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Indole alkaloid production by hairy root cultures of *Catharanthus roseus*

Dedicated to Dr. Friedrich Constabel on the occasion of his 60th birthday

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Abstract. Hairy root cultures of *Catharanthus roseus* were established by infection with six different *Agrobacterium rhizogenes* strains. Two plant varieties were used and found to exhibit significantly different responses to infection. Forty-seven hairy root clones derived from normal plants and two derived from the flowerless variety were screened for their growth and indole alkaloid production. The growth rate and morphological appearance showed wide variations between the clones. The alkaloid spectra observed were qualitatively but not quantitatively very similar to that of the corresponding normal plant roots. No vindoline or deacetyltransferase activity could be detected in any of the cultures studied. O-acetylvallesamine, an alkaloid which has not been previously observed in *C. roseus* was identified from extracts of hairy root clone No. 8. Two root clones were examined for their growth and alkaloid accumulation during a 26-day culture period. Alkaloid accumulation parallelled growth in both clones with ca. 2 mg ajmalicine and catharanthine per g dry weight being observed.

Introduction

Establishment of hairy root cultures for production of valuable secondary metabolites in vitro has recently been reported for several plant species [1–6]. Hairy root cultures formed by transformation of the plant by the soil pathogenic bacterium *Agrobacterium rhizogenes* have many advantages when compared to cell suspension cultures and untransformed root cultures. Hairy roots are potentially able to produce all root-derived products of dicotyledonous plants. Moreover, it has been possible to select highly productive hairy root clones which grow faster than the corresponding

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untransformed root cultures [3]. These cultures appear to be genetically stable [7] which has not often been the case with high-producing cell suspension cultures [8]. Because of stable high production of secondary metabolites, hairy root cultures also offer a valuable system for studying the control of gene expression and the biosynthesis of secondary metabolites [9].

Materials and methods

Bacteria and plant material

Agrobacterium rhizogenes strains 15834, A2, A2-83, A47-83, R1000 (agropine-type) and TR7 (mannopine-type) were tested for their ability to induce hairy roots on *Catharanthus roseus*. Two plant varieties were used: *C. roseus* (L.) G. Don cv. Little Delicata (LD) and a flowerless variety (FV) that differed from the normal plant by producing an unusual spectrum of indole alkaloids, the major difference in the roots being the lack of catharanthine accumulation. LD plants were raised from seed supplied by Westcan Horticulture Ltd. An FV plant was observed in a plot of LD plants and was propagated by cuttings.

Infection

The bacteria were cultured at 26 °C for two days on YT agar medium (0.3% yeast extract, 0.5% tryptone, 0.066% $CaCl_2$). Sterile LD seedlings grown on Gamborg's B5 medium [10] at 26 °C in light for 2–3 weeks were inoculated with each of the bacterial strains and incubated further in light. Detached leaves from FV plants grown in the greenhouse were surface-sterilized, inoculated with the bacteria, and cultured in the dark on MS [11] or B5 agar medium without growth hormones.

After 3–6 weeks the hairy roots which had emerged from the infection points were cut off and cultured individually on MS, B5 or on 1/2B5 (one-half dilution of the standard B5) agar medium containing 3% sucrose and 0.5 g l carbenicillin (Sigma). Rapidly growing roots without bacterial and fungal contamination were transferred to fresh medium every 2–3 weeks.

Selection of the root clones

Ca. 0.1 g of each of the root clones grown on agar medium was inoculated into 15 ml of liquid 1/2B5 medium with 0.5 g l^{-1} carbenicillin. The roots were

cultured in the dark on a gyratory shaker (60 rpm) at 26 °C. After 17 days, the growth and indole alkaloid production of the cultures (four parallel flasks) were analysed.

Liquid root cultures

The best hairy root cultures were maintained also in 30 ml liquid 1/2B5 medium with 3% sucrose and 0.5 g l carbenicillin in the dark on a gyratory shaker (60 rpm) at 26 °C and subcultured every 2–3 weeks.

