90 days of the maintenance on the background of dry biomass decrease (Figure 1, a). Correspondingly, the curve of alkaloids output was with two peaks. In both peaks (on the 60th and 110th day) the output amounted to 670-700 mg/liter of the medium (Table 1; Figure 1, b). The maximum alkaloids output for K-27 line (5C) was determined on the 60-70th days of growth, when their content in dry biomass amounted to about 1%, and the output – to 380-400 mg/liter of the medium (Figure 2, a, b; Table 1).

Table 1. The dymanics of accumulating biomass and indoline alkaloids in the course of a passage by the cells of K-27 strain of R. serpentina at different maintenance conditions

Cell line	Medium	Time, day	Dry biomass, g/liter	Alkaloids output, mg/liter
K-27 (10C)	10C	20	18.6	62
		30	34.4	205
		40	44.6	243
		50	55.5	558
		60	55.6	676
	10 Cl	20	7.6	42
		50	17.7	8
	5Cl	40	20.4	333
	Rl	30	10.1	194
K-27 (5C)	5C	30	23.7	117
		40	40.2	219
		70	36.3	387
	5Cl	50	20.5	188
	Rl	40	13.9	267

Note: Selected results are presented; complete data are in Figures 1, and 2.

While maintenaning in liquid media the primary material was callus tissue of both cell lines of 40-days growth (scheme 2). In all the variants of liquid media both strain K-27 lines grew in the form of comparatively large globules with the diameter of 0.5-2cm. The submerged culture was characterized by a comparatively short growth period, its growth ended by the 20th day in the Rl medium and by the 40th day – in other variants of liquid media (5Cl and 10Cl) (Table 1; Figures 1, 2).

The productivity of the studied lines submerged culture in nutrient media, different by their content, was different. Thus, K-27 line (10C) in the 10Cl medium accumulated less than 20 g/liter of dry biomass, and practically did not synthesize alkaloids (Table 1, Figure 1, g, h). In the Rl medium the increase of dry biomass was even less – only about 10 g/liter, but the biosynthesis of alkaloids was intensive, their content amounted to 1.3% on the 50th day of the maintenance (Figure 1, c). The 5Cl medium is thought to be the best for K-27 line out of the liquid media, where the dry biomass output amounted to more than 20g/liter, the content of alkaloids in it was more than 1.5% on the 40th day of growth, and their output at this time amounted to more than 330 mg/liter of the medium (Table 1; Figure 1, e, f).

K-27 line (5C) in the liquid media also grew worse than under usual conditions on the agarosed 5C medium (Figure 2), however, in this case alkaloids in Rl medium were accumulated almost two times more than in the control – their content in dry biomass exceeded 2%, and the output – 260 mg/liter on the 40th day of the maintenance (Table 1; Figure 2, c, d).

Depending on the growth conditions the accumulation speed of indoline alkaloids fluctuated for K-27 line (10C) from 11 mg/liter per 24 hours at standard conditions in agarosed medium 10C (control) to less than 2 mg/liter per 24 hours in liquid medium 10Cl (Figure 3, a). In the cell line K-27 (5C) alkaloids accumulated two times slower both in the control (agarosed medium 5C) and in the liquid medium 5Cl. In the liquid medium Rl the speed of alkaloid accumulation was practically equal for both lines – about 6 mg/liter per 24 hours (Figure 3). The mathematization of the obtained data, including the ones, partially presented at Figures 1-3 and in Table 1, showed that Rl medium is optimum for the maintenance of K-27 strain in the submerged culture, as productivity parameters in it were more stable

