Tropane Alkaloid Production in *Hyoscyamus* Root Cultures

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Received October 5, 1985 · Accepted December 4, 1985

Abstract

Tropane alkaloid production was studied in callus and root cultures of seven *Hyoscyamus* species. Cultured roots grew well (2- to 6-fold in 8 days) without addition of growth regulators. Hyoscyamine content in these roots varied from 0.04 % DW to more than 1.1 % DW, and scopolamine content from 0.06 % DW to 0.3 % DW, depending on the plant species. Calli grew slowly (2- to 7-fold in 4 weeks) and had much lower alkaloid content than their respective root cultures. Hyoscyamine was the main alkaloid in all callus cultures.

Alkaloid production in root cultures of *H. niger* and *H. albus* was studied under various culture conditions. When roots were cultured in medium without growth regulators, the main alkaloids produced were scopolamine (0.2 % DW) in *H. niger* and hyoscyamine (0.5 to 1.2 % DW) in *H. albus*. In *H. albus* root cultures, an inverse relationship between growth and hyoscyamine content was found.

Auxin, especially IBA at 10^{-6} M to 10^{-4} M, stimulated growth of the root cultures, which was accompanied by increased lateral root induction. Alkaloid biosynthesis, however, was inhibited as auxin concentration increased. In *H. niger* root culture, scopolamine formation was inhibited by auxin more strongly than hyoscyamine formation. These effects of auxin on growth and alkaloid biosynthesis were confirmed in *Datura*, *Atropa* and *Duboisia* root cultures. Effects of other growth regulators and several basal media were also examined.

Key words: Hyoscyamus species, Hyoscyamus niger, Hyoscyamus albus, tropane alkaloids, scopolamine, hyoscyamine, root culture, indolebutyric acid.

Introduction

Production of tropane alkaloids has been studied in callus or cell suspension cultures of many alkaloid-containing Solanaceae, but has not met with much success (see Kurz and Constabel, 1985). For example, among the plant species that we tested, only callus of *Hyoscyamus niger* contained considerable amounts of alkaloids. Although hyoscyamine content in cultured *H. niger* cells could be increased up to the level of whole plants, by selection of a high-producing cell line and improvement of culture conditions, scopolamine content remained about one tenth of that of intact leaves or roots (Yamada and Hashimoto, 1982; Hashimoto et al., 1982).

Secondary products are often formed only in restricted tissues of plants. For scopolamine biosynthesis in *H. niger*, differentiation of root tissues was necessary. The cultured roots of this plant even showed much higher content of scopolamine than

Abbreviations: NAA: naphthaleneacetic acid; IAA: indoleacetic acid; IBA: indolebutyric acid; 2,4-D: (2,4-dichlorophenoxy) acetic acid; Ha: *Hyoscyamus albus*; Hn: *Hyoscyamus niger*.

intact mature roots (Hashimoto and Yamada, 1983). Culture of differentiated tissues, such as excised roots, must be reassessed for production of secondary products, which are often not produced in undifferentiated cells.

The basic techniques of excised root culture have long been established (see a review by Butcher and Street, 1964). Not all roots from different species could be cultured successfully and the growth of cultured roots has generally been thought to be inferior to that of suspension cultured cells. Besides, studies so far on root culture were mainly devoted to the development of culture techniques; little attention has been paid to the secondary metabolites that the cultured roots produced. We have little knowledge, for example, on how growth regulators, which are often included in the culture medium of excised roots, affect the biosynthesis of secondary metabolites in root tissues.

In this paper, we report on the establishment of callus and root cultures from 7 *Hyoscyamus* species. Effect of growth regulators and culture medium on growth and alkaloid formation were studied in two fast-growing root cultures of *H. niger* and *H. albus*.

Materials and Methods

Materials

Seeds of different species were obtained from the following sources.

Kyoto Botanic Garden, Kyoto, Japan; *Hyoscyamus niger* L., *H. albus* L., *H. pusillus* L., *Datura stramonium* L. var. stramonium, *D. stramonium* L. var. inermis, *D. leichhardtii, D. innoxia* Mill. and *Atropa belladonna* L.

Royal Botanic Gardens, Kew, England; *H. niger* L. (# 297–02).

The University Botanic Gardens, Birmingham, England; *H. albus* L. (S. 1218), *H. albus* L. ssp. major (S. 1220), *H. gyorffi* (*H. niger* X *H. albus* allopolyploid) (S. 1703), *H. pusillus* (S. 1288), *H. bohemicus* (S. 1353) and *H. canariensis* (S. 1145).

Jardin Botanique, Dijon-Mairie, France; *H. albus* L.

Botanic Garden of the University, Leiden the Netherlands; *H. muticus* L.

Hortus Botanicus Pekinensis, Instituti Botanici Academiae Sinicae, China; *Datura fastuosa* L.

Root cultures of *Duboisia leichhardtii* F. Muell. were obtained as reported previously (Yamada and Endo, 1984).

Chemicals

l-Hyoscyamine hydrobromide and scopolamine hydrobromide were purchased from Nakarai Chemicals (Kyoto, Japan). Authentic samples of 6 β-hydroxyhyoscyamine hydrobromide and 3 α-[2-hydroxy-3-phenylpropionyloxy]-tropane (littorine) sulfate were generous gifts from Dr. Her Liyi of the Chinese Academy of Medical Sciences, China, and Prof. S. Yamada of Jyosai University, respectively.

