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GROWTH CHARACTERISTICS AND INDOLE ALKALOID ACCUMULATION IN *Rauvolfia serpentina* TISSUE CULTURE CULTIVATED ON NUTRIENT MEDIA OF DIFFERENT COMPOSITIONS

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Aim. To evaluate the productivity of the K-27M strain of *R. serpentina* and the content of indole alkaloids in biomass cultured on two nutrient media differing in mineral composition, sugar content, and growth regulator supplementation.

Methods. *In vitro* culture, high-performance liquid chromatography, and statistical methods.

Results. The strain was maintained on two media: the specially developed synthetic growth regulator-free 10C medium and the classical Murashige-Skoog (MS) medium, which contained vitamins and other organic compounds and was supplemented with 2 mg/L indole-3-acetic acid and 0.2 mg/L kinetin. Cultivation of the callus on 10C medium resulted in a fresh biomass yield of 596.2-677.4 g/L of medium per passage on day 60 of subculture. The corresponding dry biomass yield ranged from 43.4 to 53.5 g/L, with a dry matter content of 7.3–8.5%. In contrast, callus grown on the MS medium showed significantly lower values: fresh biomass yield decreased 1.8-fold, dry biomass yield 3.8-fold, and dry matter content 2.4-fold. The biomass of callus cultured on 10C medium contained 3.5% indole alkaloids, approximately twice the content observed in the MS-cultured strain. The 10C medium supported a higher accumulation of ajmaline (0.815–1.260%) and ajmalicine (0.017–0.025%) in the dry biomass. In the MS-cultured strain, the ajmaline content did not exceed 0.362%, while vomilenine accumulation was 1.9–2.7 times higher (0.728–0.840%) compared to the 10C medium. Reserpine content ranged from 0.006 to 0.019% on MS medium and from 0.007 to 0.011% on 10C, whereas yohimbine was detected at low levels (0.002–0.005%) on both media. Ajmaline productivity was substantially higher in cultures grown on the 10C medium (8.29.5 mg L^{-1} day^{-1}) than in those grown on the MS medium (0.70.9 mg L^{-1} day^{-1}), representing an approximately tenfold difference.

Conclusions. The specially developed synthetic growth-regulator-free nutrient medium 10C ensures high productivity in the K-27M strain of *R. serpentina* tissue culture and promotes greater alkaloid accumulation. Under these conditions, the dry biomass contains up to 3.5% indole alkaloids, including ajmaline (0.815–1.260%), ajmalicine (0.017–0.025%), vomilenine (0.273-0.434%), reserpine (0.007–0.011%), and yohimbine (0.002–0.005%). In contrast, cultivation on the classical MS medium supplemented with growth regulators results in 2–3-fold lower productivity and overall alkaloid accumulation, except for vomilenine, which accumulates at higher levels.

Keywords: plant tissue culture, *Rauvolfia serpentina* Benth. Ex Kurz, indole alkaloids, cellular biomass.

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Cardiovascular diseases are the leading cause of mortality in Ukraine. According to the WHO Regional Office for Europe, nearly 390,000 deaths in Ukraine were attributed to cardiovascular diseases in 2019. The 2019 WHO STEPS survey also documented a high burden of noncommunicable diseases and their associated risk factors in the Ukrainian population [1]. Despite the wide range of drugs currently available for treating cardiovascular diseases, the search for promising plant-derived biologically active compounds (BACs) remains highly relevant. Such compounds may serve as therapeutic and preventive agents with mild pharmacological activity.

One of the most important medicinal plants exhibiting antiarrhythmic and cardiotonic properties is *Rauvolfia serpentina* Benth. ex Kurz, a tropical shrub widely used in traditional Chinese medicine and long recognized in Ayurvedic practice. Its roots synthesize over 50 indole alkaloids, including primarily ajmaline, reserpine, ajmalicine, serpentine, vomilenine, raukaffricine, yohimbine, and others, which exhibit antiarrhythmic, antihypertensive, psychotropic, and sedative activities [2, 3]. In traditional medicine, herbal preparations based on *R. serpentina* extracts are primarily used to treat arterial hypertension, bradycardia, and other cardiovascular disorders, as well as insomnia, depression, anxiety, nausea, bronchospasm, and mental health conditions [3].

The growing demand for herbal medicines has increased the need for large quantities of plant raw materials containing target BACs. One of the most promising alternative approaches for obtaining high-quality, standardized biomass of medicinal plants is *in vitro* culture.