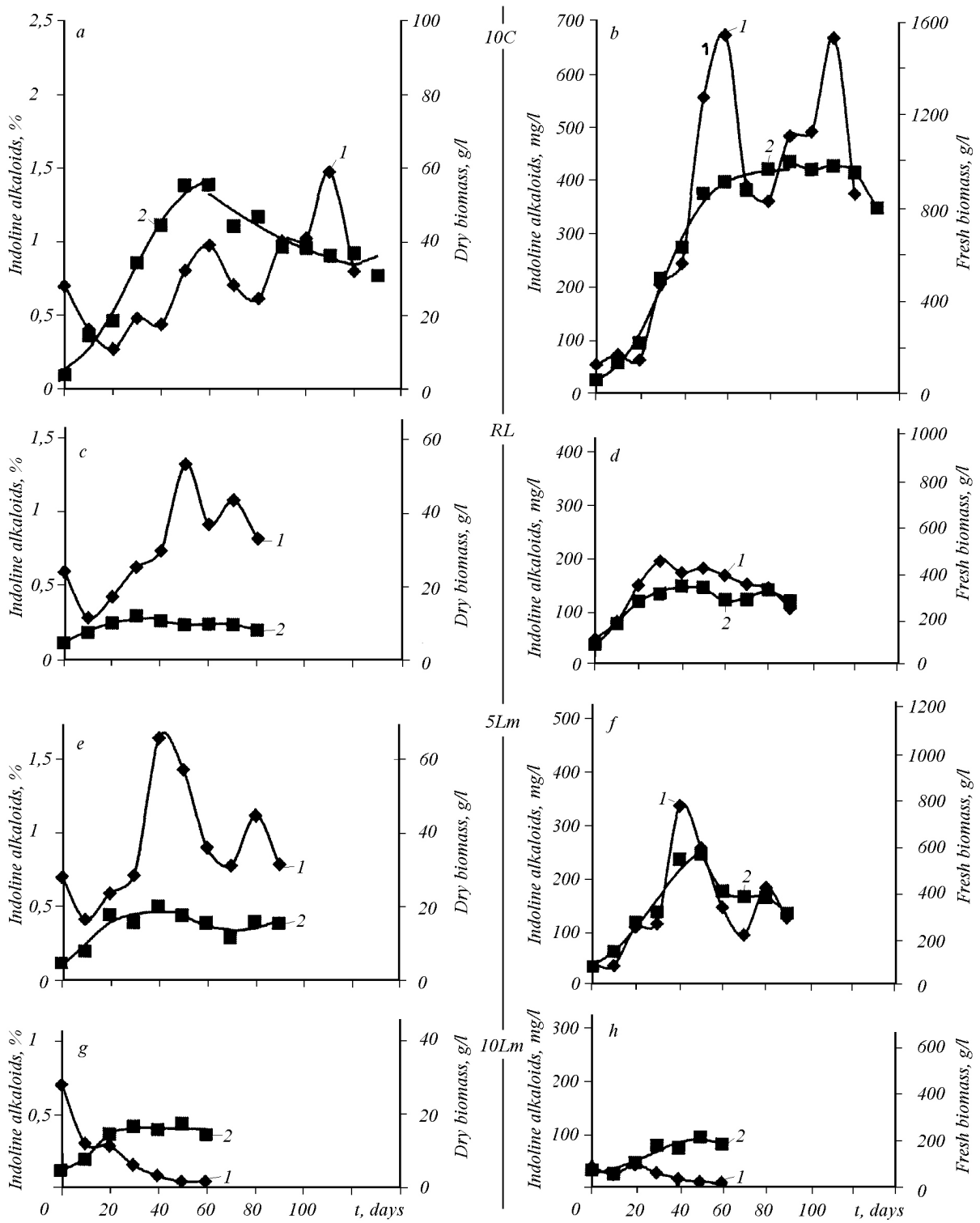


Fig. 1. The dynamics of indoline alkaloids and dry biomass accumulation in the course of the passage by the callus tissue of K-27 line (10C) of *R. serpentina* at the maintenance in different media (a, c, e, f: 1 – the content of alkaloids in dry biomass, %; 2 – the output of dry biomass, g/liter; b, d, f, h: 1 – the output of alkaloids, mg/liter; 2 – the accumulation of dry biomass, g/liter). 10C – control, agarosed medium according to [8]; Rl – liquid medium according to [13]; 5Cl – liquid medium 5C; 10Cl – liquid medium 10C.