Culture of Calli and Excised Roots

Seeds were sterilized and germinated to give sterile seedlings. Seeds obtained from different sources were treated as different lines, even if they were the same species. Usually four sterile seedlings were used in each seed line. From each seedling, a small piece of a cotyledon and a part of a young root (ca. 5 mm), including the root tip, were excised aseptically. Leaf explants were cultured at 25 °C in the dark on the LS medium (Linsmaier and Skoog, 1965) containing 10^{-5} M NAA, 5×10^{-6} M 6-benzyladenine, 3% sucrose and 1% agar. Induced calli were sub-

cultured on the same medium every 4 weeks and, at every subculture, only undifferentiated pieces of callus were transferred, until calli consisted of undifferentiated cells only were obtained. The calli used in the experiments were 3- to 6-month old since callus induction.

Excised roots of *Hyoscyamus* species were cultured on a reciprocal shaker (60 strokes/min) at 25 °C in the dark in the LS medium containing 3 % sucrose, unless otherwise noted. Excised roots of other species were cultured under the same conditions with the addition of 10^{-6} M or 10^{-5} M IBA to the medium. Root cultures were subcultured at one or two week intervals, depending on the rate of growth of the roots.

Extraction and analysis of alkaloids

Harvested calli or roots were freeze-dried, then powdered and soaked overnight in an EtOH-28 %NH_4OH (19 : 1) mixture. This macerated material was centrifuged for 5 min at 3,000 rpm. Extraction with the basic alcohol was repeated twice, and the combined alcohol extracts were evaporated to dryness at 40 °C. The dry residue was dissolved in 2.0 ml of 0.1 N HCl and the acidic aqueous solution was filtered through filter paper No. 2 (Toyo Roshi Co., Ltd, Japan). One milliliter of the filtrate was made alkaline with 0.2 ml of approx. 1 M Na_2CO_3-$NaHCO_3$ buffer (pH 10.0) and 1.0 ml of the alkaline aqueous solution was loaded onto a Extrelut-1 column (Merck Art. 15371). After 5 to 10 min, 6 ml of $CHCl_3$ was passed through the column, and the $CHCl_3$ extracts were evaporated to dryness at 35 °C. The dry residue was dissolved in a mixture of 1,4-dioxane and *N,O*-bis (trimethylsilyl) acetamide (4 : 1 v/v), into which tricosane at a concentration of 0.5 $g \cdot l^{-1}$ was added as the internal standard.

For the extraction of alkaloids from the culture medium, 2.0 ml of the medium was made alkaline with 0.2 ml of the carbonate buffer. The subsequent extraction procedures through Extrelut-1 columns were the same as described above for the extracted cells.

Alkaloids were measured with a gas-liquid chromatograph, Shimadzu Model GC-7A, with a capillary column OV-101 (25 m × 0.3 mm ∅). The column temperature was 250 °C, the carrier gas was He at a flow rate of 0.92 $ml \cdot min^{-1}$. A split ratio of 54 : 1 and the detector FID were used. Alkaloid contents (%DW) were calculated following the formula:

$$\frac{\text{alkaloid (g) in the cells} + \text{alkaloid (g) in the medium}}{\text{dry weight (g) of harvested cells}} \times 100$$

Identification of Alkaloids

Alkaloids in calli or cultured roots were identified by the following criteria: 1. the Rf values on silica-gel TLC, with a developing solvent system of $CHCl_3$/EtOH/28 %NH_4OH (85 : 15 : 4) (Yamada and Hashimoto, 1982), 2. the retention times on GLC, and 3. the mass spectra. These were compared to the values for authentic alkaloids. The mass spectra of trimethylsilyl-derivatives of alkaloids were recorded with a Shimadzu, Model QP-1000, GC-MS spectrometer. An EI mode with an ionizing energy of 70 eV and an ion source temperature of 250 °C were used. Four alkaloids were identified.

Hyoscyamine; Rf 0.35, t_R 4.51 min, MS m/z (rel. int.) 361(9), 140(8), 125(11), 124(100), 123(4), 104(4), 96(10), 95(8), 94(13), 83(21), 82(20), 73(18).

Scopolamine; Rf 0.90, t_R 5.54 min, MS m/z (rel. int.) 375(13), 193(4), 154(36), 139(10), 138(100), 137(21), 136(30), 120(12), 108(34), 104(10), 103(10), 97(19), 95(11), 94(81), 81(17), 75(15), 73(54).

6β-Hydroxyhyoscyamine; Rf 0.18, t_R 5.89 min, MS m/z (rel. int.) 449(2), 434(2), 333(4), 213(4), 212(20), 159(2), 145(2), 122(3), 103(3), 96(14), 95(100), 94(66), 75(9), 73(25).

Littorine; Rf 0.46, t_R 4.61 min, MS m/z (rel. int.) 361(11), 193(6), 140(6), 125(14), 124(100), 123(5), 96(10), 95(7), 94(12), 91(7), 83(19), 82(18), 73(23).

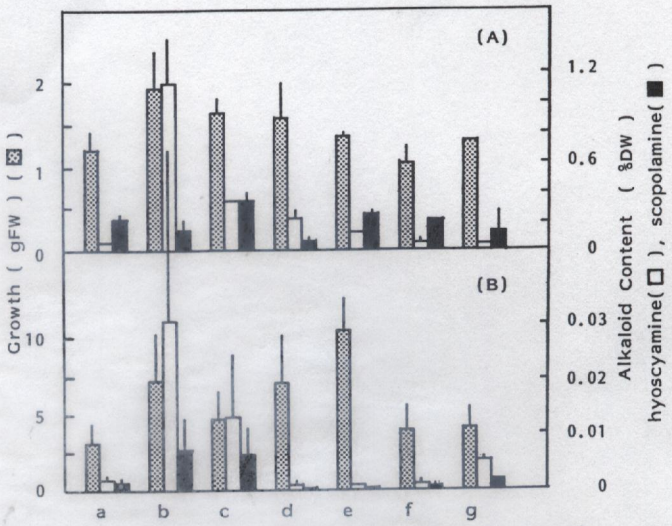

Fig. 1: Growth and alkaloid content in calli and cultured roots of various *Hyoscyamus* species. a, *H. niger*; b, *H. albus*; c, *H. gyorffi*; d, *H. pusillus*; e, *H. muticus*; f, *H. bohemicus*; g, *H. canariensis*. The bars represent the standard error of the mean values. (A) Cultured roots (0.6 g FW) were inoculated in 25 ml of the liquid medium and harvested after eight days. (B) Calli (1.4 g FW) were inoculated on 25 ml of the agar medium and harvested after four weeks.

