Production of Polyketide Pigments in Hairy Root Cultures of *Cassia* Plants

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Hairy root cultures of three Cassia plants, *Cassia torosa, C. occidentalis* and *C. obtusifolia* were induced by infection with *Agrobacterium rhizogenes* and were established to investigate their production and biosynthesis of phenolic pigments of polyketide origin. The hairy root cultures produced pigments similar to those of the mother plants; however, the contents of each pigment was varied by growth conditions. Incorporation experiments using stable isotopes in *C. torosa* hairy root cultures revealed the high biosynthetic activity of germichrysone.

Key words Leguminosae; *Cassia torosa*; hairy root culture; *Agrobacterium rhizogenes*; *C. obtusifolia*; *C. occidentalis*

Since Flores and Filner reported the potentiality of hairy root cultures of *Solanaceous* plants for the production of alkaloids which had been difficult to produce by cell or tissue cultures,[1] extensive investigations on the production of secondary metabolites by hairy root cultures which had been transformed by *Agrobacterium rhizogenes* were carried out in a wide variety of plants.[2] We initiated the studies on hairy root cultures to clarify whether this method is applicable for the production of plant constituents other than alkaloids, and preliminary results on the production of pigment and oligoside by hairy root cultures were reported briefly in our previous communications.[3]

The seeds of *Cassia torosa, C. occidentalis* and *C. obtusifolia* (Leguminosae) have been known to be mild purgative drugs in traditional oriental medicines, and phenolic pigments such as anthraquinones, xanthones and naphthopyrones of polyketides origin have been known to be their constituents. Extensive studies on the pigment contents in various parts of these plants by Kitanaka *et al.* made it feasible to compare the pigment composition among different types of cultures.[4]

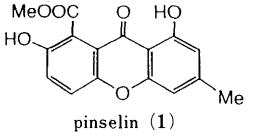
Hairy root cultures of *Cassia* plants that produce constituents of the original plants with high biosynthetic activities were expected to be a good alternative for untransformed dedifferentiated cell cultures in biosynthetic investigations of the plant constituents.[3,5] This paper describes in detail the investigation of pigment production of hairy root cultures of *Cassia* plants, and the results of feeding experiments with ^{13}C labelled acetate to clarify the time course productivity of the pigments.

Materials and Methods

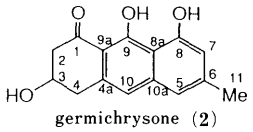
NMR spectra were measured on JEOL FX-100, GX-400 and GSX-500 spectrometers. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS 0.0) as an internal standard. MS was done on a JEOL JMS-DX 300, IR on a JASCO model 701G and UV on a Hitachi model 100-60.

Plant Materials The seeds of *Cassia torosa, C. obtusifolia* and *C. occidentalis* were obtained from the medicinal plant garden of Toho University. The seeds were washed with a detergent solution (DIF, Parker Co.) by stirring for 20 min and then rinsed with tap water. They were rinsed with sterile distilled water after being soaked in 70% aqueous EtOH for 3 min under reduced pressure, further sterilized with 1 % NaOCl (with a few drops of Tween 20, Wako Pure Chem. Ind.) for 20 min under reduced pressure, and then rinsed repeatedly with sterile distilled water. The sterilized seeds were then placed on solid Murashige-Skoog's '62 (MS; Sigma Chemical Co.) medium[6] (35 ml) in culture boxes (4 × 4 × 13 cm). Germinated seedlings were then kept under a diurnal (12 h light and 12 h dark) condition at 25 °C. For the quantitative determination of pigments of plantlets, the seedlings were cultivated for two months under the same condition. MS medium containing 3% sucrose, solidified with 0.2% of Gel rite (San-ei Co.), was used for the solid cultures and the medium without Gelrite was used for the liquid cultures throughout the experiments, unless noted.

