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PRODUCTION OF INDOLE ALKALOIDS BY *IN VITRO* ROOT CULTURES FROM *CATHARANTHUS TRICHOPHYLLUS*

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Key Word Index—*Catharanthus trichophyllus*, Apocynaceae, *Agrobacterium rhizogenes*, normal root culture, hairy root culture, indole alkaloids

Abstract—Following *in vitro* infection of an aseptic *Catharanthus trichophyllus* plant with *Agrobacterium rhizogenes* (15834), six hairy root cultures were established. These were compared with normal root cultures, derived from the same plant, with respect to production of indole alkaloids. A rapidly growing hairy root line was used for fermenter (20l) cultures. The alkaloid content of the roots obtained was examined Seventeen monomeric indole alkaloids were purified and characterized, including five hitherto undescribed substances Normal root and hairy root line cultures showed similar alkaloid composition. Analyses, performed at five-week intervals on five-week-old cultures showed variable alkaloid yields.

INTRODUCTION

Secondary metabolite production by plant cell cultures has been the subject of many investigations. Indole alkaloids such as ajmalicine, a cardio-vascular drug, or vinblastine and vincristine, two antitumour dimeric compounds, produced by *Catharanthus roseus* are important in the pharmaceutical industry [1]. Cell cultures from the same plant species have been propagated in several laboratories because of the physiologically potent indole alkaloids they are expected to produce [2]. Undifferentiated cell lines producing high yields of ajmalicine, or serpentine, which can be easily converted into ajmalicine by reduction, have been obtained by selection [3] However, a substantial problem for the industrial exploitation of such cultures has been the variability of the productivity, inherent to undifferentiated cultures [4] This variability is attributed to somaclonal variation, described as the result of genetic or epigenetic instability of such cultures [5, 6]. A straightforward solution to this problem would be to establish *in vitro* cultures of differentiated tissues or organs. Root and/or shoot cultures of *Scopolia parviflora* [7], *Duboisia* species [8], *Catharanthus roseus* [9–11], *Papaver somniferum* [12] and *Digitalis* species [13–16] were reported to have secondary metabolite compositions similar to those found in parent plant organs. In contrast, undifferentiated cell cultures of these same species had low amounts of secondary metabolites or did not produce them. Because of the ease with which fast growing hairy root cultures can be obtained, several groups have investigated their potential for *in vitro* production of secondary metabolites. Hairy roots were found to produce the same secondary metabolites as those usually synthetized in intact parent plant roots, with similar or higher yields [17–28]. Hairy roots are induced upon inoculation of the phytopathogen *Agrobacterium rhizogenes* to many dicotyledons [29]. These roots can easily be cultured under aseptic conditions. The molecular basis for hairy root is the transfer to the plant genome of specific DNA segment(s), called T-DNA for transferred DNA, originally present on a plasmid in *A. rhizogenes* [30].

In order to study indole alkaloid production, *Catharanthus roseus* G. Don [31, 32] and *C. trichophyllus* (Bak) Pich. hairy root cultures were isolated from plants inoculated with *A. rhizogenes*. In the present paper, we compare the alkaloid content of *in vitro* cultured normal roots and hairy roots, and of roots from greenhouse-grown *C. trichophyllus* plants.

RESULTS

One axenic normal root culture and six hairy root lines (lines 2, 4, 5, 9, 10 and 11), derived from independent root tips, were established from one inoculated *C. trichophyllus* plant. The transformed nature of each hairy root line was checked by Southern hybridization and dot blot hybridization with T-DNA probes (Shen *et al*, in preparation) The growth rate of normal root line and hairy root lines was compared. Growth was measured as relative weight increase over a 40-day period. Lines 2, 4, 5 and normal roots exhibited similar growth rates, lines 10 and 11 grew twice as fast, whereas line 9 had a very reduced growth rate (Shen *et al*, in preparation).