Results

Growth and Alkaloid Production in Callus and Root Cultures of 7 Hyoscyamus Species

Growth and alkaloid (hyoscyamine and scopolamine) content were studied in callus and root cultures from 7 *Hyoscyamus* species; *H. niger, H. albus, H. gyorffi, H. pusillus, H. muticus, H. bohemicus* and *H. canariensis* (Fig. 1). All excised roots grew well, even without addition of any growth regulators. After subculture of about 3 months, these roots grew 2- to 3-fold in 8 days (Fig. 1 A). *H. albus* root cultures had the highest growth rate among them. The root cultures could be divided into 3 groups, depending on the ratio of scopolamine/hyoscyamine. These were hyoscyamine-rich species (*H. albus* and *H. pusillus*), scopolamine-rich species (*H. niger, H. muticus, H. bohemicus* and *H. canariensis*) and intermediate-type species (*H. gyorffi*). Some *H. albus* root cultures had a hyoscyamine content of more than 1.2 % DW. *H. niger* root cultures had the highest ratio of scopolamine to hyoscyamine (approx. 5). Two other alkaloids were also identified in root cultures. 6β-Hydroxyhyoscyamine was found in all species and littorine in *H. albus, H. gyorffi* and *H. pusillus* (Fig. 2). All four alkaloids were found mostly in the root tissues; up to 20 % of the alkaloids in a flask were found in the culture medium. Scopolamine tended to be excreted into the medium in greater quantity than other alkaloids.

Calli generally grew slower than the corresponding root cultures (Fig. 1 B). *H. muticus* calli grew fastest, at a growth rate of about 8-fold in 4 weeks. Alkaloid contents

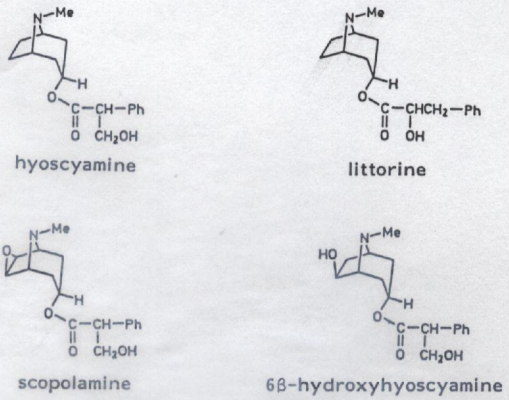

Fig. 2: Structures of tropane alkaloids identified in *Hyoscyamus* root cultures.

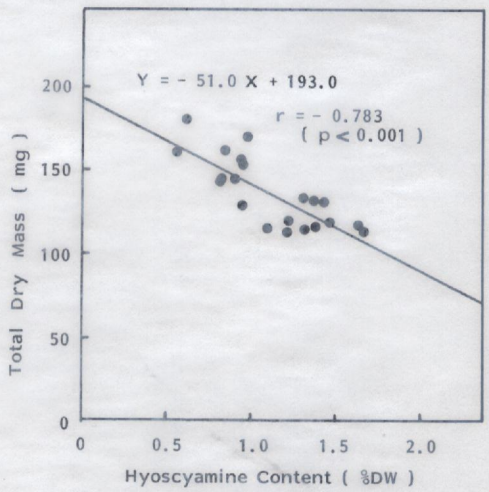

Fig. 3: Correlation between hyoscyamine content and growth in *H. albus* root cultures. Culture conditions were the same as in Fig. 1.

in all calli were much lower than those in root cultures. Hyoscyamine contents and scopolamine contents in calli were less than one-tenth and less than one-twentieth, respectively, of those in corresponding root cultures. Hyoscyamine was the main alkaloid produced in every callus, irrespective of whether the species was hyoscyamine-rich type or scopolamine-rich type.

Relationship between Alkaloid Content and Growth

Alkaloid content and final dry mass after specified periods of cultivation were measured in 33 root cultures and 54 calli of *H. niger*, and in 21 root cultures and 20

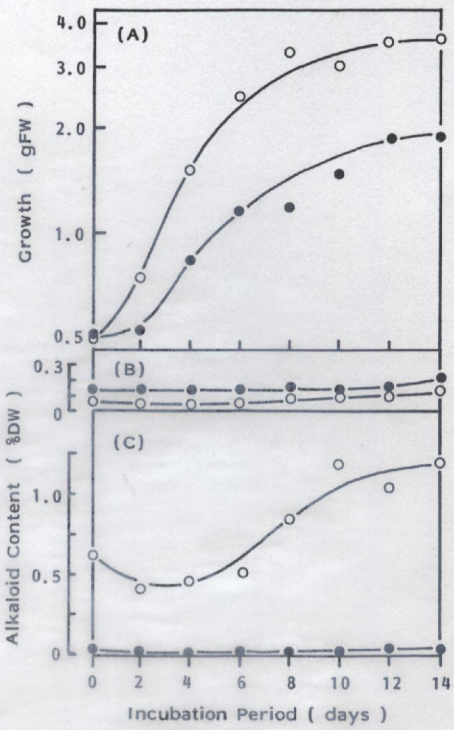

Fig. 4: Time courses of growth and alkaloid content in root cultures of *H. niger* and *H. albus*. Growth in fresh weight (A), scopolamine content (B) and hyoscyamine content (C); *H. albus* (○), *H. niger* (●).