The Department of Genetics of Cell Populations at the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine has developed and characterized *R. serpentina* cell lines and strains derived from tissue cultures that produce indole alkaloids with antiarrhythmic, antihypertensive, α-adrenolytic, sedative, and vasodilatory activities. These cell lines and strains were obtained using cell selection and selection for maintenance of the desired characteristics [4]. Preclinical studies conducted in collaboration with researchers from the Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine have demonstrated that alkaloid extracts isolated from the cellular biomass of the K-27M strain possess both antihypertensive [5] and antiarrhythmic activities [6].

One approach to enhancing the productivity of plant tissue cultures further is to develop an optimal nutrient medium that is both simple in composition and suitable for the large-scale production of BACs.

The study was purposed to evaluate and compare the productivity of the K-27M strain of *R. serpentina* tissue culture, a producer of indole alkaloids, when cultivated on the classical, widely used Murashige–Skoog (MS) medium and on the specially developed synthetic growth regulator-free 10C medium containing 10% beet sugar.

Materials and Methods

The highly productive K-27M strain of *R. serpentina* was used in this study. The culture was maintained under thermostatically controlled conditions at 27–28 °C in the dark in 250-mL glass jars containing 50 mL of agar-solidified nutrient medium. The subculture interval was 50–60 days, after which the tissue culture was transferred to fresh nutrient medium. The initial explant weight was 3.0–3.5 g per jar (equivalent to 60–70 g/L of medium). The experiment comprised six consecutive passages. At each sampling point, 10–12 culture samples (jars) were collected for analysis.

Two nutrient media were used for cultivation. The first was the classical and widely used Murashige–Skoog (MS) medium [7], supplemented with 3% sugar and the growth regulators 2 mg/L indole-3-acetic acid (IAA) and 0.2 mg/L kinetin (6-furfurylaminopurine) (hereinafter referred to as MS medium). The second was the growth regulator-free 10C medium, specially developed for *R. serpentina* tissue culture, containing 10% beet sugar [8] (Table 1).

Fresh and dry biomass yields were determined by gravimetric analysis on the 60th day of cultivation. At each sampling point, 8–10 culture jars were analyzed. The value was calculated according to the following equation:

$$DMC = m/M \times 100\%$$

where DMC is the dry matter content, m is the dry biomass, and M is the fresh biomass of the tissue culture. The data are presented as means ± standard error (SE) calculated from three independent experiments. Error bars represent the standard error of the mean.

The cellular biomass was dried in an infrared drying oven (Avangard SVM Service LLC, Ukraine) for 24 h at 55 °C and 56%

Table

Composition of the nutrient media

Component	MS medium	10C medium
Macronutrients, mg/L		
NH_4NO_3	1650	2500
KNO_3	1900	300
$(NH_4)_2SO_4$	–	300
$MgSO_4 \cdot 7H_2O$	370	1300
$Ca(NO_3)_2 \cdot 4H_2O$	–	900
Anhydrous $CaCl_2$	300	–
$NH_4H_2PO_4$	–	200
$(NH_4)_2HPO_4$	–	300
KCl	–	70
KH_2PO_4	170	–
Micronutrients, mg/L		
KI	0.9	0.9
$Na_2MoO_4 \cdot 2H_2O$	0.3	0.3
$CuSO_4 \cdot 5H_2O$	0.03	0.03
$CoCl_2 \cdot 6H_2O$	0.10	0.10
$MnSO_4 \cdot 5H_2O$	22	22
H_3BO_3	11	11
$ZnSO_4 \cdot 7H_2O$	10	10
Other components, mg/L		
$FeSO_4 \cdot 7H_2O$	27.8	28
$Na_2EDTA\ 2H_2O$	37.3	38
Thiamine hydrochloride	0.1	5
Pyridoxine	0.5	–
Nicotinic acid	0.5	–
Glycine	2.0	–
myo-Inositol	100	–
Indole-3-acetic acid (IAA)	2.0	–
Kinetin	0.2	–
Sugar	30000	100000
Agar	9000	9000
Distilled water	Make up to 1 L	

Note. The pH values of the media prior to autoclaving were 5.7 for the MS medium and 6.0 for the 10C medium.

relative humidity, with forced air ventilation applied every 15 min for 4 min.

The qualitative and quantitative alkaloid profiles were determined using an Agilent 1260 Infinity II high-performance liquid chromatograph coupled with a mass spectrometric detector (HPLC–MS). Chromatographic separation was performed on a C18 reversed-phase column using gradient elution with water and acetonitrile containing 0.1% formic acid as the mobile phase. Standard calibration solutions were prepared from ajmaline, ajmalicine, vomilenine, reserpine (free base), and yohimbine hydrochloride by dissolving accurately weighed amounts of each compound in methanol to obtain concentrations of 0.5–1.0 mg/mL. Chromatograms were reconstructed as extracted ion chromatograms (EICs) using the protonated molecular ions ([M+H]+) for each alkaloid. To quantify the target compounds, the chromatographic peak areas were compared to those of the corresponding reference standards. For alkaloids other than ajmaline, ajmalicine, vomilenine, reserpine, and yohimbine, the estimated content was calculated from the ratio of their chromatographic peak areas to those of the reference standards, assuming similar ionization efficiencies for structurally related alkaloids. The data are presented as means ± SE from three independent experiments.