Indole alkaloid production by hairy root fermenter cultures

A rapidly growing hairy root line (line 10) was selected for culture in fermenters to examine its growth rate and alkaloid content. The results of two bioreactor runs (R_1 and R_2) are presented. The fresh weight of hairy root line 10 increased from an original inoculum (10 g each) by 200 (R_1) and 250 (R_2, Fig 1) times over 6.5-week and 9 5-week

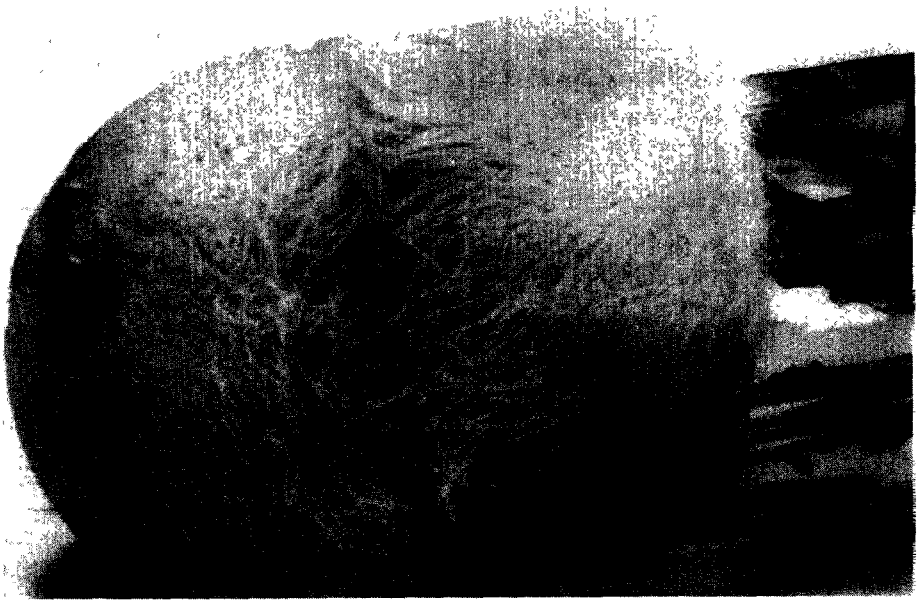

Fig 1 Photograph showing the densely packed biomass (diameter 23 cm, height 33 cm, 2 5 kg fr wt) of *C trichophyllus* hairy root line 10 obtained in a 20 l fermenter (R_2) (See Experimental for culture conditions)

Table 1 Alkaloid content in hairy root line 10 fermenter cultures*

Alkaloid†	mg	Yield % total crude alkaloid content‡
Horhammericine (**1**)	88	2 73
Vindolinine (**2**)	10	0 31
Lochnericine (**3**)	163	5 06
Echitovenine (**4**)	44	1 37
Ajmalicine (**5**)	20	0 62
Tabersonine (**6**)	95	2 95
Epivindolinine (**7**)	10	0 31
Pericalline (**8**)	8	0 25
Akuammicine (**9**)	76	2 36
Akuammigine (**10**)	13	0 40
Minovincinine (**11**)	16	0 50
Anthraserpine (**13**)	2	0 06
Dimethoxyanthraserpine (**14**)	13	0 40
Pseudoanthraserpine (**15**)	1	0 03
Desanthraserpidine (**16**)	1	0 03
Dimethoxydesanthraserpidine (**17**)	1	0 03

* Pooled biomass from R_1 and R_2 fermenters

† Purified compounds

‡ The crude extract contained unidentified components and large part of alkaloids was lost during successive purifications

culture periods, respectively Harvested hairy roots from the two fermenters were freeze-dried, pooled and the alkaloids extracted in the classical manner The crude alkaloid yield relative to dry matter was 0 92% The mixture was then fractionated through a Sephadex LH20 column. Final purification by CC on alumina and by prep. TLC on silica gel allowed the isolation of 17 alkaloids **1** to **17** (Table 1) These were characterized by their physical and spectral data and by comparison with authentic samples. Serpentine (**12**) was also extracted but was not isolated, it was converted to ajmalicine **5** by borohydride reduction In addition to the known compounds **1** to **12,** five novel alkaloids were isolated. Two of them, anthraserpine (**13**) and dimethoxyanthraserpine (**14**) were found to contain the 11-methoxy *epiallo*-yohimbine skeleton, esterified at C-18 with 2-acetamidobenzoic acid and 2-acetamido-4,5-dimethoxybenzoic acid, respectively, on the basis of spectral data Isolated in trace