calli of *H. albus*. Correlation between alkaloid content and final dry mass was calculated in each culture, but statistically significant correlation was obtained only in root cultures of *H. albus*. The correlation coefficient (r) between hyoscyamine content, the main alkaloid in *H. albus* root cultures, and root growth was -0.783 ($p < 0.001$) (Fig. 3). The negative correlation means that faster-growing roots contained less alkaloids.

Alkaloid Production in Root Cultures of H. niger and H. albus

Among root cultures of 7 *Hyoscyamus* species, *H. niger* (Hn), with high scopolamine/hyoscyamine ratio, and *H. albus* (Ha), with good growth and high hyoscyamine content, were chosen for further studies. The root cultures which were used in the following experiments had been subcultured for more than 6 months and, in the course of subculturing, Ha root cultures became faster growing and contained less alkaloids. Fig. 4 shows the time courses of growth and alkaloid content in Hn and Ha root cultures. Cultured Ha roots grew very fast (6-fold in about a week) and entered a stationary phase after 10 days in culture, whereas cultured Hn roots grew 3-fold in

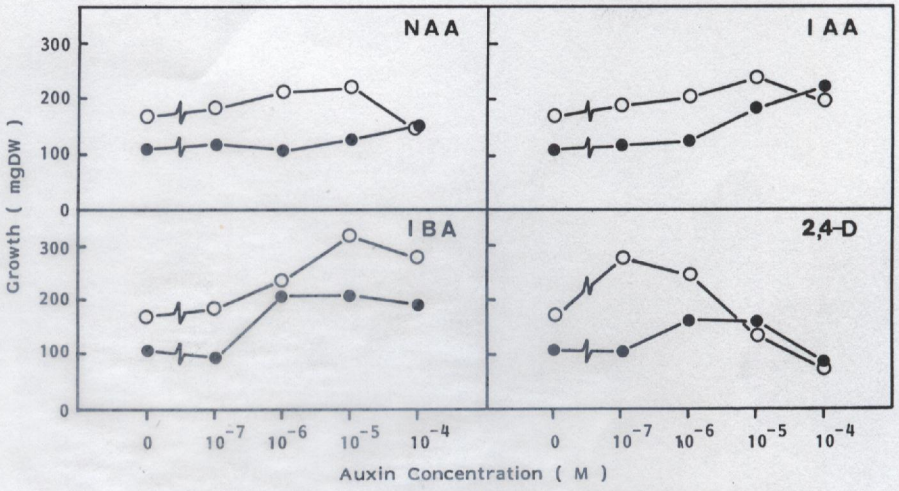

Fig. 5: Effect of auxin on growth in root cultures of two *Hyoscyamus* species. *H. albus* (○), *H. niger* (●). Cultured roots (0.5 g FW) were incubated in 25 ml of the medium with auxin for one week.

about 10 days. As already shown in Fig. 1, the main alkaloids were scopolamine (0.15–0.20% DW) in cultured Hn roots and hyoscyamine (0.5–1.2% DW) in cultured Ha roots. Fluctuations in alkaloid content at different growth phases were evident in hyoscyamine content in Ha; the alkaloid content was the lowest when roots were growing exponentially and increased substantially at an early stationary phase.

Effect of Auxin on Growth and Alkaloid Content

Four auxins (NAA, IAA, IBA and 2,4-D), each at concentrations from 10^{-7} M to 10^{-4} M, were tested for their growth promoting activities in Hn and Ha cultured roots (Fig. 5). Of the auxins tested, IBA was most effective for promoting growth of both cultures. At optimal concentrations, IBA increased the root dry weight twice as much as in cultures without auxin. At 10^{-6} M or 10^{-5} M IBA, cultured Hn roots grew 8-fold in one week and, at 10^{-5} M IBA, cultured Ha roots grew 9-fold in one week. NAA and IAA were less effective. 2,4-D promoted growth of Ha roots at low concentrations and inhibited growth of both cultures at 10^{-4} M.

When cultured with increasing concentrations of auxin, lateral roots were frequently induced and, at supra-optimal auxin concentrations, clusters of root primordia were formed (e.g. Fig. 6, where Hn roots were cultured with IAA). Under high concentrations of auxin, these root primordia did not grow any further. Elongation of main roots and the number of induced lateral roots or root primordia were measured after root segments, each with one root tip, were incubated with different concentrations of auxins (Fig. 7). Auxin inhibited elongation of cultured roots, but promoted induction of root primordia at optimal concentrations. For example, IBA