Results and Discussion

The study was conducted using the highly productive K-27M strain of *R. serpentina* tissue culture. The general appearance of the tissue culture grown on MS medium and on 10C medium on the 60th day of subculture is shown in Fig. 1.

The fresh and dry biomass yields, as well as the dry matter content of the K-27M strain, varied depending on the composition of the nutrient medium (Fig. 2). During cultivation



A *B*

Fig. 1. ***Rauvolfia serpentina*** **tissue culture (K-27M strain) grown on MS medium supplemented with 2 mg/L IAA and 0.2 mg/L kinetin (*A*) and on 10C medium (*B*) on the 60th day of subculture**

on MS medium over five consecutive passages, the fresh biomass yield gradually increased and reached a maximum of 514 g/L of medium in the final passage. The average dry biomass yield was 15.0 g/L of medium, while the dry matter content ranged from 3.5 to 4.2% (Fig. 2, *A*).

Cultivation of the callus on the 10C medium resulted in substantially higher productivity, with fresh biomass yields of 596.2–677.4 g/L of medium, dry biomass yields of 43.4–53.5 g/L, and a dry matter content of 7.3–8.5% (Fig. 2, *B*).

Overall, after five passages, the mean values exceeded those obtained on MS medium by 1.8-fold for fresh biomass yield, 3.8-fold for dry biomass yield, and 2.4-fold for dry matter content.

The total indole alkaloid content in the biomass cultivated on a 10C medium reached 3.5% of dry weight, which was approximately twofold higher than that of the biomass grown on MS medium (1.7%). In addition, tissue