amounts, the gross structures of three other congeners of the same series, pseudoanthraserpine (**15**), desanthraserpidine (**16**), and dimethoxydesanthraserpidine (**17**), have been proposed [33]. Several yohimbine-type bases and a number of minor unidentified components were also isolated. Tetrahydroalstonine (**18**), vincaleukoblastine, cataphylline, vindorosine and trichophylline, reported [34–37] in *C. trichophyllus* roots, were not detected. We examined also the alkaloid content of the growth medium after 10-, 20-, 30-, and 40-day periods, for several cultures of the same line The alkaloids were retained almost entirely in the root tissue, less than 0.01 g/l of crude alkaloids being released in the medium (data not shown).

Alkaloid analyses of normal roots and hairy roots

The major alkaloids **1–6, 14,** purified from fermenter cultures (see above), were used as internal standards for HPLC. The alkaloids **12** and **18** were available in our laboratory. The alkaloid content of roots of four greenhouse-grown plants (1–4) was examined (Table 2); they showed variable compositions. To avoid the genetic heterogeneity between different plants and in order to compare indole alkaloid production, of *in vitro* normal root and hairy root cultures, we analysed the alkaloid content of five-week-old root cultures, derived from the same parent plant. Analyses were performed on three subcultures (A–C) for each root line, at five-week intervals. The results are given in Table 3.

DISCUSSION

Catharanthus trichophyllus plant roots and cultured roots showed similar alkaloid patterns They produced a rather broad alkaloid spectrum, usually found in *C. trichophyllus* roots [34–37], with representatives from the corynantheine-heteroyohimbine- (**5, 10, 12**), the yohimbine- (**13–17**), the vallesamine- (**8**), the strychnos- (**9**) and

Table 2. Alkaloid content of greenhouse-grown plant roots (10^{-3} mg/g fr. wt)

Plant roots	1	2	3	4	5	6	12	14	18	Σ
1	80	115	10	119	126	28	41	63	7	589
2	83	108	26	41	33	65	19	122	tr	497
3	122	135	45	369	51	85	28	155	16	1006
4	237	212	51	355	227	43	118	262	17	1522

tr.: trace amounts (< 5).
Σ: total of alkaloid (**1–6, 12, 14, 18**) amounts.

Table 3. Alkaloid content of three (A, B, and C) *in vitro* normal root and hairy root five-week-old cultures, at five-week intervals (10^{-3} mg/g fr. wt)

Root system		1	2	3	4	5	6	12	14	Σ
Normal root line										
	A	165	40	308	103	7	20	10	52	705
	B	96	149	73	26	tr	8	tr	53	405
	C	214	293	294	60	tr	7	tr	59	927
Hairy root lines.										
2	A	158	30	125	65	tr	16	tr	15	409
	B	173	191	96	66	tr	17	5	128	676
	C	218	237	182	68	—	8	tr	83	796
4	A	102	12	100	38	—	tr	tr	24	276
	B	206	162	125	52	14	38	8	202	807
	C	167	161	109	33	tr	15	11	96	592
5	A	253	26	167	68	tr	16	5	47	582
	B	108	61	20	22	—	7	5	64	287
	C	197	150	76	48	tr	19	9	91	590
9	A	91	7	tr	6	5	—	tr	tr	109
	B	57	5	5	8	tr	8	tr	32	115
	C	64	14	12	14	tr	15	10	128	257
10	A	126	6	87	43	tr	19	tr	36	317
	B	130	93	22	36	tr	17	8	100	406
	C	155	71	35	40	tr	18	7	93	419
11	A	109	8	115	72	8	50	10	55	427
	B	96	58	45	52	18	66	17	158	510
	C	183	126	114	45	tr	25	9	82	584

—undetected; tr: trace amounts (< 5)
Σ: total of alkaloid (**1–6, 12, 14**) amounts.