Indole alkaloid analysis

Root samples were extracted according to the protocol established by Kurz & Constabel [12]. Alkaloids were screened by thin-layer chromatography (Whatman silica gel 60A TLC-plates, fluorescent at 254 nm). As migration solvents, methanol/ethyl acetate (10:90) was used for 1-dimensional TLC-analysis and dichloromethane/ethanol (12:1) and methanol/ethyl acetate (20:80) for 2-dimensional TLC-analysis. Alkaloids were detected by spraying with ceric ammonium phosphate reagent [13].

High-performance liquid chromatography (HPLC) was used for quantitative analysis of ajmalicine and catharanthine [14]. The values presented are the average of two parallel cultures.

For structure elucidation, alkaloids were purified by using a multistep 1-dimensional TLC method with methanol/ethyl acetate (10:90), hexane/ether (1:1) and ether or dichloromethane/methanol (8:1) as migration solvents as required. The purified alkaloids were subjected to MS and/or NMR analysis. Also their UV spectra were measured.

Ei mass spectra were obtained with a Finnegan model 4000 spectrometer which was run at 70 eV. ^{1}H-NMR spectra were obtained with a Bruker 360 MHz spectrometer. Samples were dissolved in deuterochloroform and tetramethylsilane was employed as internal standard.

Spectroscopic data for pericalline (apparicine) and O-acetylvallesamine were reported in Joule et al. [15] and Perera et al. [16]. Authentic samples of other alkaloids were available from our reference collection.

Detection of opines

Opine analysis was performed by a modified method of Petit et al. [17]. Mannopine (Sigma Chemical Co.) was used as a standard.

Enzyme assays

The activities of tryptophan decarboxylase (TDC) and deacetyltransferase (DAT) in some of the root clones were assayed by using protocols as in Eilert et al. [18] and De Luca et al. [19], respectively.

Sucrose analysis

Sucrose analyses were performed by HPLC using a Bio-Rad Aminex carbohydrate HPX-87C 330 × 7.8 mm column which was equipped with a Bio-Rad micro-guard carbo-C guard column. The solvent used was water at a flow rate of 0.8 ml min^{-1} and a column temperature of 80 °C. The injection size was 20 μl and detection was achieved with a refractive index detector.

Establishment of cell cultures of the flowerless variety

For comparison, cell cultures were also established from leaves of the FV plants. Callus cultures were started from sterilized leaf segments using B5 agar medium with either 2 mg l^{-1} 2,4-D or 1 mg l^{-1} 2,4-D and 1.5 mg l^{-1} kinetin. After 3 passages, cell suspension cultures were established using B5 medium with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin. The ability of these cultures to produce alkaloids was studied as described by Constabel et al. [20].

Results

Establishment of hairy root cultures

Hairy roots emerged after 3–6 weeks of incubation of infected leaves or plantlets. Only strain TR7 was totally unable to induce hairy roots. Strains 15834, A2, A2-83 and A47-83 were all very active in inducing hairy roots in LD plants (infection rate 60–80%). For the FV plants, strains 15834, A2 and A2-83 were most active (infection rate 30–40%). Therefore, these strains were used in further work.

Root tips of ca. 280 (LD) and 350 (FV) hairy roots induced by the bacterial strains were excised and cultured individually on MS, B5 or 1/2B5 agar medium for 2–3 passages every 2 weeks. In the case of normal plants, about 30% of the total hairy roots tested continued to grow on the phytohormone-free medium after an initial lag phase of about one month. The