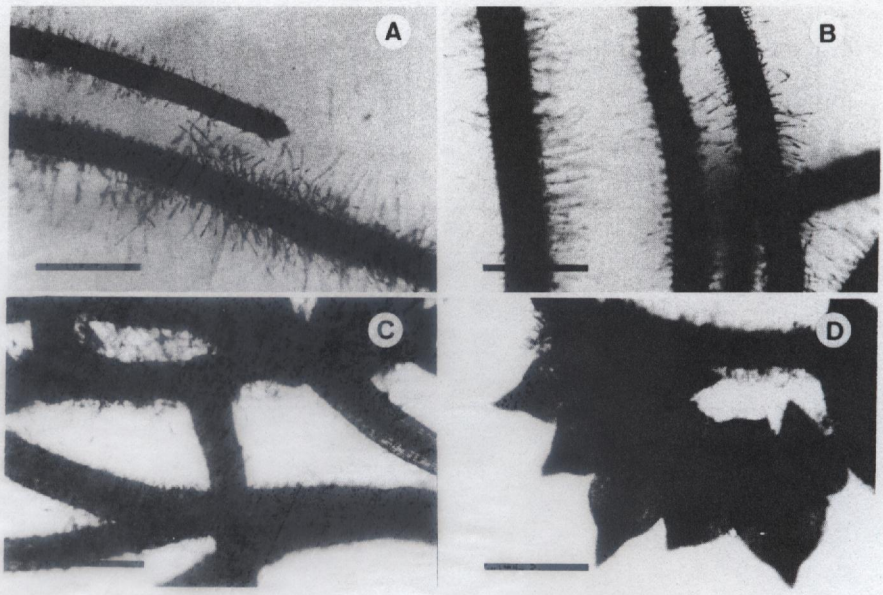

Fig. 6: *H. niger* roots cultured in the medium supplemented with IAA. IAA concentrations: (A) 10^{-7} M (B) 10^{-6} M (C) 10^{-5} M (D) 10^{-4} M. Culture conditions were the same as in Fig. 5. The bars represent 1.0 mm.

at 10^{-6} M increased the number of root primordia formed per each root segment from 4 to 23 and decreased elongation of main roots from 12 mm to 1 mm in Hn cultured roots. It is concluded that auxin promoted growth of root by inducing lateral roots and root primordia.

Fig. 8 shows the effects of auxin on alkaloid content in cultured roots. All 4 kinds of auxin inhibited formation of the alkaloids in cultured Ha roots. Higher concentrations of auxin generally decreased alkaloid content further, but at high 2,4-D concentrations of 10^{-5} M and 10^{-4} M, hyoscyamine content recovered to two-third of the content in auxin-free medium. In cultured Hn roots, auxin severely inhibited scopolamine formation but increased hyoscyamine content. At the auxin concentration of 10^{-4} M, hyoscyamine became the main alkaloid in the roots.

These interesting effects of auxin on the formation of hyoscyamine and scopolamine in Hn roots were further examined (Fig. 9). When root cultures that had been subcultured without auxin were first transferred to a medium containing 10^{-6} M IBA, the roots grew faster and the scopolamine content decreased during the period of good root growth (Fig. 9 A). Hyoscyamine content increased and remained higher than that of roots cultured without auxin throughout the culture period. However, root cultures which were subcultured in a medium containing 10^{-6} M IBA for more than 3 passages, and then transferred back to the auxin-free medium (Fig. 9 B), showed reduced growth rate. Scopolamine content increased and hyoscyamine content decreased soon after transfer. The higher scopolamine content and the lower

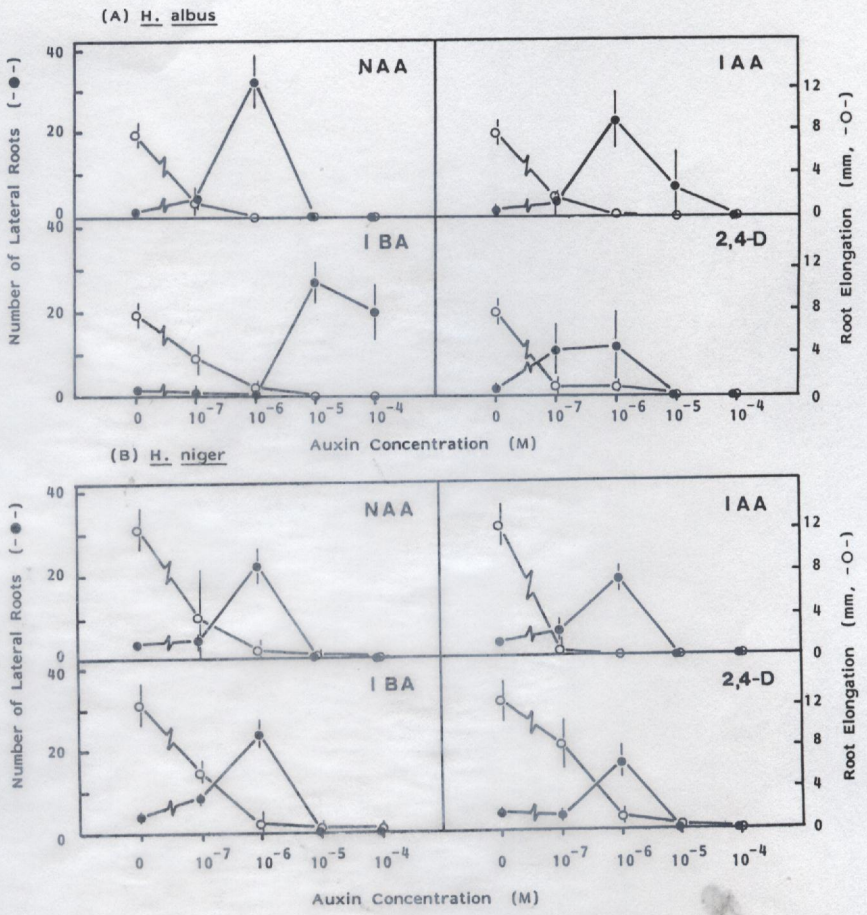

Fig. 7: Effect of auxin on root elongation and lateral root initiation in root cultures of two *Hyoscyamus* species. (A) *H. albus* (B) *H. niger*. -●-, number of lateral roots or root primordia. -O-, increase in root length of a primary root. Four root segments (1.0 cm in length), each with one root tip, were placed in a Petri dish (9 cm in diameter) containing 7 ml of LS medium, and incubated for 9 days with occasional shaking. Experiments were done in duplicates.

hyoscyamine content were maintained throughout the culture period. Since hyoscyamine is a precursor of scopolamine, we conclude that the biosynthetic pathway from hyoscyamine to scopolamine is inhibited by auxin more strongly than the pathway up to hyoscyamine in cultured Hn roots and that this inhibition is reversible.