the aspidospermine- **(1–4, 6, 7, 11)** groups [38] Thus, in roots from this species, almost all the biogenetic pathway of the monoterpenoid indole alkaloids is functional Members of the *Iboga*-type representing the last step of the indole alkaloid biosynthesis evolution in Apocynaceae [39–40] were, however, not observed The yield of *Corynanthe*-type alkaloids **(5, 12)** was lower in cultured roots This difference could be due to the fact that *in vitro* cultured roots were grown in the light Indeed, production of ajmalicine by *C roseus* cell suspensions has been reported to be higher when these were grown in the dark rather than in the light [9]. In addition to already described alkaloids, we found five compounds that had not been observed previously They have been identified as anthraserpine derivatives The presence of these in normal root cultures confirms that hairy root transformation does not influence the pattern of alkaloid biosynthesis in *C trichophyllus*

In this study we show that, except for one line which grew poorly and showed marked tendency to callussing, root cultures, normal or transformed, produce similar amounts of alkaloids This result suggests the expression of hairy root T-DNA has no direct influence on secondary metabolite production, although it may have an indirect one by controlling the rate of growth This feature may be of importance for many plant species for which fast-growing normal root cultures cannot be obtained [32] Positive influence of growth rate on productivity of secondary metabolites has been reported by others [26]

In spite of research conducted over more than 30 years [41], the commercial feasibility of secondary metabolite production by *in vitro* culture methods still has to be demonstrated However, we believe that root culture has an important potential Indeed measurements of secondary metabolite production by hairy root cultures show that productivity of these is within the range of that of roots taken from whole plants [17, 18, 22, 23, 25, 27 and this work] If variability in yields has been observed (this work), it is encouraging to note that this is also well within the variability range of whole plants and that the spectrum of metabolites produced is similar to that of whole plants At this point the numerous reports published on the usefulness of hairy root cultures for secondary metabolite production give a rather encouraging impression The variability, from one line to another [19, 27] and within different cultures of the same line, although real is certainly much less than for undifferentiated cultures The fact that reproducible yields have been obtained by some workers [19] suggests that variability in yields can be controlled by adjusting culture parameters

These features of secondary metabolite production by root cultures probably reflect the fact that they are much closer to real plant organs than cultured cells to cells from whole plants Without engaging into a complete discussion of the differences between cell and root cultures we believe it is fair to say that root cultures are genetically more stable than cell cultures We think that this is due to the fact that the constraints of root meristem neoformation, for which we have evidence that it is a clonal process (Van Sluys and Tempé in preparation), efficiently achieves elimination of variant cells The first hairy root cultures were established some eight years ago [42] and by both criteria of opine production and T-DNA structure [43] they have proven to be perfectly stable over this period

One important aspect of *in vitro* culture production that is rarely dealt with in publications, although it is a real problem at the level of production, is the poor compatibility between mass production techniques and maintenance of proper physical and physiological state of cultured material. Availability of nutrients in large scale suspension cultures is often a limiting factor to which simple solutions like increasing agitation are not applicable because of the fragility of plant cells This problem has been dealt with mainly by modifying fermenter design or operation in order to minimize damage to cells With this respect, root cultures appear to be particularly suited since it is possible to obtain high yields of cultured material in a standard bioreactor designed for bacterial cultures Other workers have reported good adaptation of root cultures for fermenter production [20, 26] The important factors here appear to be the mechanical strength of the roots and also their growth pattern With respect to this, hairy roots may be at advantage over normal roots because of their high rate of branching and eventually their lack of geotropism which will allow efficient occupation of the bioreactor volume by a tridimensional network of roots. It should, however, be kept in mind that roots of some plants are difficult to grow in liquid cultures and that therefore this solution may not be of general applicability With this respect, *C trichophyllus* roots appear to be much better adapted to fermenter cultures than *C. roseus* roots whose fragility was a major problem

Among the improvements to come we see genetic engineering as potentially the most successful Indeed cloning of genes encoding enzymes involved in secondary metabolite synthesis [44] opens the way to genetic manipulation for higher production Double transformation strategies already available [45] would allow to obtain in one step genetically manipulated hairy roots