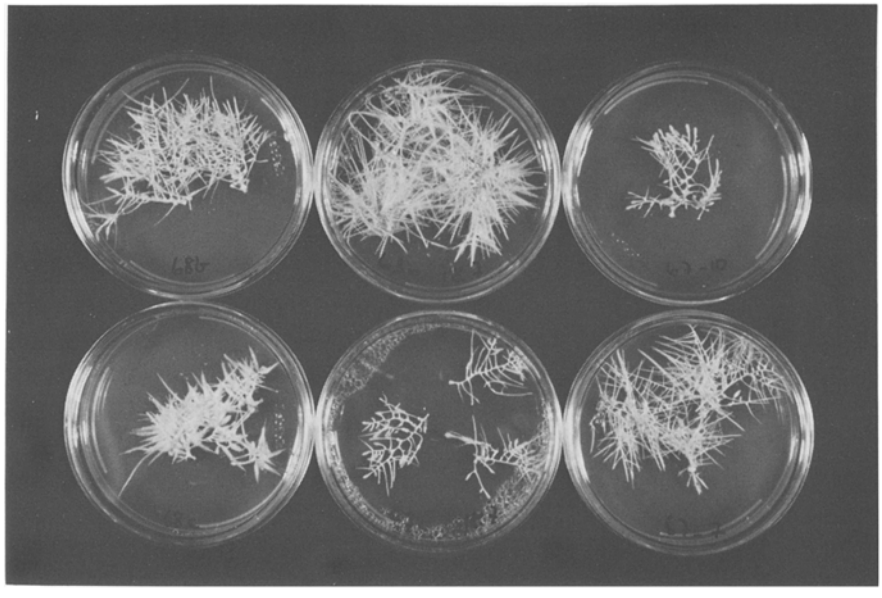


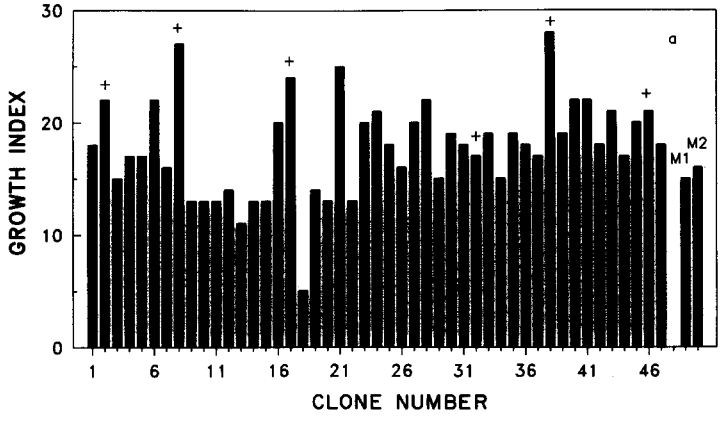
Fig. 1. Hairy root cultures of *Catharanthus roseus* showing the divergence in the growth between different clones derived from the same variety.

growth of the other 70% was arrested, although some roots grew slowly at the first transfer. In the case of FV plants the number of hairy roots that started to grow was very low: only 11% of the roots tested started growing initially, while after several subcultures only 2.5% continued their growth. 1/2B5 medium was found most suitable for growth of roots without callus development.

Finally, a total of 87 hairy root clones derived from the LD plants and 9 clones from the FV plants were successfully cultured on 1/2B5 agar medium in the dark. The cultures were characterized by increased lateral branching, profusion of root hairs and absence of geotropism, characteristics which are generally typical of transformed root cultures. There were, however, remarkable differences in appearance between the cultures (Fig. 1).

Selection of root clones for further study

Forty-seven rapidly growing clones derived from LD and two derived from the FV plants were examined for their growth and alkaloid contents in liquid 1/2B5 medium. The growth index (g fresh weight per g inoculum after 17 days growth) varied between 5 and 28 (Fig. 2a). Eighteen hairy root clones