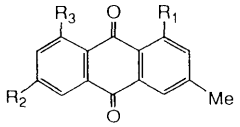
Induction and Culture of Hairy Roots The bacterial strains used throughout this study were *Agrobacterium rhizogenes* 15834 and A4, which were grown on YEB-medium.[7] Four or five days' cultures of the above strains were used for the infection experiments. Twelve- to twenty-day old aseptically grown young plantlets were directly infected with the bacteria using a hooked needle on the stems of the seedlings. Bacterial mass was smeared onto the wounded sites of the plantlets by the hooked needle. When the infection was successful, outgrowths of fine white hairy roots were observed after a week, and the roots reached up to 1 to 2 cm in length within 2 weeks. Segments of the hairy roots were excised and cultured on the solid MS medium containing Claforan (Hoechst Japan; 300 mg/ml). Thus obtained hairy root cultures were free of bacterium, and were successively subcultured on the same medium (23 ml) without antibiotic in Petri-dishes (90 × 20 mm) at 25 °C under the dark for one month. To establish a liquid culture, some segments of the hairy roots grown on Petri-dishes were transferred into the MS medium (100 ml) in Erlenmeyer flasks (500 ml), and were cultivated at 25 °C with 100 rpm in the dark. The growing hairy roots in liquid medium were subcultured every 4 to 6 weeks. Gall-like tissue sometimes appeared along with hairy root culture on the solid medium, and was found to



pinselin (1)



germichrysone (2)



chrysophanol (3) : R_1, R_3=OH; R_2=H
emodin (4) : R_1, R_2, R_3=OH
physcion (5) : R_1, R_3 =OH; R_2=OMe
8-*O*-methylchrysophanol (6) : R_1=OH; R_2=H; R_3=OMe
1,8-di-*O*-methylchrysophanol (7) : R_1, R_3=OMe; R_2=H

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be maintainable without adding any plant growth regulators when it was excised from the hairy roots.

Detection of Opines Hairy roots (about 100 mg fresh weight) were ground with a glass rod in a test tube containing distilled water, then centrifuged at 3000 rpm for 5 min. Twenty to 50 μl of the supernatant of crashed tissue were spotted on a paper (Whatman 3MM) together with samples of mannopine and agropine synthesized by the method of Tate *et al.*[8] and was electrophorased in a buffer of formic acid–acetic acid–H_2O (5:15:80 by vol., pH 1.8) at a constant voltage of 400 V. Opines were visualized by alkaline silver nitrate reagents after drying the paper.

Identification of Pigments Harvested hairy roots were extracted separately with 80% acetone to identify pigments, and the extracts were partitioned with AcOEt and H_2O. The AcOEt layer was evaporated under reduced pressure and the residue was applied to silica gel column chromatography using benzene–AcOEt as a solvent. Pinselin (**1**), germichrysone (**2**), chrysophanol (**3**), emodin (**4**), physcion (**5**)[9] and 8-*O*-methylchrysophanol (**6**) were identified by the direct comparison with the authentic samples. An unknown pigment isolated from the *C. obtusifolia* cultures was identified as 1,8-di-*O*-methylchrysophanol (**7**) by direct comparison with a synthetic sample. The synthetic **7** was prepared in a yield of 65% by the methylation of chrysophanol using dimethyl sulfate and K_2CO_3 at 75 °C for 12 h. mp 197—199 °C (from benzene). MS *m/z*: 282. UV λ_{max}^{MeOH} nm: 257, 390. IR ν_{max}^{KBr} cm^{-1}: 1655, 1590. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.48 (3H, s, 3-C$\underline{H}_3$), 3.99, 4.00 (2×3H, 2×s, 1-OC$\underline{H}_3$), 7.10 (1H, br s, 2-$\underline{H}$), 7.29 (1H, d, J=8 Hz, 7-$\underline{H}$), 7.62 (1H, t, J=8 Hz, 6-$\underline{H}$), 7.65 (1H, d, J=1 Hz, 4-$\underline{H}$), 7.83 (1H, dd, J=8, 1.1 Hz, 5-$\underline{H}$).

Quantitative Determination of Pigments Hairy roots (200 mg each) grown on Petri-dishes were harvested after a month of culture and extracted twice with AcOEt at 60 °C for 2 h. The extracts were concentrated and diluted with MeOH to an appropriate volume to quantify the pigments with HPLC (Tosho HLC 803C/ CCPM, Tosoh Co.) using a UV detector (UV 8000, Tosoh) at 254 nm. An appropriate volume of the sample solution in MeOH was injected into a column (TSK gel LS 410K, Tosoh Co.) (4.6×300 mm) and eluted with MeOH–H_2O–AcOH (95:5:0.5) with a flow rate of 0.7 ml/min. **1**, **2**, **3**, **4**, **5**, **6** and **7** gave peaks at retention times of 6.0, 6.8, 14.0, 22.3, 11.5, 12.30 and 8.0 min, respectively. Standard calibration curves were made from solutions of a known concentration of authentic samples. The values shown in Tables II and III are the average of experiments with three to five cultures.

