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ALKALOID PRODUCTION IN CULTURED ROOTS OF THREE SPECIES OF *DUBOISIA*

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Key Word Index—*Duboisia leichhardtii*; *D. myoporoides*; *D. hopwoodii*; Solanaceae; regeneration; root culture; hyoscyamine; scopolamine; nicotine; nornicotine.

Abstract—Cultured roots were obtained from calluses of *Duboisia leichhardtii*, *D. myoporoides* and *D. hopwoodii*. Cultured roots of all these species produced both tropane and pyridine-type alkaloids. The selected cultured roots of *D. leichhardtii* showed high contents of tropane alkaloids (hyoscyamine 0.53%, scopolamine 1.16%, on a dry weight basis).

INTRODUCTION

The genus *Duboisia* consists of three species, *D. leichhardtii* F. Muell, *D. myoporoides* R. Br. and *D. hopwoodii* F. Muell. The former two species are important commercial sources of the tropane alkaloids hyoscyamine and scopolamine. The main alkaloids of *D. leichhardtii* and *D. myoporoides* are hyoscyamine and scopolamine, respectively. In contrast, the third species, *D. hopwoodii*, produces nicotine and nornicotine, both of which are pyridine alkaloids normally found in tobacco plants [1]. Although the characteristic alkaloid spectra of the three *Duboisia* species have been studied chiefly by analyses of plant leaves, recently both tropane and pyridine-type alkaloids were detected in the roots of these three species [2, 3].

We report in this paper the alkaloid spectra of cultured roots derived from calluses of *D. leichhardtii*, *D. myoporoides* and *D. hopwoodii*; we compare the spectra with those found in intact plants; and we discuss the culture conditions and selection methods favourable for tropane alkaloid production.

RESULTS AND DISCUSSION

Young calluses of *Duboisia* species tend to form adventitious roots on the subculture media described in the Experimental. This root-differentiating ability, however, decreased during long-term cultures. When the roots formed were excised and cultured in darkness on a reciprocal shaker (60 strokes/min) in liquid B5 medium [4] containing 10^{-5} M IBA (indole-3-butyric acid), the roots showed rapid growth. Transfers were made every 4 weeks by inoculating the rapidly growing roots (*ca* 1 cm in length) to a fresh medium.

Normal tropane alkaloids have not been detected in callus tissues of *D. myoporoides* [5], of the *D. leichhardtii–D. myoporoides* hybrids [6], or of *D. leichhardtii* [7]. We also found that in calluses of the three *Duboisia* species, tropane and pyridine alkaloids were not detected. Shoots were regenerated when the calluses were transferred to an agar medium containing 10^{-5} M 6-benzyladenine. In these shoot-organizing calluses of the three *Duboisia* species, neither tropane nor pyridine alkaloids were detected, but cultured roots differentiated from the non-producing calluses produced both types of alkaloids. This result suggests that the alkaloid production is associated with organogenesis of roots in *Duboisia* species.

The alkaloid spectra of cultured roots subcultured 2–4 times after root initiation were compared with those of leaves and roots of intact plants (Table 1). In the leaves of *D. leichhardtii* and *D. myoporoides*, the main alkaloids were the tropane alkaloids hyoscyamine and scopolamine, and the nicotine content was very low in these leaves. However, the roots of these two species contained relatively large amounts of nicotine. Considering the result that shoot-organizing calluses of *Duboisia* did not produce detectable amounts of the alkaloids, these data suggest that in *D. leichhardtii* and *D. myoporoides*, tropane and pyridine alkaloids are synthesized in the roots exclusively and that selective transportation of tropane alkaloids from the roots to the aerial parts of the plants occurs whereas most of the nicotine remains in the roots. Another possibility is that rapid degradation of nicotine takes place in the aerial parts of these two plants. In contrast, the ratio of pyridine alkaloids to tropane alkaloids in the aerial parts of *D. hopwoodii* is similar to that in the roots, which indicates that the selective transportation of tropane alkaloids suggested for *D. leichhardtii* and *D. myoporoides* does not take place in *D. hopwoodii*. Alternatively, the tropane alkaloids are degraded in the aerial parts. Nornicotine was found only in the leaves of *D. hopwoodii*. The demethylation reaction from nicotine to nornicotine seems to occur in the aerial parts of this species.