GROWTH INDEX
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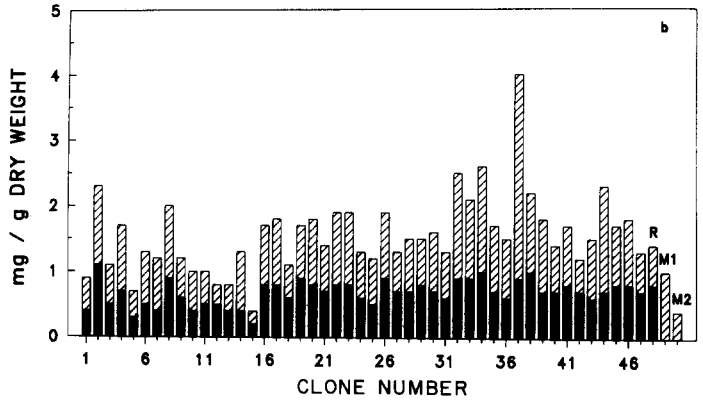
mg / g DRY WEIGHT
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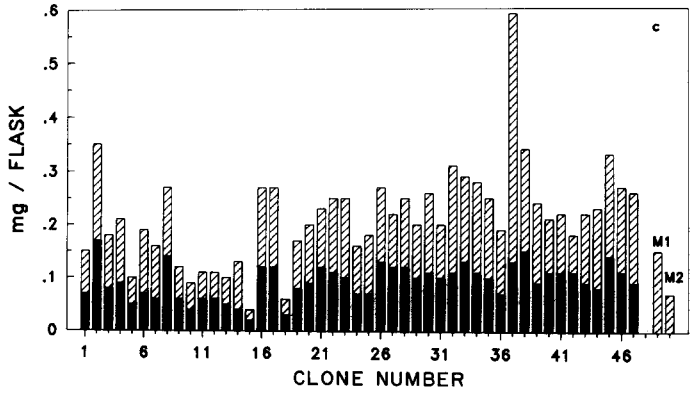
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CLONE NUMBER

Table 1. Mannopine production of hairy root cultures of *Catharanthus roseus.*

Hairy root clone No.	Mannopine detected
2	+
8	++
17	+
32	+
37	−
38	−
46	+
M1	+
M2	++

+detected, − not detected

(LD plant) grew without any callus development, six of which with especially long and branched roots (+). All the cultures produced a wide variety of indole alkaloids (> 20) as judged by TLC and HPLC. Six of the cultures tested produced less than 1 mg g^{-1} DW ajmalicine and catharanthine (Fig. 2b). In most of the clones (35) ajmalicine and catharanthine production was 1–2 mg g^{-1} DW, which is comparable to the production of these alkaloids in normal plant roots grown in soil (Fig. 2b). Generally, the differences in ajmalicine and catharanthine production between clones were not very remarkable, except for clone No. 37, which produced significantly higher amounts of ajmalicine. This clone also possessed a different appearance with its roots being thicker.

The volumetric production (mg ajmalicine and catharanthine per flask) of the six clones with the best growth behaviour (Nos. 2, 8, 17, 32, 38 and 46) was more than 250 μg per flask (16.7 mg l^{-1}) in 17 days (Fig. 2c). These root clones as well as clone No. 37 were assayed for opine production (Table 1). Only in clones Nos. 37 and 38 could no opines be detected. Further confirmation of the transformed nature of these root cultures remains to be performed.

The hairy root clones derived from the FV plants (M1 and M2) tended to

←

Fig. 2. Growth rate and indole alkaloid production of *C. roseus* hairy root cultures. Forty-seven hairy root clones (M1 and M2) derived from normal plants (Nos. 1–47) and two clones derived from the flowerless variety were each cultured in 15 ml of liquid 1/2B5 medium in the dark at 26 °C for 17 days. (*a*) Growth index represents the fresh weight after 17 days growth per fresh weight of inoculum, + = clones with long unbranched roots. (*b*, *c*) Amount of catharanthine (solid bar) and ajmalicine (light bar) accumulated by the various clones after 17 days. R = roots from a field-grown plant. Values present average of 4 parallel cultures.

form callus in liquid medium. However, they produced a wide range of indole alkaloids. These clones also produced large amounts of mannopine (Table 1).

In all of the hairy root cultures tested, only 2–5% of the ajmalicine and catharanthine was detected in the growth medium.