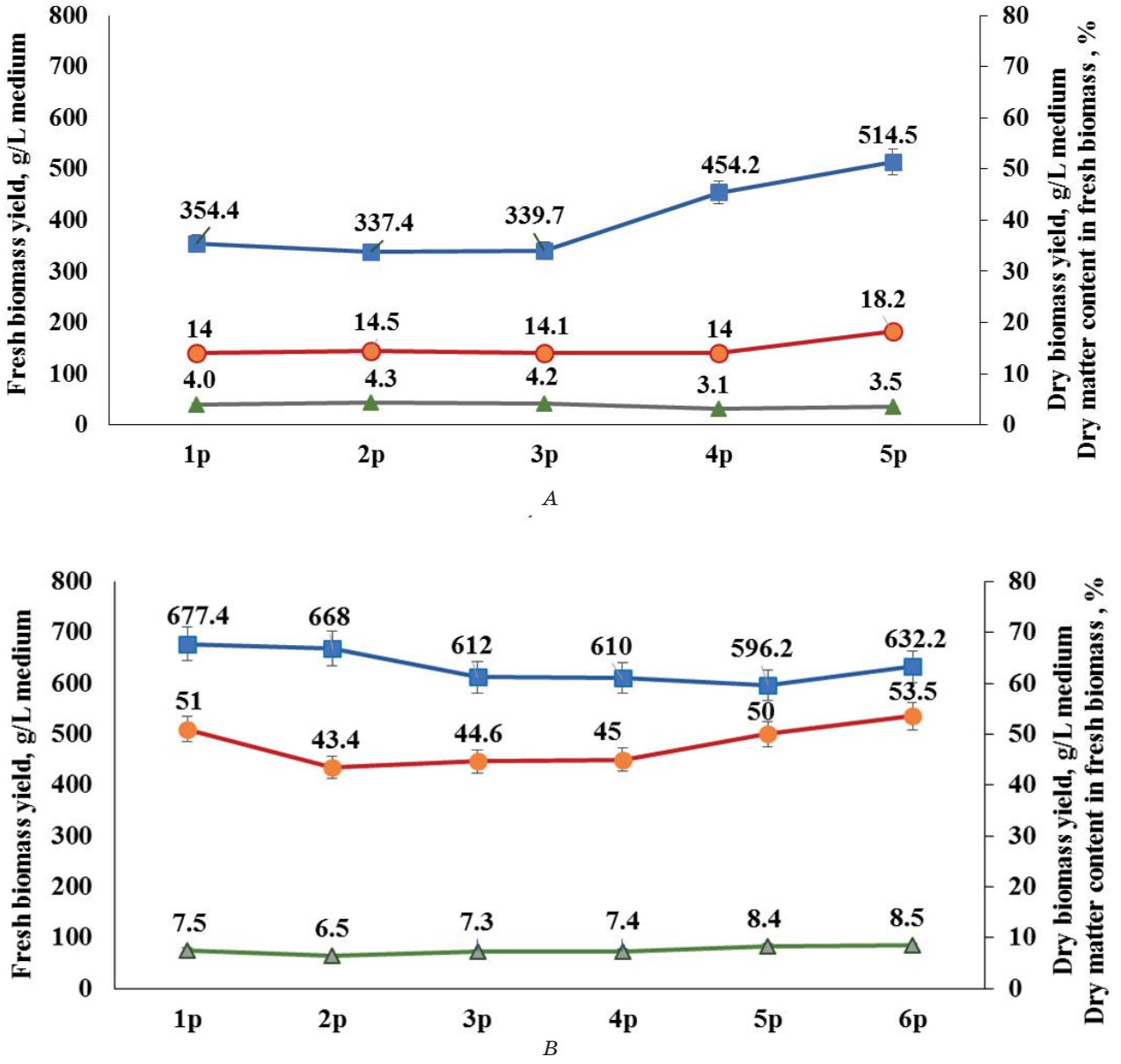


Fig. 2. **Biomass production of K-27M strain cultivated on MS medium supplemented with 2 mg/L IAA and 0.2 mg/L kinetin (*A*) and on 10C medium (*B*) during six consecutive subcultures**

The *X*-axis shows the number of subcultures; the yield of fresh biomass is indicated by a blue line, the yield of dry biomass by a red line, and the dry biomass content in the fresh biomass by a green line. Data are presented as means ± SE from three independent experiments. Error bars represent SE.

grown on 10C medium accumulated higher amounts of ajmaline (0.815–1.260% of dry weight) and ajmalicine (0.017–0.025% of dry weight) (Fig. 3, *A*, *B*).

In contrast, biomass cultivated on MS medium accumulated higher amounts of vomilenine, with concentrations ranging from 0.728 to 0.840% of dry weight, representing a 1.9–2.7-fold increase compared with tissue grown on 10C medium (Fig. 3, *A*).

Reserpine and yohimbine were present at considerably lower levels than the other alkaloids. The highest reserpine content (0.018–0.019% of dry weight) was detected in biomass cultivated on MS medium, whereas slightly lower levels (0.007–0.011%) were observed in tissue grown on 10C medium. Yohimbine content remained within the range of 0.002–0.005% of dry weight in all samples (Fig. 3, *B*). Ajmaline productivity was substantially higher in cultures grown on the 10C medium (8.29.5 mg L^{-1} day^{-1}) than in those grown on the MS medium (0.70.9 mg $L^{-1}day^{-1}$), representing an approximately tenfold difference.

Various nutrient media differing in mineral composition have been reported for the cultivation of *R. serpentina* callus cultures, including agar-solidified and liquid Murashige–Skoog (MS) medium [7], WPM [9], Gamborg's B5 medium [10], WRC medium [11], LS medium [12], and several other formulations [13–15], supplemented with different nutrients and plant growth regulators [14, 17]. Among these, MS-based media containing full- or half-strength macro- and micronutrients, supplemented with 3% sucrose and various concentrations of plant growth regulators, have been used most frequently [16, 17]. These media have been successfully employed for callus induction and the production of indirect regenerants [15], including leaf-derived callus cultured on MS medium supplemented with 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 mg/L 6-benzylaminopurine (BAP) [16, 17], as well as with 2.5 mg/L 2,4-D [13] and cytokinins [19, 20].

