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ALKALOID PRODUCTION IN *CATHARANTHUS ROSEUS* CELL CULTURES: INITIAL STUDIES ON CELL LINES AND THEIR ALKALOID CONTENT*

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Key Word Index—*Catharanthus roseus*; Apocynaceae; cell culture; indole alkaloids; variability.

Abstract—Several hundred serially cultured cell suspensions derived from three cultivars of periwinkle (*Catharanthus roseus*) were established in Gamborg's B5 medium and then transferred to Zenk's alkaloid production medium. Total alkaloid concentration ranged from 0.1 to 1.5% of dry weight. Alkaloids present were of the corynanthe, strychnos and aspidosperma types, with the greatest diversity arising during the third to the fifth week of subculturing. The alkaloid content appeared both specific for, and reproducible in, individual cell lines.

INTRODUCTION

Plant cell cultures are a potential source of medicinally important substances [1–4]. Those of the Madagascar periwinkle (*Catharanthus roseus*) have received particular attention because of the physiologically potent indole alkaloids they are expected to produce [5]. Compounds with sedative effects, such as serpentine, have been detected in periwinkle cultures in relatively high concentrations [6]. However, the anti-cancer agents, vinblastine and vincristine, have yet to be produced in periwinkle cell cultures, although precursors including one aspidosperma alkaloid, vindoline, have been reported [5].

The production of alkaloids in cell cultures can be regarded as a differentiation process governed both by environmental conditions as well as by the genotype of the material in question [7]. While the environmental conditions which might induce cultured cells to synthesize and accumulate alkaloids have been fairly well described, the selection of a promising genotype requires continued investigation.

The present study was undertaken to delineate the variability of serially cultured callus and cell suspension cultures of *C. roseus* derived from highly uniform explants, that is anthers of buds identical in the developmental stage. The only variables introduced were the use of three separate cultivars and treatment of the buds with a mutagen. In a supplementary study, the synthesis and accumulation of alkaloids were related to the growth of those periwinkle cultures which were selected for their particular alkaloid content [8].

RESULTS

Callus cultures

Callus grown from anthers on Murashige and Skoog's medium [9] generally originated at the cut of the filament and in the anther walls, and so consists of diploid tissue. Callus grown from microspores and initially consisting of haploid cells will be described in a future communication. When grown to a size of 1–2 g fresh weight, *ca* 2 cm in diameter, the callus was cut into small pieces and serially subcultured on fresh agar medium or transferred to Gamborg's liquid medium [10] giving rise to a cell suspension.

Cell suspension cultures

Cell suspensions generally were greenish, sometimes clumpy cultures. A few serially cultured suspensions (cell lines) were white and grew at a rate which required transfer to fresh medium twice a week in order to prevent ageing [8]. These cultures consisted of small cell aggregates and single cells. Each culture was variable to the extent that the cells varied in diameter, large ones assuming the dimensions of laticifers (> 100 μm in length). The cultures contained 70–80% diploid cells, the rest were polyploid, a few giant cells being highly polyploid. One cell line was exceptionally uniform in showing aggregates of up to 15 cells of small diameter (< 30 μm), rich in starch grains, growing rapidly, being diploid throughout. This cell line did not produce any alkaloids.

Alkaloid production

Alkaloid production varied with the cell line and age of the subculture and ranged from 0.1 to 1.5% of cell dry weight. The relative amounts of alkaloids produced were fairly constant under conditions given and appeared cell-line specific. All subcultures of cell lines grown in 7.5 l. Microferm bioreactors followed essentially the pattern shown in Fig. 1. After incubation with actively growing cell suspension, the mitotic index (MI) dropped to zero within 24 hr and remained there for 2–3 days. Thereafter, the index rose sharply and reached its maximum (MI 1.8–3.0) within 2 days and declined again gradually over the following 10–15 days to zero. The cell dry weight over the culture period increased by a factor of 8–10 while the variation in pH stayed within half a unit.