Indole alkaloid production

All hairy root clones produced a wide variety of indole alkaloids. TLC comparison (2-dimensional) of the alkaloid mixture obtained from roots of field-grown *C. roseus* with the alkaloid mixture obtained from hairy roots indicated that their compositions were qualitatively very similar with more than 20 alkaloids being common to both types of samples from a majority of the clones. The roots of the FV plants did not accumulate catharanthine, nor did the corresponding root clones. Discernable differences were however noted between roots of normal field-grown plants (FG) and hairy root

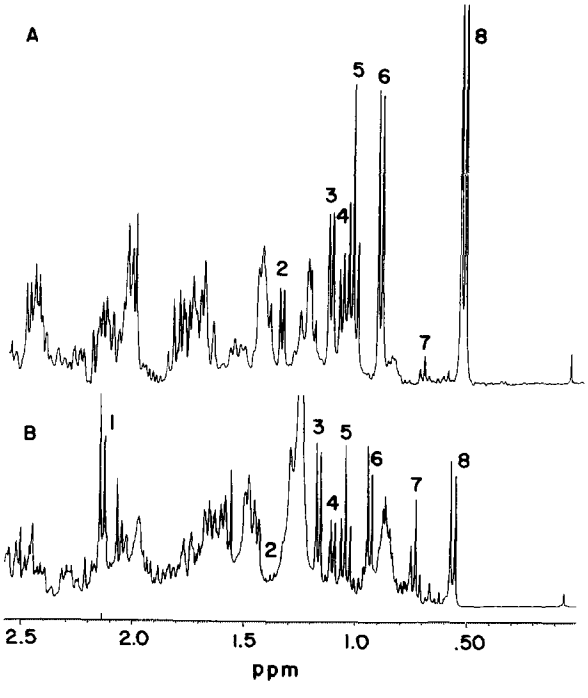

Fig. 3. ^{1}H-NMR spectra (δ 2.5 to δ 0) of the crude alkaloid derived from (*A*) roots of field-grown LD plant, (*B*) hairy root clone No. 8 (derived from LD plant). Alkaloids responsible for some of the observed resonances were (1) O-acetylvallesamine, (2) tetrahydroalstonine, (3) ajmalicine, (4) horhammericine, (5) catharanthine, (6) vindolinine, (7) lochnericine, (8) epivindolinine.

cultures (HRC) derived from them, as at least two extra spots were observed in the TLC samples. One of these was determined to be due to O-acetylvallesamine, an alkaloid which has not previously been observed in *C. roseus*. The alkaloid responsible for the other spot was not isolated.

Although the alkaloid profiles observed from roots of FG were qualitatively similar to those observed from the HRC, quantitatively there were great differences as indicated by the ^{1}H-NMR spectra of the corresponding alkaloid mixtures (Fig. 3). In the FG sample, ajmalicine, catharanthine, vindolinine and epivindolinine were found to be the predominant components. In the HRC sample, alkaloids such as lochnericine, pericalline and yohimbine were also major components. O-acetylvallesamine, which was found only in the HRC sample, was also a significant component. How much of these quantitative differences could be ascribed to environmental or developmental influences and how much to the transformed genome remains to be determined.

Table 2 lists the alkaloids which were purified and identified by using extracts of hairy root clone No. 8. Monoterpenoid indole alkaloids belonging structurally both to Corynanthe (1–4 and 12, 13), Aspidosperma (5–10) and Iboga (11) families could be identified. Alkaloids 1–10 were detected also in root clones M1 and M2 derived from the flowerless plants.

Table 2. Indole alkaloids identified from extracts of hairy root clone No. 8.

Alkaloid	Basis of identification[a]
1. Strictosidine lactam	1, 2, 3a
2. Yohimbine	1, 3b, 4, 5
3. Ajmalicine	1, 2, 3a, 4
4. Tetrahydroalstonine	1, 4
5. Tabersonine	1, 2, 3a, 4
6. Horhammericine	1, 4
7. Lochnericine	1, 2, 3a, 4
8. Venalstonine	1, 4
9. Vindolinine	1, 2, 3a
10. 19-epi-vindolinine	1, 2, 3a
11. Catharanthine	1, 2, 3a, 4
12. Pericalline (apparicine)	4, 5
13. O-acetylvallesamine	3b, 4, 5

[a] Basis of identification:
1. TLC (R_F and spraying, compared to authentic samples)
2. HPLC (retention time compared to authentic samples)
3a. UV spectrum (compared to authentic samples)
3b. UV spectrum (compared to literature)
4. MS
5. NMR