The basic effect of auxin in cultured Hn and Ha roots was promotion of growth and inhibition of alkaloid formation. We, then, examined whether this effect could be extended to other alkaloid-producing root cultures (Table 1). Root cultures of *Datura leichhardtii, D. stramonium* var. inermis and var. stramonium, *D. innoxia, D. fa-*

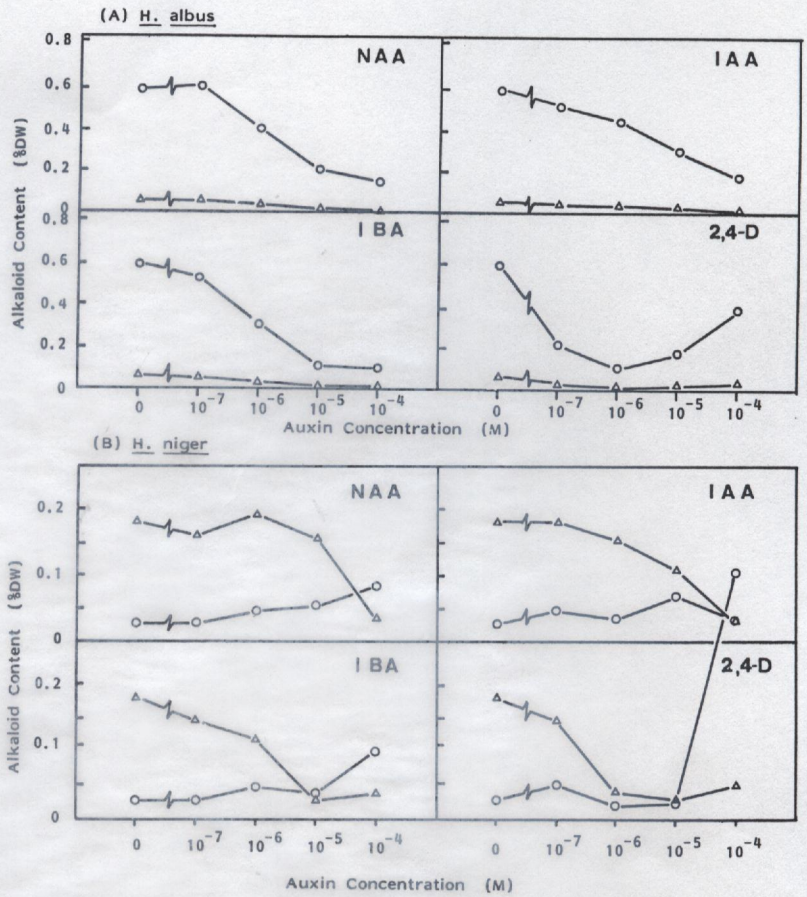

Fig. 8: Effect of auxin on alkaloid content in root cultures of two *Hyoscyamus* species. (A) *H. albus* (B) *H. niger*. –○–; hyoscyamine, –△–; scopolamine. Culture conditions were the same as in Fig. 5.

stuosa, *Atropa belladonna* and *Duboisia leichhardtii* were regularly subcultured in a medium containing 10^{-6} M or 10^{-5} M IBA, because they could not maintain growth in an auxin-free medium. Two lines of Hn root cultures were also maintained in a medium containing 10^{-6} M or 10^{-5} M IBA. When these root cultures were transferred into an auxin-free medium, growth rates generally decreased but alkaloid content increased in most of the cultures. Total alkaloid amounts produced in a flask also increased in about half of the cultures.

Effect of Other Growth Regulators on Growth and Alkaloid Content

Other growth regulators were also studied for their effect on growth and alkaloid content in Hn and Ha root cultures. The examined growth regulators were kinetin

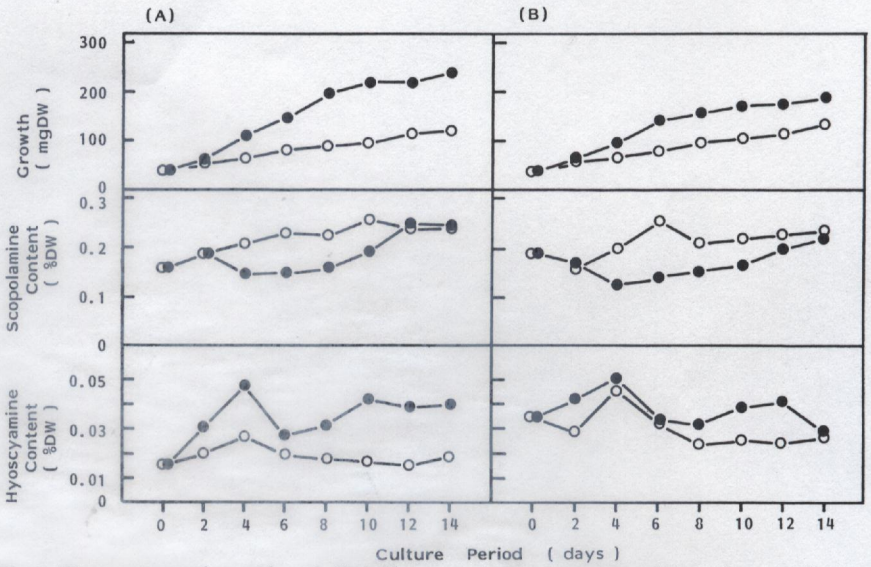

Fig. 9: Time courses of growth and alkaloid content in *H. niger* root cultures after transfer into medium with or without 10^{-6} M IBA. Roots, which had been subcultured in the auxin-free medium (A) or in the medium with 10^{-6} M IBA (B), were transferred into the medium with (–●–) or without (–○–) 10^{-6} M IBA.