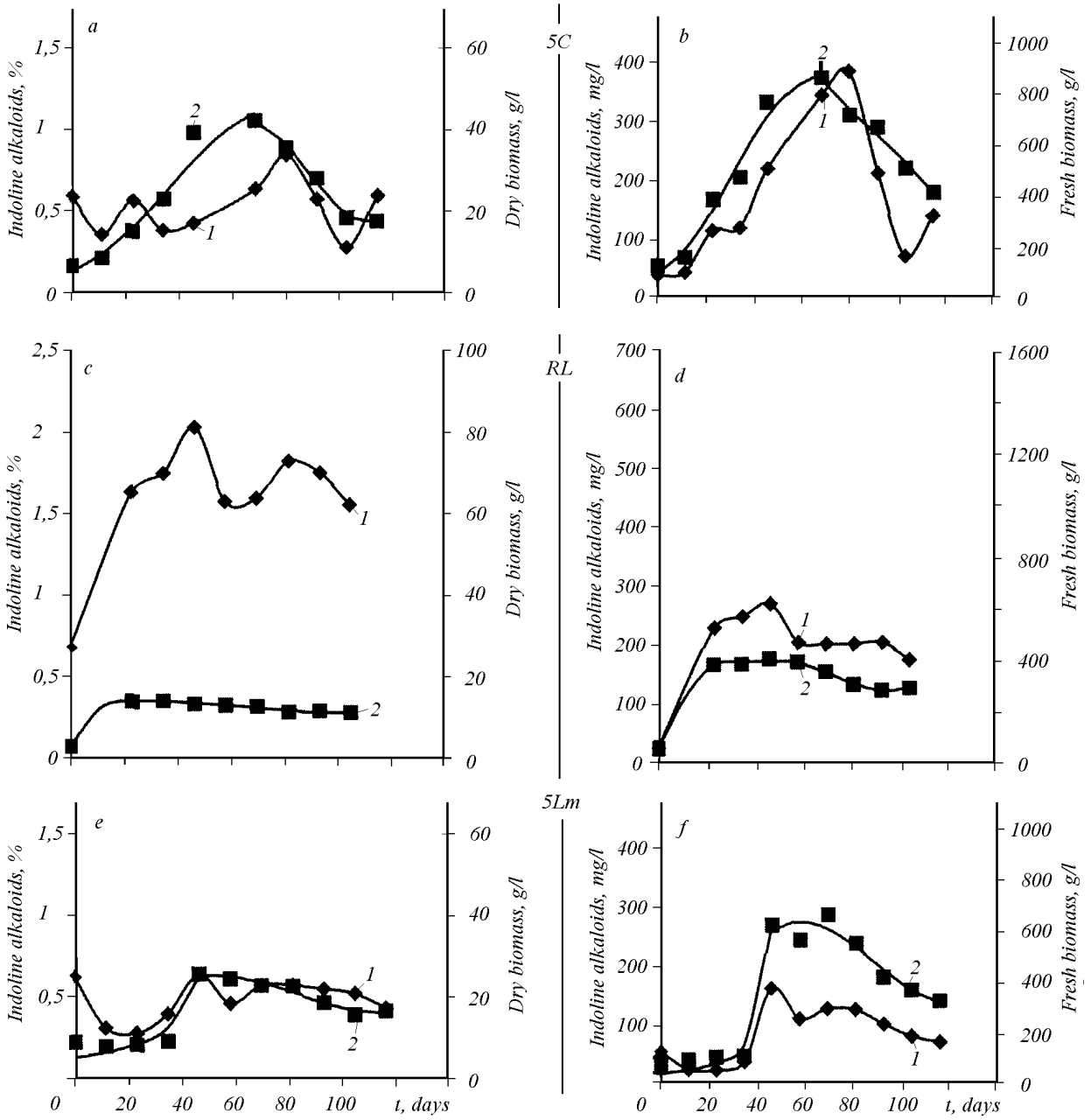


Fig. 2. The dynamics of indoline alkaloids and dry biomass accumulation in the course of the passage by the callus tissue of K-27 line (5C) of R. serpentina at the maintenance in different media (a, c, e: 1 – the content of alkaloids in dry biomass, %; 2 – the output of dry biomass, g/liter; b, d, f: 1 – the output of alkaloids, mg/liter; 2 – the output of dry biomass, g/liter). 5C – control, agarosed medium [5]; Rl – liquid medium according to [13]; 5Cl – liquid medium 5C.