Feeding Experiments Feeding experiments were carried out on the liquid cultures of *C. torosa* hairy roots. (1-^{13}C) Sodium acetate (total 100 mg) was fed daily to the 2 flasks of the 32-d-old hairy root culture in the liquid MS medium (100 ml in 500 ml flask) for 8 d. When the pulse administration of the isotope was started, the fresh MS medium (25 ml each) was added to the flasks to maintain a good nutritional condition. Hairy roots (21.3 g) were harvested and extracted with AcOEt, and the AcOEt extract was worked up as usual to yield germichrysone (**2**, 9.6 mg), which was submitted for ^{13}C-NMR measurement at 100.6 MHz in d_6-acetone. The average value of incorporation ratios based on peak height enhancement was 9.4%, when the peak height of C-4 was used to normalize both labeled and natural abundance spectra. (1,2-$^{13}C_2$) Sodium acetate was fed to a flask of 37-d-old *C. torosa* hairy root culture. A total of 150 mg (1,2-$^{13}C_2$) sodium acetate was fed daily for 7 d. The hairy roots (2.25 g) were harvested on the 47th day, and were worked up as usual to yield **2** (*ca.* 1 mg), then submitted for ^{13}C-NMR at 125.65 MHz in d_6-acetone. The signals were assigned by using ^{13}C–^{13}C coupling constants and were tabulated in Table I. The specific incorporation ratio of the acetate into **2** was 5.9—8.5% per one carbon estimated from the peak heights or area integrals of ^{13}C-NMR signals as described in the Table I.

Time Course Incorporation Experiments with ^{13}C Sodium Acetate Two flasks of the 25-d-old hairy root culture were fed with (1,2-$^{13}C_2$) sodium acetate (90 mg) and incubated for 4 h, then the culture was washed with fresh medium and transferred to the fresh medium. The harvest at the change of the medium was called 4 HR, and the harvest 6 h after the change of the medium was called 10 HR and so on. Each harvest (1.1—3.3 mg) was extracted with AcOEt for 40 min at 50 °C and then the extracts were worked up to measure ^{13}C-NMR. The time course change of the incorporation ratios of acetate into germichrysone (**2**) was estimated by the satellite signal intensity of C-11 of **2**, and was described in Fig. 2.

TABLE I. The ^{13}C-NMR of Germichrysone Labeled with (1,2-$^{13}C_2$) Sodium Acetate on the Incorporation Experiment in the Hairy Root Culture of *Cassia torosa*

	δ (d_6-Acetone)	J_{C-C} (Hz)[a]	J_{C-C} (Hz)[b]	Incorporation ratio of sodium acetate (%)
C1	204.25	41 (C1–C2)	54 (C1–C9a)	6.8[c]
C2	46.90	40 (C1–C2)	38 (C2–C3)	7.8[c]
C3	66.20	37 (C3–C4)	37 (C3–C2)	7.1[c]
C4	38.57	37 (C3–C4)	41 (C4–C4a)	8.5[d]
C4a	136.68	64[e]		
C5	118.68	55 (C5–C10a)	57 (C5–C6)	
C6	144.25	43[e]		
C7	112.75		55 (C7–C6), 65 (C7–C8)	
C8	158.58	64 (C8–C8a)	65 (C8–C7)	6.9[d]
C8a	111.42	65 (C8–C8a)	63 (C9–C8a), 59 (C10a–C8a)	
C9	163.53	66 (C9–C9a)	61 (C9–C8a)	6.3[c]
C9a	110.67	65[c]		
C10	118.42	62 (C10–C4a)	55 (C10–C10a)	7.9[d]
C10a	140.70	quintet avr. J_{C-C}=59[e]		
C11	22.0	42		5.9[d]

a) Couplings by intact acetate units. *b*) Couplings by multiple incorporated acetate units. *c*) Value calculated using a NMRI program on the VAX 3200/VMS system. *d*) Value calculated based on the peak height output of the JEOL instrument. *e*) Signal complexities restrained interpretation.