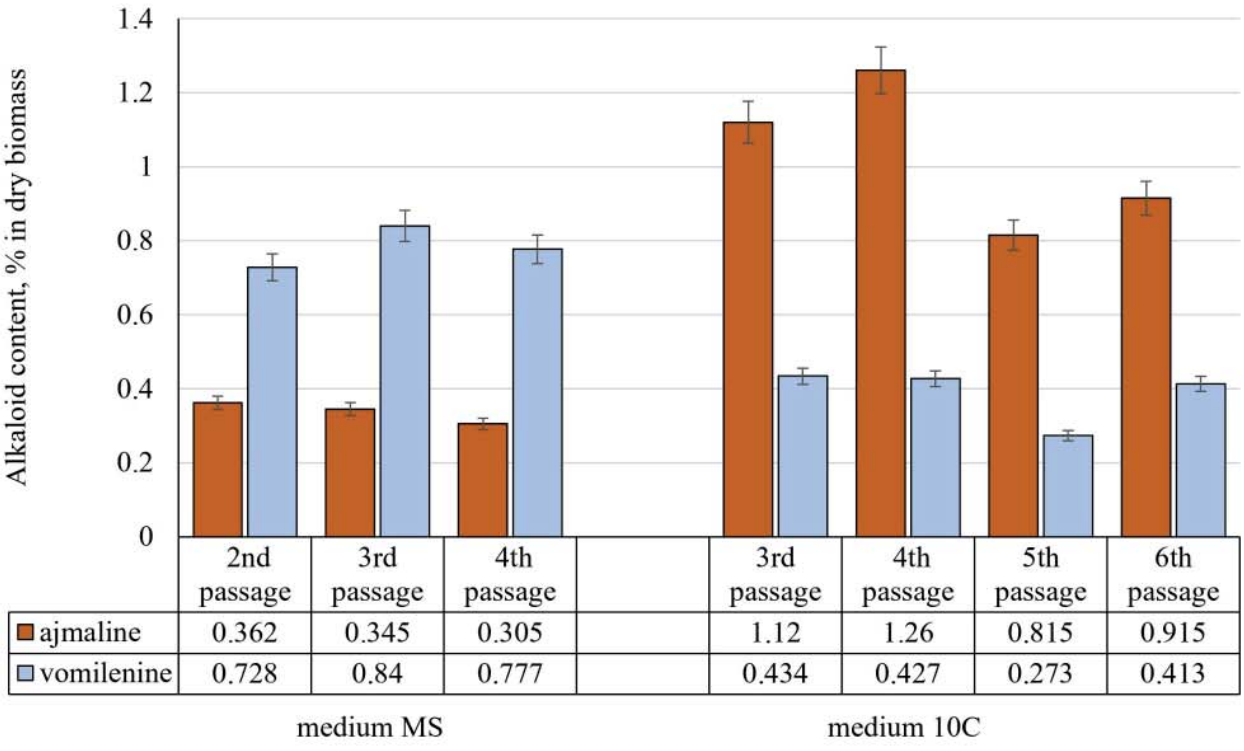
Overall, the available literature indicates that most studies on *R. serpentina* tissue culture have focused on optimizing the conditions for callus induction and quantifying indole alkaloid production [13–15]. For example, Panwar and Guru (2015) demonstrated that tissue cultures accumulated considerably less reserpine than indirect regenerants [18]. Similarly, Zafar et al. (2017) reported that cultivation of leaf-derived *R. serpentine* callus on MS medium supplemented with 1.0 mg/L 2,4-D and 0.25 mg/L BAP in the presence of $AlCl_3$ as an elicitor resulted in maximum fresh and dry biomass yields of 1.32 g and 0.16 g, respectively, while the reserpine content reached 0.129 mg/g dry weight [16]. Comparable results were obtained using $CdCl_2$ as an elicitor: fresh and dry callus biomass reached approximately 1.29 g and 0.16 g, respectively, accompanied by increased accumulation of reserpine and ajmalicine [17].

Significantly higher productivity has been reported for *R. serpentina* hairy root cultures. According to Pandey et al. (2014) [20], dry biomass production reached 24.4 ± 2.11 g/L after 40 days of cultivation. After 60 days, the contents of indole alkaloids were 0.094 ± 0.008% reserpine, 0.507 ± 0.048% ajmaline, and 0.628 ± 0.062% yohimbine on a dry weight basis. The authors also demonstrated that supplementing a growth regulator-free half-strength MS medium containing 3% beet sugar increased the dry biomass yield of hairy root cultures by 17.3% compared with the control, while the ajmaline content increased by 61.98% [20].

The first *R. serpentina* tissue culture was established by Raisa Butenko in 1964 on MS medium. It accumulated only small amounts of alkaloids, with a maximum ajmaline content of approximately 0.1%, whereas intact plant roots contained about 0.3% ajmaline [21]. However, a long-term maintenance of this non-morphogenic tissue culture through successive subcultures was unsuccessful.