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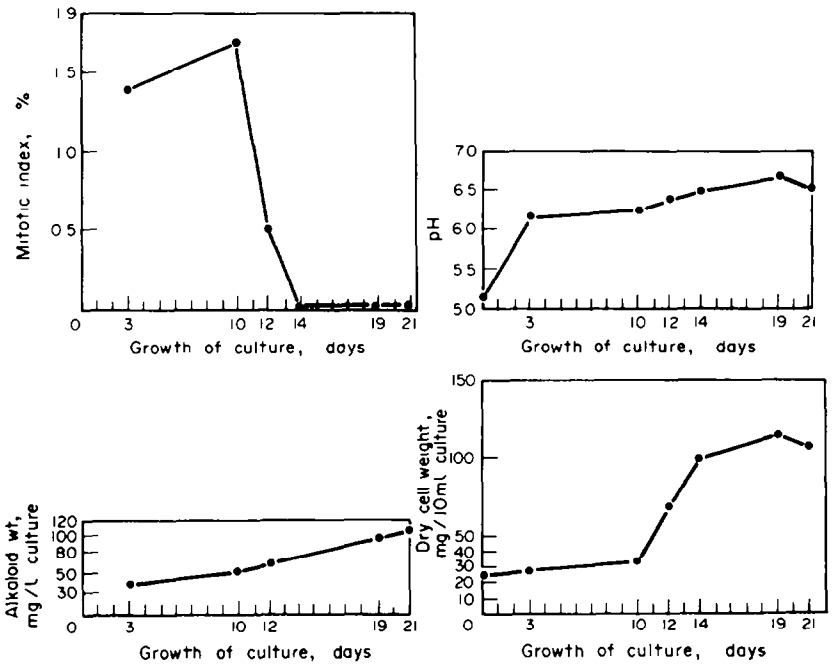


Fig. 1. Growth characteristics of *Catharanthus roseus* cell suspension cultures grown in 7.5 liter Microferm bioreactors.

Alkaloid analysis

Analysis of 458 cell lines revealed 312 lines to accumulate alkaloids detectable by TLC methods. Alkaloids identified were ajmalicine (**1**), vallesiachotamine (**2**), belonging to the corynanthe family of alkaloids; akuammicine (**3**, strychnos type); and vindolinine (**4**), hörhammerinine (**5**), hörhammericine (**6**) and lochnericine (**7**) all of the aspidosperma type. In general, the alkaloids occurred in a variety of combinations. It was of interest that the combinations were not random but certain combinations appeared at higher frequency than others. For example 6.7% had corynanthe- or strychnos-type alkaloids only (i.e. **1**, **2**, **3**); 13.8%, strychnos–aspidosperma type only; 23.1%, corynanthe–aspidosperma alkaloids only; while 13.1% contained all three types of alkaloids. It should be noted that 9.6% had only corynanthe alkaloids; 13.8% only the strychnos type and 10.5% contained only the aspidosperma type. Of the 312 lines producing alkaloids, a total of 76.6% were capable of accumulating aspidosperma alkaloids and 46.2% strychnos while 56.7% yielded corynanthe-type alkaloids.

It is of interest that the corynanthe-type alkaloids (**1**, **2**) formed earlier in the biosynthetic pathway, and indeed acting as precursors for the aspidosperma series, are found in the same cell population with members of the latter group.

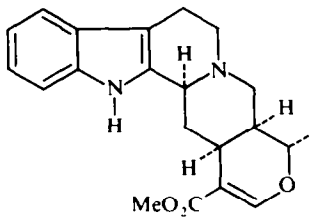
During an 8 week culture period, alkaloids have been found as soon as 2 weeks after inoculation. With some cell lines, alkaloids **1**, **2**, **4**, **5** and **6** were observed to occur from the third week. The alkaloid content, therefore, does not seem to grow in complexity with time; rather, alkaloids may be catabolized, since after 6–8 weeks of culture the occurrence of alkaloids diminished rapidly. Most cell lines showed a maximum accumulation of alkaloids in the third–fifth week of culture (Table 1).

When the transfers of cell lines from agar to liquid and production medium and subsequent TLC analyses were repeated with 120 lines, the alkaloid composition was found to be unchanged. This observation would indicate that the alkaloid composition is a characteristic feature of a particular cell line. It may be noted that cell lines passing their *second year* in culture have not lost their capability to accumulate alkaloids.