The production of indole alkaloids in hairy root clones M1 and M2 was also compared to alkaloid production of cell cultures derived from the same plant (flowerless variety). According to 2-dimensional TLC, the cell cultures produced 10–15 different alkaloids after growing in alkaloid production medium for 17 or 24 days. None of the cell lines produced catharanthine. In 3 out of 8 cell lines tested, ajmalicine levels were higher than in root clones M1 and M2. For example, one of the cell lines produced 47.5 mg l^{-1} ajmalicine in 24 days as compared to 9.8 mg l^{-1} in clone M1 and 4.4 mg l^{-1} in clone M2 in 17 days.

Growth and alkaloid accumulation of hairy root cultures Nos. 2 and 8

Figs. 4 and 5 show the growth and alkaloid accumulation of two hairy root cultures during a 26-day culture period. Clone No. 2 grew slightly slower than clone No. 8, the doubling times being 3 days and 2 days in the exponential growth phase, respectively. On the other hand, clone No. 2 produced slightly more ajmalicine and catharanthine (mg g^{-1} DW) than clone No. 8 and the TDC activity was higher in clone No. 2 than in clone No. 8 throughout the growth. No deacetyltransferase activity was detected during the culture period.

In both cultures, alkaloid accumulation occurred parallel to growth. The maximum volumetric productivity (1.4 mg ajmalicine + catharanthine l^{-1} day^{-1} in clone No. 2 and 1.5 mg l^{-1} day^{-1} in clone No. 8) was reached after 15 days, although the alkaloid yield was highest (29 mg l^{-1} and 24 mg l^{-1}, respectively) in the late stationary phase at 26 and 21 days, respectively.

Discussion

The infection rate and the ability of the transformed roots to grow further on a nutrient medium appeared to be highly dependent on the plant variety used. The importance of the plant origin in establishing hairy root cultures has been reported previously [6]. In the case of *C. roseus* cv. Little Delicata, hairy root cultures could be established relatively easily, although conventional MS and B5 media were not suitable for growth without dilution. All the established hairy root clones produced a wide variety of indole alkaloids. Except for O-acetylvallesamine, all the other alkaloids listed in Table 2 have been reported to be present either in plant roots (1, 3, 4, 7, 9–12) [21] or in cell suspension cultures (1–7, 9–11) [22] of *C. roseus*. In our work the alkaloid spectra of the hairy root cultures were shown to resemble only