Results and Discussion

Hairy Root Cultures The hairy root cultures of *Cassia torosa*, *C. obtusifolia*, and *C. occidentalis* were established as described in Materials and Methods. Some of the roots grew without showing positive geotropism. All hairy roots that were induced from the stems of *C. torosa*, *C. obtusifolia*, and *C. occidentalis* showed positive agropine and mannopine production. The hairy roots of *C. torosa* and *C. occidentalis* were composed of black axes and bright yellow tips 1—2 cm long with a black root cap on the top as in normal roots. The hairy roots of *C. obtusifolia* were a pale brown colour with the same thickness as that of *C. torosa* and *C. occidentalis*.

Gall-like tissues that appeared on the hairy root cultures were transferred on the solid MS medium and the transformed callus cultures were established to compare pigment composition with the hairy root cultures.

Difference in Pigment Formation among the Cultures of Untransformed Roots, Hairy Roots and Transformed Callus of *C. obtusifolia* The hairy root cultures of *C. obtusifolia* contained five anthraquinones, chrysophanol (**3**), emodin (**4**), physcion (**5**), 8-*O*-methylchrysophanol (**6**) and 1,8-di-*O*-methylchrysophanol (**7**) as shown in Table II. The variety in pigment composition of these cultures was reproducible; however, the content of some pigments was lower than the detectable range. Among those pigments, **7** was found only in these cultures and has not been reported as to plant origin. All the transformants contained **7** as a major constituent except for the HR(A4) culture, in contrast to the reported composition of the pigments in the roots of field-grown plants.[10] Secondary metabolites in mother plants sometimes differ from those of the corresponding cell cultures. A report of *C. obtusifolia* callus cultures would be a good example.[11] Transformed *C. obtusifolia* callus cultures contained the same pigments,

Table II. Contents of Pigment (μg/g fr. wt.) in Various Hairy Root Cultures and Plant of *Cassia obtusifolia*

	Chrysophanol (3)	Emodin (4)	Physcion (5)	8-*O*-Methyl chrysophanol (6)	1,8-Di-*O*-methyl chrysophanol (7)
HR(15834)[a]	14	19	Trace[d]	Trace[d]	69
HR(A4)[a]	24	22	Trace[d]	Trace[d]	14
HR callus[b]	4	8	20	2	25
Plant roots[c]	15	21	12	21	23
Plant stem & leaf[c]	Trace[d]	6	Trace[d]	Trace[d]	14

a) HR(15834): hairy root culture transformed by *Agrobacterium rhizogenes* ATCC15834, HR(A4): hairy root culture transformed by *A. rhizogenes* A4. *b*) Callus formed from hairy root induced by *A. rhizogenes* 15834. Pigment determined after 60 d culture. *c*) Roots and aerial part of plantlets grown for 1 month under a 12 h light: 12 h dark at 25 °C. *d*) <2(μg/g fr. wt.), noted as trace.

Table III. Effect of Medium on the Content of Pinselin (**1**) and Germichrysone (**2**) (μg/g fr. wt.) in the Hairy Root Cultures of *C. occidentalis*[a]

Strain	Medium[b]	Sucrose[c]	Pinselin (1)	Germichrysone (2)
HR(15834)	NN	10	39	437
HR(A4)	NN	10	96	450
HR(15834)	MS	10	35	348
HR(A4)	MS	10	54	259
HR(15834)	NN	30	111	1275
HR(A4)	NN	30	145	1250
HR(15834)	MS	30	66	742
HR(A4)	MS	30	62	666

a) Pigment contents were measured after 1 month culture on the solid media under the dark condition. *b*) NN; Nitsch-Nitsch '56, MS; Murashige-Skoog '62. *c*) Sucrose concentration (g/l).

but in a different proportion, and **7** was a main pigment in the culture. The *in vitro* culture of *C. obtusifolia* plantlets on the MS medium proved that the composition of pigments of the untransformed roots was similar to the hairy root and transformed callus culture (Table II).