EXPERIMENTAL

Bacterial strain A rhizogenes strain 15834 came from our collection [46] The strain was cultured on LB agar medium [47] at 28 for two days before inoculation of plants

Plant material and establishment of sterile plants in vitro *C trichophyllus* seeds used in this study were collected from greenhouse-grown plants The original seed stock was collected near lake Alaotra in Madagascar (Boiteau N 3921) The seeds were surface sterilized by immersion in a soln of $Ca(OCl)_2$ (90g l) for 30 min followed by thorough rinsing in sterile H_2O Seeds were sown on Mo agar medium [46] and maintained at 22 under 16 hr fluorescent light

Establishment and culture of hairy root lines Aseptic plantlets were inoculated *in vitro* by wounding with a scalpel, previously dipped into a fresh culture of *A rhizogenes* strain 15834 Roots appeared at the inoculation site after 4 weeks Different root tips were excised and individually placed on Petri plates containing Mo agar medium with antibiotic (cefotetan, Apacef^R, 100 μg/ml) After 2 or 3 subcultures, root tips (2 cm long) of 6 hairy root lines, selected by the highest growth rate and derived from the same parent plant were transferred to the same medium without antibiotic At 4- or 5-week intervals, each hairy root line was transferred to Mo agar medium in Petri dishes Other cultures were grown in liquid medium in Petri dishes as standing cultures or in 200 ml flasks on an orbital shaker (150 rpm)

Establishment of normal root culture An untransformed root of the *C trichophyllus* plant, used for the establishment of the six hairy root line cultures, was excised and cultured in Mo (agar)

medium. Normal roots were subcultured at 4- or 5-week intervals.

Hairy root fermenter cultures Two inocula were prepared by growing 3 g fr. wt of hairy root line 10 in 200 ml Mo medium, for 8 days at 22°. Roots (10 g fr wt) were transferred each to two Biolafitte bioreactors (R_1 and R_2) containing 18 l Mo medium (pH 6). The fermenters were aerated at 18 l/min and maintained under agitation (100 rpm/min) at 25° in daylight Cultures were harvested after 6.5 and 9 5 weeks yielding, respectively, 2063 g and 2510 g fr wt or 149 2 g and 200 9 g dry wt after freeze-drying

Isolation of alkaloids. Extraction of freeze-dried hairy root line 10 (350 g) in the classical manner gave 3 22 g of crude alkaloids which were dissolved in $CHCl_3$–MeOH (3·7). The resulting soln was filtered through a Sephadex LH20 column Alkaloids were sepd and further purified by CC on Al_2O_3 followed by prep. TLC on silica gel and crystallization. Structures were determined by use of spectrometric data (UV, IR, MS, NMR) and confirmed by comparison with those of authentic samples. The major alkaloids, purified from these fermenter cultures, were used as standards for HPLC expts.

Root cultures for alkaloid analysis. For each line studied, 5-week-old cultures were established in Mo liquid medium (100 ml) in Petri dishes without agitation at 22°, 16 hr photoperiod. Three cultures (A, B, and C) were carried out at 5-week intervals

Plant material for alkaloid analysis. From seedlings, *C. trichophyllus* plants were grown in Vermiculite under greenhouse conditions for one year.

Alkaloid analysis Freeze-dried samples from root cultures and greenhouse-grown plants were extracted using standard procedures by percolation with MeOH–NH_4OH, followed by sepn of alkaloids into acidic soln and re-extn into basic soln with CH_2Cl_2 Solvent was removed and the crude alkaloids (20 μg) in MeOH soln (1 mg/ml) were analysed by HPLC using an analytical reverse-phase C_8 column (SFCC, Hypersil 13 5 cm × 4.2 mm) The column was eluted with a binary solvent gradient of KCl 100 mM (pH 2.2)/MeCN at a flow rate of 1ml/min. The composition of the gradient was 3 1 to 1:1 over 20 min It was then held at 1:1 for 5 min before re-equilibration with 9:31 and return to initial conditions before rerunning. The effluent from the column was monitored using a UV absorption detector set at 280 nm

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