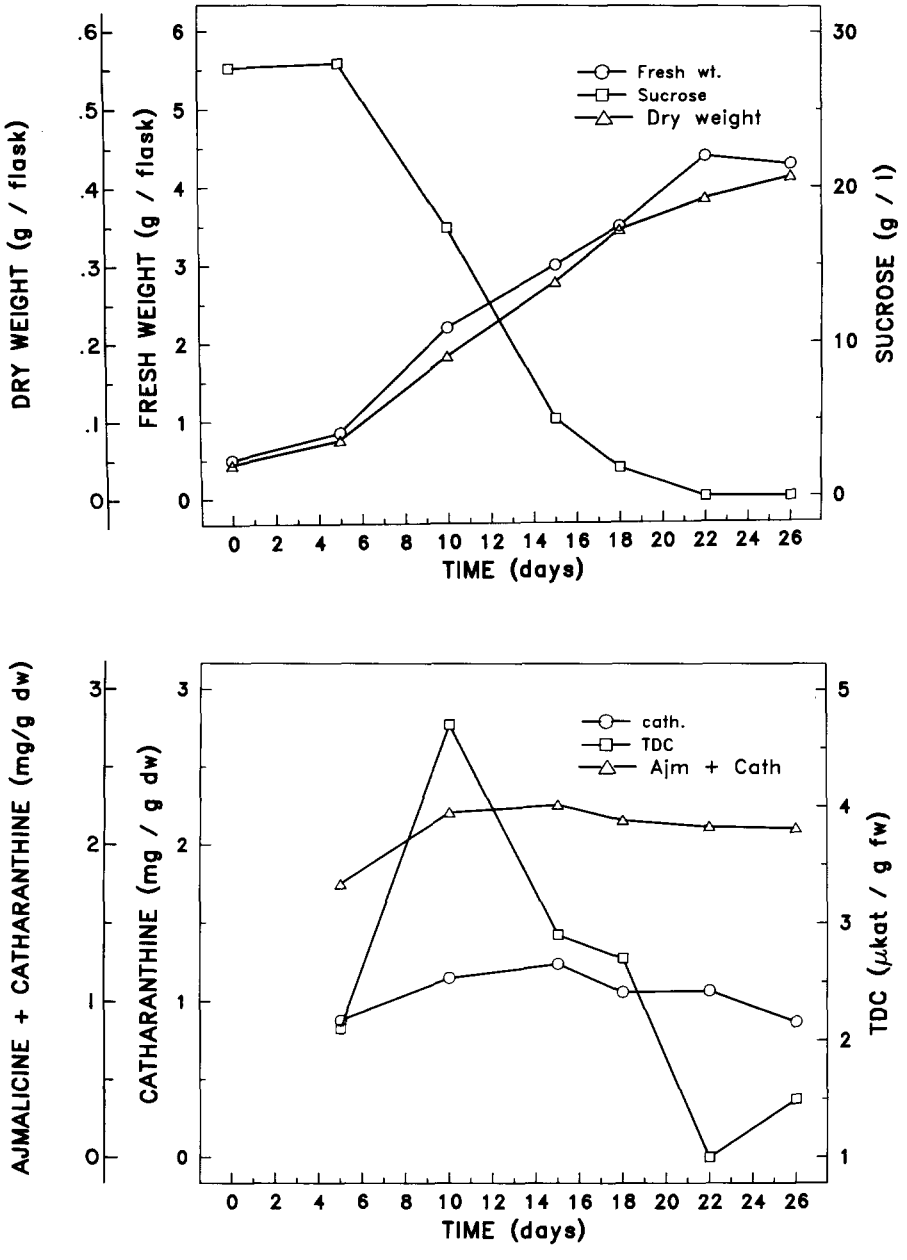

Fig. 4. Growth and alkaloid accumulation of hairy root clone No. 2 during a 26-day culture period in 30 ml liquid 1/2B5 medium with 3% sucrose. (*top*) Increase in fresh and dry weight and the concentration of sucrose in the medium. (*bottom*) Catharanthine and ajmalicine production and tryptophan decarboxylase (TDC) activity.