Effects of Medium Composition on Pigment Formation in *C. occidentalis* Hairy Root Cultures Pinselin (**1**) was reported to be the main pigment in the intact roots or callus cultures of *C. occidentalis*,[12] whereas germichrysone (**2**) was found in a much higher amount than **1** in the hairy root cultures using the solid MS medium (Table III). The effect of nutrimental elements on pigment formation was studied on the *C. occidentalis* culture, since the pigment pattern of the *C. occidentalis* hairy root culture was simpler and reproducible at least on the solid MS medium. We studied the effects of medium composition on the pigment formation in *C. occidentalis* hairy root culture. Nitsch and Nitsch '56 (NN) medium[13] was used instead of the MS medium to examine the effect of nitrate concentration on pigment formation; however, no drastic changes were found in the pattern of pigment formation between these two media, except for a higher content of pigment in the NN medium (Table III). This may reflect a general tendency that the reduced concentration of a nitrogen source induces an increase in anthraquinonoid pigment formation.[14] Two concentrations of sucrose (1% and 3% w/v) were then tested to examine their effect on pigment production. The contents of **1** and **2** decreased to 0.55—0.68 fold at a lower concentration of sucrose (1%)

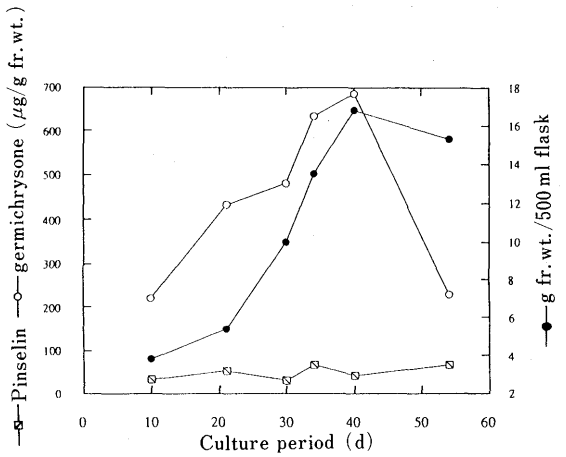

Fig. 1. Germichrysone (—○—) and Pinselin (—▨—) Content in the Hairy Root Culture (—●—) of *C. torosa* in the Liquid Medium

in both media without alteration of the pigment ratios. The results in *C. occidentalis* were well in accord with other reports on the positive effect of sucrose concentration on the production of secondary metabolites in plant cell cultures.[15]

Time Course Pigment Production in Hairy Root Cultures of *C. torosa* The two characteristic pigments of *C. torosa* hairy root cultures were pinselin (**1**) and germichrysone (**2**). A xanthone derivative, **1**,[16] was the main constituent in the field grown plant roots of *C. torosa*, while **2**, a tetrahydroanthracene derivative, was the main constituent in the seedlings and untransformed cell cultures of this plant.[17] The content of **1** varied from 443 to 1345 μg per g fresh weight, and the content of **2** varied from trace to 853 μg per g fresh weight in the hairy root cultures on the solid MS medium. This may be caused by the aging of the cultures, and ordinary **1** content increased higher in older cultures. The established liquid culture of *C. torosa* hairy roots appearing in Fig. 1 presented a different results. The culture showed a fairly long lag phase of 20 d, and then seemed to enter a stationary phase after about 20 d of an active growth period. **2** was the main pigment, with 161 to 687 mg per g fresh weight in the liquid cultures throughout the culture period in the time course investigation of pigment contents in Fig. 1, where the ratios of **1** *vs.* **2** were 0.06—0.1-fold.

In the liquid medium, the amount of **2** was associated with growth, and was somewhat less than that of untransformed cell cultures with plant growth regulators.[18] This forms a contrast to the observation in the liquid hairy root cultures of *C. obtusifolia*, in which the contents of anthraquinones varied by aging.[19] In the roots of the aseptically cultivated plantlets of *C. torosa*, **1** was again the main pigment (669 μg per g fresh weight), while the content of **2** was 107 μg per g fresh weight. Except for chrysophanol (**3**) and emodin (**4**), other constituents in the roots reported so far were not observed in detectable amounts in the hairy roots grown on the MS medium.[23] No significant difference in the pattern of the pigment content was observed between the hairy roots derived by the infection of *A. rhizogenes* 15834 or A4.