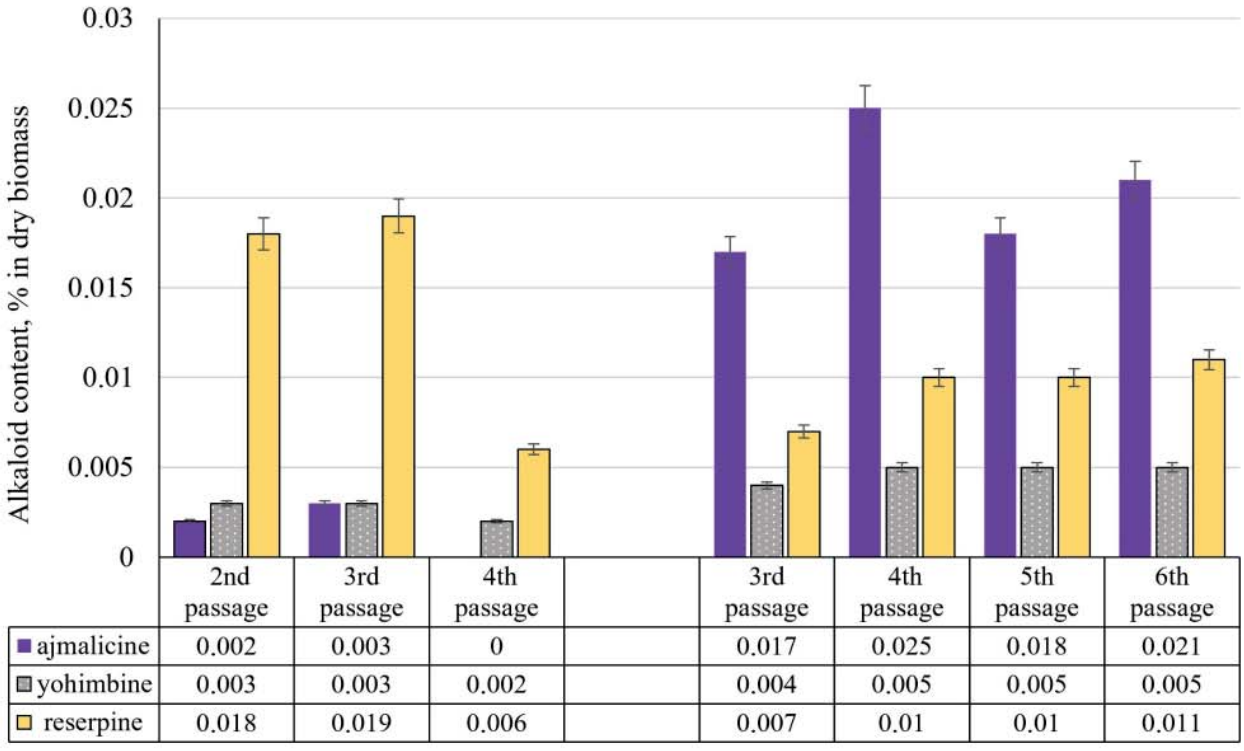
In the present study, cultivation of the K-27M strain on 10C medium resulted in 2–4-fold higher productivity than cultivation on MS medium. The total indole alkaloid content of the biomass increased approximately twofold, primarily due to a 2.7–3.5-fold increase in ajmaline content (from 0.305–0.362% to 0.815–1.260% of dry weight) and a 5.7–8.3-fold increase in ajmalicine content (from 0.003% to 0.017–0.025%).

The higher accumulation of vomilenine in biomass cultivated on MS medium (1.9–2.7-fold higher than on 10C medium) may be consistent with reduced conversion by the biosynthetic pathway of ajmaline. Ajmaline-type alkaloids, which are characteristic of the family Apocynaceae, share a common biosynthetic origin and exhibit close structural similarity [23]. Vomilenine is a biosynthetic precursor of ajmaline. Its conversion to ajmaline involves hydrogenation of two double bonds, deacetylation of the C-17 acetoxy group, and methylation of the indole nitrogen.



	2nd passage	3rd passage	4th passage		3rd passage	4th passage	5th passage	6th passage
ajmaline	0.362	0.345	0.305		1.12	1.26	0.815	0.915
vomilenine	0.728	0.84	0.777		0.434	0.427	0.273	0.413

A



	2nd passage	3rd passage	4th passage		3rd passage	4th passage	5th passage	6th passage
ajmalicine	0.002	0.003	0		0.017	0.025	0.018	0.021
yohimbine	0.003	0.003	0.002		0.004	0.005	0.005	0.005
reserpine	0.018	0.019	0.006		0.007	0.01	0.01	0.011

B

Fig. 3. **Changes in the contents of ajmaline and vomilenine (*A*), and ajmalicine, yohimbine, and reserpine (*B*) in dry biomass of the K-27M strain cultivated on MS medium supplemented with 2 mg/L IAA and 0.2 mg/L kinetin and on 10C medium**

Data are presented as means ± SE from three independent experiments. Error bars represent SE.