and characterized by less fluctuation degree, than at the maintenance in the liquid media of the same composition [16]. K-27 line (5C) was more productive in the Rl medium, accumulating about 2% of ajmaline (Figure 2, c). However, at long-term maintenance in the 5C medium, which is unusual for it, K-27 strain is characterized by non-stable productivity and lower output of alkaloids than in the 10C medium. Therefore, in the further experiments the productivity was studied in the submerged culture at two-stage maintenance of K-27 strain.

The essence of these experiments is that K-27 strain tissue, which is cultivated in the 10C medium by a surface way since 1982, was grown at 5C medium, which is simpler by its composition (30-45 days), also by a surface way, and

Table 2. The dynamics of ajmaline accumulation in dry biomass of K-27 strain tissues of R. serpentina at its two-stage maintenance in the submerged culture (summary data of three experiments with 5 repeats in each of them)

Growth time, days	Control (agarosed medium 10C accord. to [8])	Maintenance in liquid medium Rl according to [13]			
		After one passage in agarosed medium 5C accord. to [5]		After 6 passages in agarosed medium 5C accord. to [5]	
		Ajmaline content, %	Stimulation effect, %	Ajmaline content, %	Stimulation effect, %
5	0.40	0.40	102.5	0.58	145.0
10	0.39	0.49	125.6	0.62	159.0
15	0.35	0.69	197.1	0.84	240.0
20	0.32	1.00	312.5	1.12	350.0
25	0.40	1.08	270.0	1.59	397.5
30	0.48	1.08	225.0	1.75	364.6
35	0.61	1.12	183.6	1.81	296.7
40	0.73	1.01	138.4	1.83	250.7
45	0.85	1.07	125.9	1.78	209.4
50	0.92	1.12	121.7	1.75	190.2
60	0.98	1.15	117.4	1.79	182.7

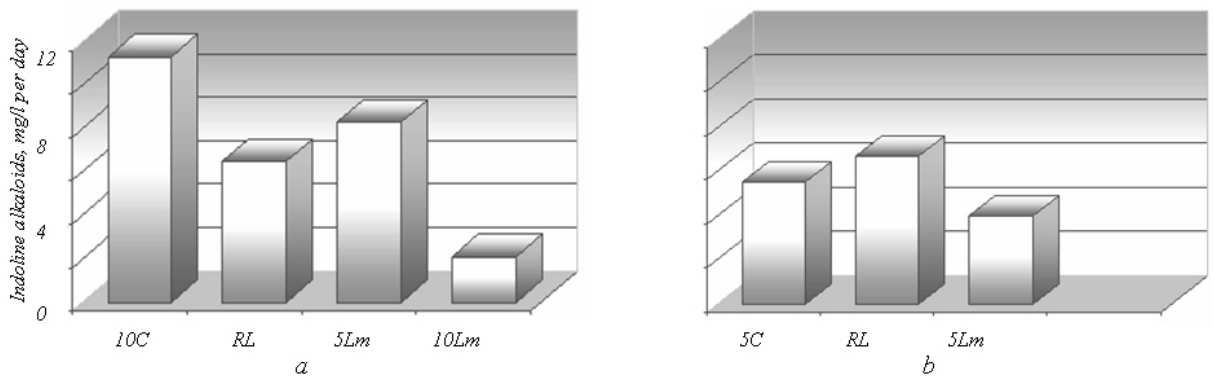


Fig. 3. The speed of indoline alkaloids accumulation (mg/liter per 24 hours) by cell line K-27 (10C) (a) and cell line K-27 (5C) (b) of *R. serpentina* in different maintenance conditions (the speed of alkaloids accumulation is shown for growth points, in which the productivity was maximum): 10C – control, agarosed medium [8], passage duration – 60 days; 5C – control, agarosed medium according to [5], passage duration – 70 days; Rl – liquid medium [13], passage duration – 30 (a) and 40 days (b); 5Cl – liquid medium 5C, passage duration – 40 (a) and 50 days (b); 10Cl – liquid medium 10C, passage duration – 20 days.