Incorporation Experiments The high yield of pigments

in the hairy root cultures of *C. torosa* prompted us to examine their biosynthetic activity. The incorporation experiments were conducted with the hairy root culture of *C. torosa* in the MS liquid medium because of its feasibility for feeding. A preliminary experiment using (1-^{13}C) sodium acetate gave high incorporation ratios, averaging 9.4% per site of germichrysone (**2**). ^{13}C–^{13}C doubly labelled acetate was introduced to investigate the fate of intact C–C bonds during the biosynthetic process and for the complete assignment of signals.[24] Another advantage to using the doubly labelled acetate is to determine the accurate enrichment of the appropriate sites in a target molecule by calculating the intensities of the corresponding satellite signals to natural abundance one. Then, the feeding experiment with doubly labelled (1,2-$^{13}C_2$) sodium acetate showed an incorporation ratio that ranged from 5.9 to 8.5% by calibrating the intensities of natural abundance with the satellites resonances (Table I). The multiple coupling shown in Table I could be explained by the labelled acetate being directly incorporated into **2** without extensive dilution.[25] The stage of the hairy root cultures used in these feeding experiments were the late log or stationary phase. (Fig. 1) This observation reveals that the *de novo* synthesis of **2** was still very active in the hairy roots at these stages. However, the cell culture of *C. torosa* that induced **2** biosynthesis by physical stress as it was transferred into fresh medium, or "inoculation", showed a high incorporation ratio only at that stage, and it then showed low activity after the late-log to stationary phase.[18]

Next, the hairy root culture in the liquid medium was exposed to (1,2-$^{13}C_2$) sodium acetate for 4 h to examine germichrysone (**2**) biosynthesis by observing the time course changes of the specific incorporation ratio of acetate into **2**. The incorporation ratio reached a maximum (4.2%) at about 12 h after incubation and then gradually decreased to 2% levels in 24 h and reained for 2—4 d (Fig. 2). The maximum incorporation ratio at 12 h suggests that the biosynthesis of **2** was induced by manipulating feeding and changing the medium, as was observed at the time of inoculation in undifferentiated tissue culture,[19] since multiple incorporation of acetate into **2**, as shown in Table I, suggests that a considerable proportion of added sodium acetate was immediately utilized without being pooled as a metabolized form. The multiple incorporation of acetate into **2** was also seen after the 12 h harvest as well. On the other hand, changes in the incorporation ratio could be interpreted in this way: the biosynthetic activity of using labelled acetate reached a steady level after the induction of biosynthesis triggered by manipulatiing feeding and changing the medium.[20]

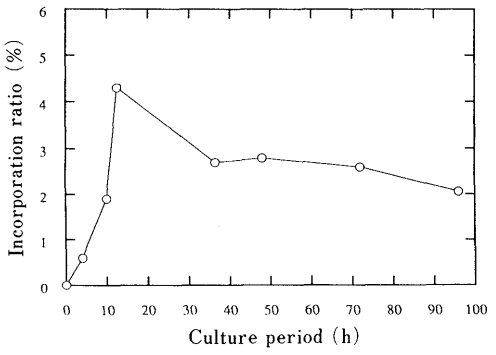

Fig. 2. Changes of Incorporation Ratio of (1,2-$^{13}C_2$) Sodium Acetate into Germichrysone in the Hairy Root Culture of *C. torosa*

Comparative studies of pigment formation in mother plants, corresponding cell cultures and transformed cultures, clearly demonstrated that the secondary metabolism of *Cassia* species was not altered by the transformation process and produced the almost the same kinds of secondary metabolites as their mother plants under the same growth conditions, though the ratio of content varied by cultures.

Conclusion

This high uptake rate of the precursors shown in the hairy root culture of *C. torosa* proved its potential as a system for studying the biosynthesis of secondary metabolites in a higher plant, which has previously been hampered by its low incorporation of precursors in intact plants.[21] The induction method that stimulates the biosynthesis of secondary metabolites with an elicitor often implies the cessation of cell growth, but the biosynthesis of germichrysone (**2**) in both of the above cultures is triggered by fresh media and followed by active growth of the cells to produce a sufficient amount of secondary metabolites. The root culture, which is a much more complicated system than a cell culture, keeps its high biosynthetic activity through a broader range of the culture period. Recently, the hairy root cultures of *Datura* and *Nicotiana* were used for *in vivo* NMR studies of the secondary metabolism of nitrogen.[22] This may indicate the potential for the use of a variety of root cultures.

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