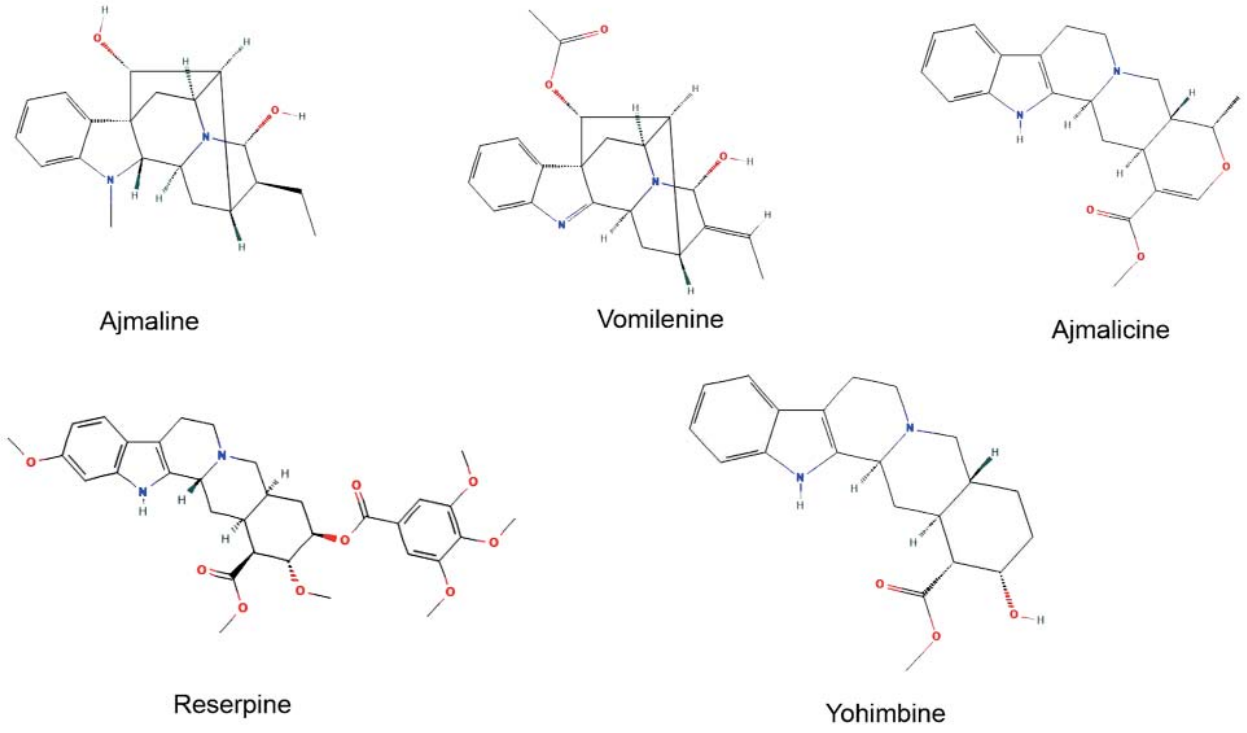


Fig. 4. **Chemical structures of the indole alkaloids**

In this pathway, vomilenine is converted sequentially into 1,2-dihydrovomilenine, acetylnorajmaline, and norajmaline, ultimately yielding ajmaline. Previous studies have demonstrated that ajmaline biosynthesis depends on the availability of vomilenine [25, 26]. Therefore, the elevated vomilenine content observed in biomass cultivated on the MS medium may reflect reduced conversion of this precursor into downstream intermediates and, ultimately, ajmaline [26]. It may be hypothesized that transferring the culture from growth regulator-free 10C medium to MS medium supplemented with 2 mg/L IAA and 0.2 mg/L kinetin alters the culture's physiological state, thereby affecting the ajmaline biosynthetic pathway and resulting in greater accumulation of vomilenine (Fig. 4).

Effects of plant growth regulators on the productivity of *R. serpentina* tissue cultures and the accumulation of BACs have also been reported. In particular, cultivation of tissue culture biomass of line A on 25 variants of the 5C medium containing different combinations of plant growth regulators over five consecutive subcultures demonstrated that kinetin, regardless of concentration, and IAA at concentrations of 1–10 mg/L suppressed alkaloid biosynthesis [21].