Table 1. Alkaloid production in cell lines during an 8 week culture period

% lines exhibiting alkaloids	Time taken (weeks)
51	2
64	3
75	4
83	5
15	6
10	7
3	8

Given cell-line-specific alkaloid composition, an analysis of the relationship between it and experimental variables appeared desirable. For clarification of this approach it may be restated that plants employed for explantation consisted of about equal numbers of pink and white flowering specimens and a lower number of 'Red Dot' plants. Also, equal numbers of cuttings were subjected to mutagen treatment. A third variable, root formation with callus cultures, was incidental. Table 2 shows the number of cell lines featuring specific alkaloids as a function of the three variables described. As a result it appeared that, generally, the source of the cell line, the treatment with mutagen and the capacity for rooting of the callus were not related to the particular alkaloid spectrum produced by respective cell lines. An exception may be found with cell lines which accumulated aspidosperma



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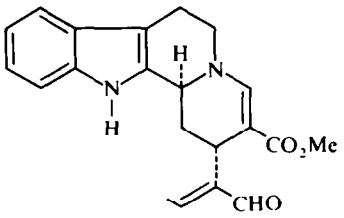

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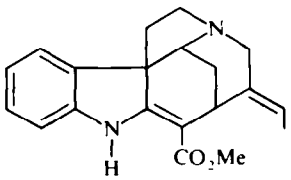

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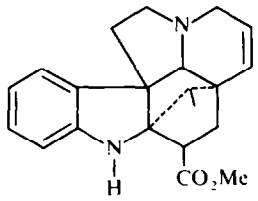

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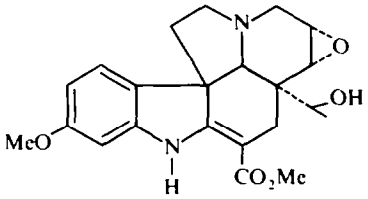

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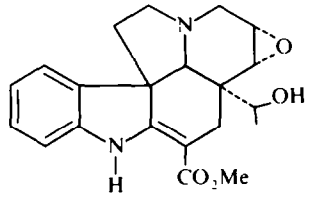

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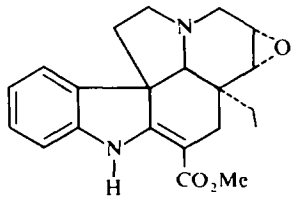

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Table 2. Alkaloid specificity of cell lines as a function of cultivar origin and mutagen treatment

Cultivar*			Alkaloids	Mutagen treatment			Rooting	
pfl	wfl	RD	isolated	None	EMS	EMS + 2,4-D†	−	+
3	3	1	**7**	2	3	2	1	6
0	1	0	**1**	1	0	0	0	1
3	8	3	**4**	3	6	5	6	8
2	0	0	**5,6**	1	0	1	0	2
24	11	11	**1,4**	10	21	15	11	35
0	1	0	**1,3**	0	0	1	0	1
18	0	2	**4,5,6**	2	10	8	0	19

* pfl = cv *roseus*, wfl = cv *alba*, RD = cv Red Dot.
† 5×10^{-6} mol of 2,4-dichlorophenoxyacetic acid.

alkaloids (**4**, **5**, **6**). These appear to have originated from the pink flowering periwinkle cultivar only.

DISCUSSION

More than 400 serially cultured calli and cell suspensions of periwinkle were established and screened for the production of indole alkaloids. Cultures often featured several alkaloids, some of which could be identified. Alkaloid composition appeared to be cell-line specific.

The rationale for an approach of industrial exploitation of cell culture systems by tapping natural variability of the biosynthetic potential in callus and cell suspension culture has been perceived, since a few cell lines of various species were observed to accumulate unusual amounts of secondary metabolites: *Haplopappus gracilis* [11], *Morinda citrifolia* [12], and *Lithospermum erythrorhizon* [7]. Roller described the variability for capability and capacity for alkaloid production in periwinkle cell cultures [13]. Heins investigated the biotransformation capacity of *Digitalis* cell cultures in a similar manner [14]. The experiments described here confirmed the value of screening a great number of serially subcultured calli and cell suspensions for superior cell lines. A few lines showed high concentration of total alkaloids, a few others did not present any traces of alkaloids.

Variability among cell lines generally does not only extend to initial calli but may also occur upon long term subculturing and, thus, may affect the stability of cell lines. Loss of biosynthetic potential is quite often encountered in cell culture systems. As this variability is thought to coincide with the rate of mitoses, periwinkle stock cultures were maintained as slowly growing callus. So far, a loss of capability to accumulate alkaloids has not been observed in lines analysed repeatedly.