then the obtained tissue was transferred to Rl medium continuing the maintenance at constant stirring. The investigation results of the productivity at such a maintenance method showed that ajmaline content in the dry biomass on the second stage (in liquid medium with a special composition) increases 2-3 times in the comparison with the control (K-27 strain in 10C medium). Due to this the term of the callus tissue maintenance, accumulating 1% and more of ajmaline, decreases considerably – to 20-30 days, i.e. practically 3-fold (Table 3).

Still more interesting data were obtained at prolonging the first stage of two-stage maintenance (cultivation of sowing tissue of K-27 strain at 5C medium) to 5-10 passages (the time of one passage is 35-45 days). And only after this, the tissue was transferred to Rl medium. In this case the speed of ajmaline accumulation increased even more

considerably and in comparison with 10C medium (control) the content of this alkaloid increased 3-4-fold on the 20-35th days of the maintenance, amounting to 1.8% (Table 2).

Thus, the obtained data proved that agarosed medium 10C according to [8] is the optimum cultural medium for the maintenance and keeping of highly-productive K-27 strain of *R. serpentina* in long-term collection. To accelerate and make the obtaining of large volumes of cell biomass, containing 1-1.8% of ajmaline on the 20-35th day of the maintenance, more technological, two-stage maintenance of callus tissues should be used. The first stage is the maintenance of collection material of K-27 strain (K-27 line (10C)) at the agarosed 5C medium according to [5], and the second one is the maintenance of callus tissue in liquid cultural medium Rl according to [13] with some modifications on the shakers (shake-flask propagators) or in bioreactors (fermenters). Preliminary maintenance in 5C medium may last for one passage (30-45 days), but the increase of the passages number to 5-10 increases the alkaloids accumulation 3-4-fold (Table 2).

Conclusions. 1. 10C medium [8] is the optimum cultural medium for the maintenance in the collection of highly productive hormone-independent strain K-27 of *R. serpentina* in a surface way.

2. Liquid medium Rl [13] was considered the most suitable for the submerged maintenance of K-27 strain.

3. Two-stage maintenance of K-27 strain is an effective way of the maintenance for the accumulation of indoline alkaloids. On the first stage callus tissues are grown at the agarosed medium 5C [5], and on the second one – in the liquid cultural medium Rl with some modifications.

4. The elaborated way of two-stage maintenance of callus tissues of R. serpentina increases the level of ajmaline accumulation in the early terms 3-4 fold and allows decreasing the time from the growth beginning to the harvest output from 60-80 days to 20-40 days when about 1.8% of ajmaline is accumulated in the dry biomass.

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Можилевская Накопление индолиновых алкалоидов клеточными линиями раувольфии змеиной при поверхностном и глубинном выращивании

Резюме

Изучено накопления индолиновых алкалоидов, в том числе аймалина, в биомассе культуры тканей раувольфии змеиной на примере гормононезависимого высокопродуктивного штамма К-27 при его поверхностном и глубинном выращивании на разных по содержанию минеральных компонентов и сахарозы питательных средах. Установлены оптимальные составы сред для обоих способов выращивания. Подобраны условия двухэтапного выращивания в поверхностной, а затем в глубинной культуре на простых по составу питательных средах, что повышает уровень накопления алкалоидов в ранние сроки в 3—4 раза и позволяет сократить время от начала роста каллусных тканей до съема урожая с 60–80 до 20–40 сут.

Ключевые слова: аймалин, индолиновые алкалоиды, культура тканей растений, Rauwolfia serpentina, клеточные линии – продуценты алкалоидов.