Conclusions

A comparative study was conducted to evaluate the productivity of the K-27M strain of *R. serpentina* grown on two types of nutrient media: the synthetic 10C medium and the classical Murashige–Skoog (MS) medium supplemented with organic compounds. The specially developed 10C medium supported higher biomass production and significantly greater accumulation of ajmaline and ajmalicine than the widely used MS medium supplemented with 2 mg/L IAA and 0.2 mg/L kinetin. Ajmaline productivity was substantially higher in cultures grown on the 10C medium (8.2–9.5 mg L^{-1} day^{-1}) than in those grown on the MS medium (0.7–0.9 mg L^{-1} day^{-1}), highlighting the potential of the 10C medium for enhancing ajmaline production in *R. serpentina* K-27M cultures. Conversely, when biomass enriched in vomilenine is desired, the K-27M strain should be cultivated on the MS medium supplemented with 2 mg/L IAA and 0.2 mg/L kinetin.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

I.I. Konvalyuk — Conceptualization, Methodology, Investigation, Writing—Original Draft Preparation, Writing—Review and Editing; O.A. Bieda — Data Curation, Methodology, Investigation, Writing—Original Draft Preparation; L.P. Mozhylevska — Methodology, Investigation, Data Curation; G.Yu. Myryuta — Methodology, Investigation, Data Curation; S.M. Yarmolyuk — Conceptualization, Methodology, Data Curation, Writing—Review and Editing; V.A. Kunakh — Conceptualization, Methodology, Investigation, Data Curation, Writing—Review and Editing, Supervision.

All authors have read and agreed to the published version of the manuscript.

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ОСОБЛИВОСТІ РОСТУ ТА НАКОПИЧЕННЯ ІНДОЛЬНИХ АЛКАЛОЇДІВ У КУЛЬТУРІ ТКАНИН *Rauvolfia serpentina* ЗА ВИРОЩУВАННЯ НА РІЗНИХ ЗА СКЛАДОМ ЖИВИЛЬНИХ СЕРЕДОВИЩАХ

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Мета. Дослідити продуктивність штаму К-27М *R. serpentina* та вміст індольних алкалоїдів в його біомасі за вирощування на двох різних за складом мінеральної основи, цукру та фітогормонів живильних середовищах.

Методи. Культура *in vitro*, високоефективна рідинна хроматографія з мас-спектрометричною детекцією, статистичні методи.

Результати. Культуру тканин вирощували на двох середовищах: на спеціально розробленому синтетичному безгормональному середовищі 10С та класичному середовищі Мурасіге-Скуга (МС), що містить вітаміни та інші органічні добавки, доповненого 2 мг/л ІОК та 0,2 мг/л Кін. За вирощування калюсу на середовищі 10С вихід сирої біомаси становив 596,2–677,4 г/л середовища за пасаж (60-ий день росту), сухої — 43,4–53,5 г/л, вміст сухої біомаси у сирій — 7,3–8,5%. За вирощування на середовищі МС, доповненому фітогормонами, ці показники були нижчими: вихід сирої біомаси у 1,8 раза, сухої — у 3,8 раза, вмісту сухої біомаси у сирій — у 2,4 раза. Сумарний вміст індольних алкалоїдів був вдвічі вищим у біомасі, вирощеній на середовищі 10С порівняно з МС та становив 3,5%. У тканині, отриманій на середовищі 10С, був високий вміст аймаліну (0,815–1,260% від сухої маси) та аймаліцину (0,017–0,025%). У калюсі, вирощеному на середовищі МС, вміст аймаліну не перевищував 0,362%, при цьому накопичення воміленіну зростало у 1,9–2,7 раза (0,728–0,840%) порівняно із отриманою біомасою на середовищі 10С. Вміст резерпіну у тканині коливався в межах 0,006–0,019% за вирощування на середовищі МС та 0,007–0,011% — на 10С, йохимбін присутній у невеликих кількостях (0,002–0,005%) у всіх зразках. Встановлено, що продуктивність за аймаліном була значно вищою у культурі тканин за вирощування на середовищі 10С (8,2–9,5 мг·л$^{-1}$ добу$^{-1}$), порівняно з калюсом на середовищі МС (0,7–0,9 мг·л$^{-1}$·добу$^{-1}$), що відповідає приблизно десятикратній різниці.

Висновки. Встановлено, що спеціально розроблене синтетичне агаризоване живильне середовище 10С без регуляторів росту забезпечує високу продуктивність штаму К-27М культури тканин *R. serpentina*. За таких умов суха біомаса містить 3,5% індольних алкалоїдів, зокрема аймалін (0,815–1,260%), аймаліцин (0,017–0,025%), воміленін (0,273–0,434), резерпін (0,007–0,011%) та йохимбін (0,002–0,005%). Показники продуктивності культури та накопичення індольних алкалоїдів у біомасі за вирощування на класичному середовищі МС, доповненому фітогормонами, були у 2–3 рази нижчими порівняно з 10С за винятком вмісту воміленіну.

Ключові слова: культура тканин рослин, *Rauwolfia serpentina* Benth. Ex Kurz, індольні алкалоїди, клітинна біомаса.

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