and 6-benzyladenine as cytokinins, gibberellic acid (GA_3), abscisic acid, ethephone as an ethylene producer, sodium benzoate as an inhibitor of ethylene biosynthesis and *p*-chlorophenoxyisobutyric acid and 1-naphthoxyacetic acid as anti-auxins. These regulators were studied at concentrations from 10^{-7} M to 10^{-4} M. None of the chemicals gave significant and consistent stimulation or inhibition of growth and alkaloid content in either root culture.

Effect of the Basal Medium on Growth and Alkaloid Content

The basal media studied were Linsmaier-Skoog's medium, Gamborg's B5 medium (Gamborg et al., 1968), White's medium (White, 1963) and Knop's medium (Knop, 1965) (Fig. 10). Both root cultures grew best in B5 medium and had higher alkaloid content in White's or Knop's medium, which were not favorable for growth. Alkaloid production per flask was similar in 4 basal media for cultured Ha roots, whereas higher alkaloid production was achieved in B5 and White's medium for cultured Hn roots.

Discussion

Alkaloid-producing solanaceous plants accumulate alkaloids in almost every part of the plant, but the main site of alkaloid biosynthesis is believed to be roots. This is based mostly on the results of reciprocal graft hydridization between species of different genera (Waller and Nowacki, 1978). Only a few root cultures have been re-

Table 1: Effect of IBA on growth and Alkaloid Synthesis in Various Root Cultures.

Species	Line	Basal Medium	IBA conc. (M)	Growth Index	Alkaloid Content (% DW)		Alkaloid Amount (μg/flask)	
					hyoscyamine	scopolamine	hyoscyamine	scopolamine
Hyoscyamus niger	Hn 11	LS	10^{-6}	5.0	0.024	0.113	40	190
			0	2.0	0.019	0.212	16	178
	Hn 17	B5	10^{-5}	6.6	0.019	0.009	38	18
			0	5.4	0.030	0.048	47	75
Atropa belladonna		LS	10^{-6}	3.8	0.128	0.014	172	19
			0	2.1	0.270	0.016	221	13
		B5	10^{-5}	2.9	0.040	0.003	42	3
			0	3.0	0.134	0.004	142	4
Duboisia leichhardtii★	K-4B	LS	10^{-5}	8.6	0.081	0.046	234	134
			0	1.9	0.312	0.274	287	252
		B5	10^{-5}	4.0	0.125	0.073	205	105
			0	1.6	0.397	0.555	235	173
	1753-4	LS	10^{-5}	1.8	0.110	0.040	151	55
			0	1.1	0.164	0.064	146	57
	S 3232-2	B5	10^{-5}	3.5	0.143	0.207	273	358
			0	2.0	0.200	0.354	205	260
Datura leichhardtii★		LS	10^{-6}	2.7	0.188	0	259	0
			0	2.3	0.184	0	201	0
D. stramonium var. inermis★		LS	10^{-6}	1.3	0.109	0	94	0
			0	2.0	0.201	0	207	0
D. stramonium var. stramonium★		LS	10^{-6}	1.4	0.257	0	260	0
			0	1.8	0.423	0	445	0
D. innoxia★		LS	10^{-6}	2.7	0.232	0.041	258	46
			0	1.3	0.382	0.051	248	33
D. fastuosa		LS	10^{-6}	5.0	0.123	0.107	208	181
			0	2.6	0.108	0.101	112	104

Roots of 0.5 g FW were cultured in the basal medium with or without IBA for 1 week or 2 weeks (indicated with ★). LS: Linsmaier-Skoog; B5: Gamborg. Growth Index: $\frac{\text{Harvest FW}}{\text{Inoculum FW}}$

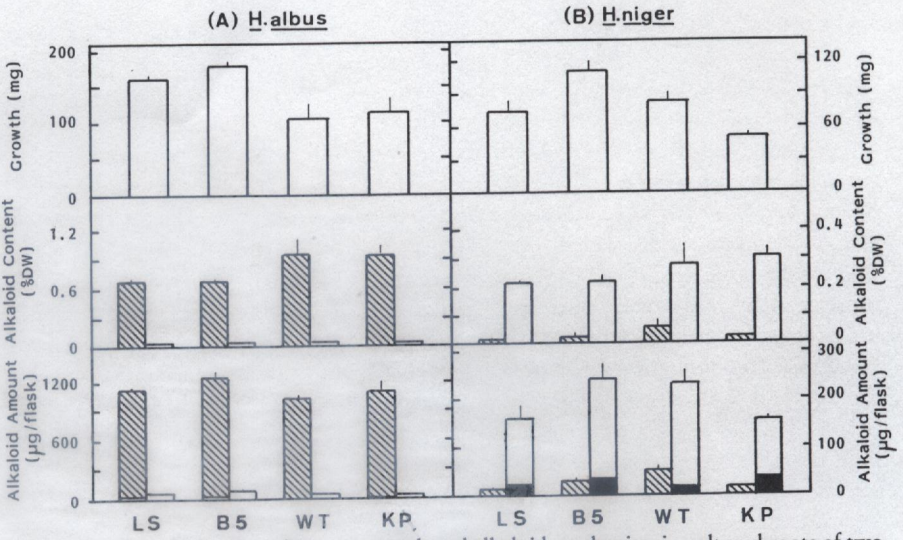

Fig. 10: Effect of the basal medium on growth and alkaloid production in cultured roots of two *Hyoscyamus* species. (A) *H. albus* (B) *H. niger* ▨ hyoscyamine, □ scopolamine; both in the cells. ■ alkaloids in the medium, LS; Linsmaier-Skoog's medium, B5; Gamborg's B5 medium, WT; White's medium, KP; Knop's medium.