Cell suspensions of periwinkle often featured several alkaloids. Individual compounds found in such groups may belong to corynanthe, strychnos, or aspidosperma families of alkaloids. This observation does *not* permit us to conclude that all the alkaloids are found co-occurring in *one* cell. Rather, the spectra may reflect the diversity of cells in one suspension (population). Cells of one suspension, indeed, differ in cell size, differentiation and ploidy. Any cell suspension showing pigmentation by anthocyanin and betalain will illustrate the point [2, 11, 15].

Quantitative analysis of alkaloid spectra found in periwinkle cell lines has not been completed. Medicinally desirable bisindole alkaloids like vinblastine and vincristine have not been detected. The occurrence of lochnericine, vallesiachotamine, hörhammerinine, hörhammericine and vindolinine in cell cultures has not been reported earlier.

EXPERIMENTAL

Cell cultures. Seeds of periwinkle (*Catharanthus roseus* (L.) Don.) were planted in a mixture of Vermiculite, peat and sand, supplemented with Osmocote fertilizer pellets and grown in a greenhouse in photoperiods of 16 hr/day of daylight and, if necessary, white fluorescent and incandescent light of 40 W/m^2. As soon as the plants had developed two pairs of leaves, they were potted and transferred to a growth room at 26° at day and 22° at night with white light, 20 W/m^2, in photoperiods as before. Prior to flowering, cuttings were treated with 0.1% EMS (ethane sulfonic acid methyl ester) in test-tubes over a period of 4 days. The tubes were replenished daily. Thereafter the cuttings were harvested, the inflorescences bagged and stored in a refrigerator at 4–6° for 2 days. These inflorescences were then sterilized by immersion in 70% EtOH for 10 min and washed with sterile H_2O. Buds, approximately 1.5–2.0 mm in length, were selected, and anthers removed under a dissecting microscope. The anthers were incubated, 10 per 2 ml, floating on liquid 0.1 MSC medium, i.e. medium after Murashige and Skoog [9], fortified with 0.1 mg/l NAA (α-naphthalene acetic acid) and 1 g/l. casein hydrolysate (NZ-Amine, Humko-Sheffield Co., Norwich, N.Y.) in 60 × 15 mm petri dishes. The dishes were sealed with Parafilm and stored in plastic boxes in dim light at 27°. Within 3–4 weeks *ca* 30% of the anthers formed greenish callus preferentially at the anther wall. Some of the anthers produced white callus which burst from the mass of microspores. Karyological investigation revealed dividing haploid cells. Derivatives of the daughter cells, however, were found to be diploid and approximately 5% polyploid. Sometimes tracheids formed in abundance. In view of the variability of cell lines, as observed later, no attempt was made to select for haploid callus. After 6–8 weeks individual calli were cloned by transfer to 30 ml of nutrient medium solidified with 0.8% agar in jars. Once the calli had grown to a clump of cells *ca* 2 cm in dia, a few small pieces were subcultured on agar medium, the rest subcultured in 50 ml of liquid Gamborg's B5 medium [10] and continuously shaken. Subcultures on agar medium were kept at 27° in continuous light, 10 W/m^2, and transferred to fresh medium at monthly intervals.

Alkaloid synthesis. To test cell lines for their ability to synthesize indole alkaloids, subcultures were transferred to 500 ml Erlenmeyer flasks containing 250 ml of alkaloid production medium [6], and agitated on gyratory shakers (130 rpm) at 27° in continuous light 10 W/m^2. Samples were withdrawn at weekly intervals and analysed for their alkaloid content. In order to obtain large amounts of cell material from cell lines containing alkaloids of interest, cultures were grown in alkaloid production medium in 7.5 l. Microferm-Bench top bioreactors (New Brunswick Scientific Co. Inc., Edison, N.J.) equipped with sintered disc spargers (5 cm o.d.). To minimize the shearing effect, the bioreactors were stirred at 200 rpm and aerated with 35 ml/min air per l. culture. Samples were taken every second day and analysed for mitotic index (MI), pH and dry wt. The alkaloid content was analysed at irregular intervals, but with increasing frequency after the mitotic index started to decline.

Alkaloid extraction and identification. A detailed account of a standard extraction procedure is given in the following publication [8], which also describes the identification of the individual alkaloids.

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