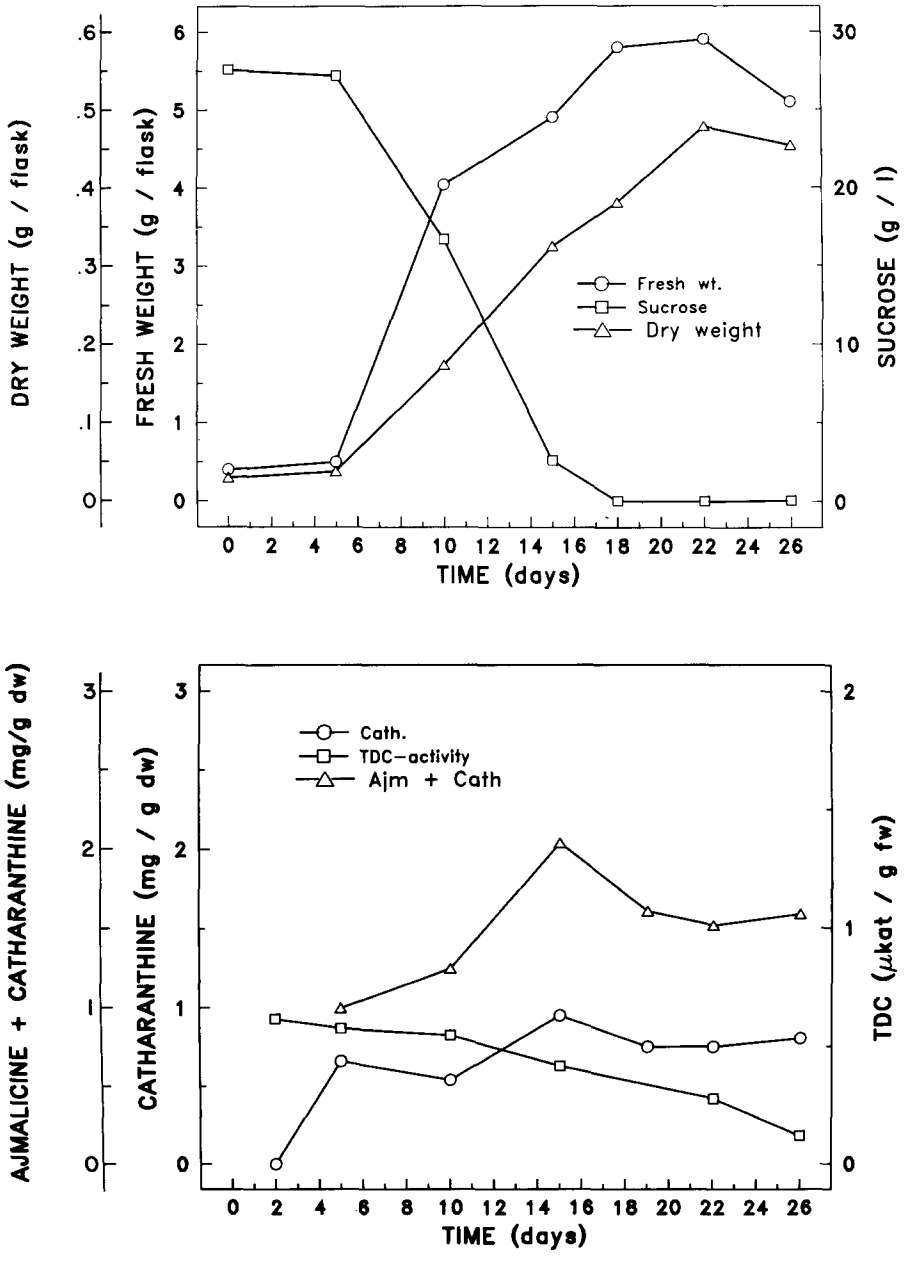

Fig. 5. Growth and alkaloid accumulation of hairy root clone No. 8 during a 26-day culture period in liquid 1/2B5 medium with 3% sucrose. (*top*) Increase in fresh and dry weight and the concentration of sucrose in the medium. (*bottom*) Catharanthine and ajmalicine production and tryptophan decarboxylase (TDC) activity.

qualitatively the spectra observed in corresponding normal plant roots grown in vivo.

Bisindole alkaloids or their precursor vindoline could not be detected in hairy root cultures nor roots of field-grown plants by the methods used, although many other alkaloids belonging to the Aspidosperma group were present. The absence of acetyltransferase activity, which is associated with the enzyme catalysing the last step of vindoline biosynthesis [23], indicates that hairy root cultures derived from either plant are unable to synthesize vindoline. This is in contrast to the results published previously by Parr et al. [24] who reported the detection of vinblastine by a combination of HPLC and radioimmunoassay ($0.05\,\mu g\,g^{-1}$ DW). Svoboda et al. [21] previously reported the identification of vinblastine in normal roots of *C. roseus* but we could not corroborate this result in our plants. On the other hand the role of light in the induction of late vindoline biosynthetic enzymes has been demonstrated [25].

The levels of ajmalicine and catharanthine reported here are comparable to those published previously for a *C. roseus* hairy root culture [24]. In cell suspension cultures, similar ajmalicine and catharanthine levels were reached by adding elicitors [26]. However, hairy root cultures also produce a wide variety of other indole alkaloids in significant amounts. Moreover, hairy root cultures are genetically more stable [7, 8] which facilitates optimization of growth and alkaloid production. The production of significant quantities of many different types of indole alkaloids by hairy root cultures of *C. roseus* suggest their use as a valuable tool for studying biosynthetic pathways of indole alkaloid production.

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