ported to grow well *in vitro* and produce alkaloids comparable to the levels of whole plants; nicotine in *Nicotiana tabacum* (Solt, 1957), nicotine and anabasine in *N. glauca* (Solt et al., 1960) and atropine in *Atropa belladonna* (Mitra, 1972). When excised and cultured without auxin, *Hyoscyamus* roots grew well and produced considerable amounts of alkaloids (Fig. 1). In these cultures, alkaloid content was often higher than, and their compositions were sometimes different from, those reported for the leaves (e.g. Oswald and Flück; 1964, Pelt et al., 1967 and Ghani et al., 1972) and the mature roots (see Hashimoto and Yamada, 1983). The quantitative and qualitative changes of alkaloids in *Hyoscyamus* cultured roots might be due to the absence of transportation of alkaloids to aerial parts and the difference in developmental stages between cultured roots and intact mature tissues. It is interesting that the scopolamine content in cultured roots of *H. gyorffi*, an allopolyploid between *H. niger* and *H. albus*, exceeded that in both parents. High conversion capacity from hyoscyamine to scopolamine in *H. niger* and high capacity of hyoscyamine biosynthesis in *H. albus* may be combined into this allopolyploid. A similar result has been reported for scopolamine content in a hybrid between *Dutura ferox* and *D. stramonium* (Romeike, 1961).

Tropane alkaloids were not produced in large amounts in *Hyoscyamus* calli (Fig. 1), as reported also in calli of other alkaloid-containing species (see Yamada and Hashimoto, 1982; Kurz and Constabel, 1985). The low scopolamine content in these calli supports our previous conclusion, in an hyoscyamine-rich *H. niger* cell line, that root organization is necessary for efficient expression of the capacity to epoxidize hyoscyamine (Hashimoto and Yamada, 1983).

Many reports suggest that, in undifferentiated cells, an inverse relationship between primary metabolism, such as growth, and production of secondary metabolites exists (Böhm, 1977 and Lindsey and Yeoman, 1983). In general, fast-growing cultures, or cells at the growth phase of active cell division, accumulate only low levels of secondary products. On the other hand, in differentiated or organized cultures, such as root cultures, both an inverse and a proportional relationship between primary and secondary metabolism was reported. Nicotine production in tobacco root cultures was proportional to root dry weight, whereas anabasine production accelerated as the culture period prolonged (Solt, 1957 and Solt et al., 1960). It was thus proposed that nicotine production depends on some activity in the root tip, but anabasine production is a property of mature root tissues. During active growth, from 2 to 6 days after inoculation, Ha roots produced only low levels of hyoscyamine and, after growth of the roots slowed down, hyoscyamine content increased to more than 1% DW (Fig. 4). Analysis of Ha root cultures after 8 days of cultivation showed a relatively high negative correlation ($r = -0.783$, $p < 0.001$) between hyoscyamine content and total root dry mass (Fig. 3). This inverse relationship is also indicated by the fact that, after 8 months of subculturing Ha root cultures, the rate of growth accelerated from 3-fold to 6-fold in 8 days, while the hyoscyamine content decreased from 1.0–1.2.% DW to 0.6% DW. In Hn root cultures, however, no such inverse relationship has been observed for more than 2 years.

Effects of auxin on root growth seemed much weaker and ranged in wider concentrations in Fig. 5 than in Fig. 7; many lateral roots and root primordia were observed over a wide range of auxin concentrations (from 10^{-6} M to 10^{-4} M) in the experiment of Fig. 5, whereas root primordia were generally induced only at a narrow range of auxin concentrations (mainly at 10^{-6} M) in Fig. 7. This is probably because of the difference in the ratio of root tissues to the volume of the culture medium between two experiments. In the former experiment, 500 mg FW of roots were cultured in 25 ml of the medium, but in the latter experiment less than 4 mg FW of roots were inoculated in 7 ml of the medium.

Among several growth regulators tested, only auxin affected growth and alkaloid biosynthesis of root cultures. The basic effects of auxin were increase of root dry weight by inducing more lateral roots and inhibition of alkaloid biosynthesis, especially the conversion of hyoscyamine to scopolamine. An extractable enzyme activity which converts hyoscyamine to 6β-hydroxyhyoscyamine was also suppressed in roots cultured with IBA (Hashimoto and Yamada, in preparation). Promotion of active cell division and, possibly, inhibition of cell elongation by auxin (Burström, 1942) are correlated with a decreased level of alkaloids.

Reversible inhibition of tropane alkaloid biosynthesis by higher concentrations of auxin seems to be a general phenomenon in solanaceous root cultures (Table 1). These results suggest that a two-step culture of alkaloid-rich roots should be useful for an efficient production or biotransformation of tropane alkaloids. In a two-step culture, roots are propagated at an auxin concentration optimal for fast growth, then transferred into a production medium where auxin is omitted to induce higher bio-

synthetic capacity. These «second-stage» cultures should be useful for studies on the enzymology and pathways of secondary metabolite synthesis.

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