The alkaloids produced in cultured roots were similar to those in the roots of intact plants; the contents, however, were lower. That the ratios of scopolamine to hyoscyamine in the cultured roots of *D. leichhardtii* and *D. myoporoides* were higher than those in the roots of intact plants might result from the difference in the stage of development between the cultured roots and the intact

Table 1. Alkaloid spectra of *Duboisia*

	Alkaloid spectra	Pyridine alkaloid / Tropane alkaloid	Scopolamine / Hyoscyamine
D. leichhardtii	0 1 2% DW		
Plant leaves		0.01	0.64
Plant roots		0.42	0.39
Cultured roots		1.00	1.24
D. myoporoides			
Plant leaves		0.02	2.59
Plant roots		1.65	0.63
Cultured roots		0.61	3.60
D. hopwoodii			
Plant leaves		5.58	0.50
Plant roots		3.23	0.56
Cultured roots		5.59	0.42

Hyoscyamine, scopolamine, nicotine, nornicotine.

plant roots. Cultured *Hyoscyamus niger* roots derived from suspension cells exhibited higher scopolamine production than normal roots [8].

Typical time courses for the cultured roots are shown in Fig. 1. The doubling times measured for the cultured roots of *D. leichhardtii*, *D. myoporoides* and *D. hopwoodii* were 30, 117 and 73 hr, respectively. Alkaloid production decreased in the early stage of incubation but began to increase when roots reached the logarithmic phase of growth, and it continued after the growth had stopped in *D. leichhardtii* and *D. hopwoodii* roots. Especially the amount of scopolamine increased remarkably in *D. leichhardtii* roots, even after day 16, when the amount of hyoscyamine had reached a plateau. This result suggests that the enzyme activities catalysing epoxidation from hyoscyamine to scopolamine continued until the end of the culture period, whereas the enzyme activities of hyoscyamine biosynthesis decreased after day 16. In contrast, *D. myoporoides* roots showed relatively stable alkaloid synthesis throughout the culture period. *D. leichhardtii* and *D. myoporoides* roots released scopolamine to the media, and hyoscyamine was also discharged from the roots of *D. leichhardtii*. However, the alkaloids found in the media comprised only a small proportion of the total alkaloid production in the roots.

Cultured roots of *D. leichhardtii* showed the highest level of tropane alkaloid production and the most rapid growth among the three *Duboisia* species. Therefore we undertook the preliminary selection of *D. leichhardtii* roots for high tropane alkaloid production. First, all the root lines obtained were analysed, then the root lines which showed the highest contents were selected, and 10–15 roots (*ca* 1 cm) of these lines were incubated in four to five flasks. The roots were cultured for 4 weeks, after which they were analysed and re-selected. We obtained a high alkaloid-producing line by repeating this selection procedure ten times. The contents of hyoscyamine and scopolamine in this line were 0.53 and 1.16% on a dry weight basis, respectively. These values were *ca* three times more than the contents of low-producing lines. The sum of hyoscyamine and scopolamine contents in this line is twice as high as that in intact plant roots and is almost comparable to that in intact plant leaves.

We also studied which culture conditions of *D. leichhardtii* roots were favourable to alkaloid production. A high sucrose concentration in the medium stimulated nicotine production; the nicotine content in roots cultured in B5 medium supplemented with 5% sucrose was three times higher than that in the 3% sucrose medium. In Linsmaier–Skoog medium [9], *D. leichhardtii* roots produced 1.8 times more nicotine than in B5 medium. These changes, however, did not affect the production of hyoscyamine and scopolamine, which are commercially more important than nicotine. Changes of neither the mineral salt nor vitamin concentration in B5 medium ($\times 3$, $\times 1$, and $\times 1/3$ concentration of standard medium) showed significant effects on the alkaloid production (data not shown).

The effects of aeration on alkaloid production in *D. leichhardtii* roots are shown in Table 2. Roots were inoculated into 25 ml of liquid B5 medium in Erlenmeyer flasks of different volumes (300, 100 and 50 ml) and in test-tubes of 28 mm diameter. A large flask has a larger water surface than a small flask when the same volume of medium is in it; therefore the former is better aerated. In the test-tubes, the roots sank to the bottom and both the growth rate and the alkaloid production were low. The roots in the 50 ml flasks showed the best growth for alkaloid production but the 100 ml flasks were the most favourable. Thus the optimum aeration rate for alkaloid

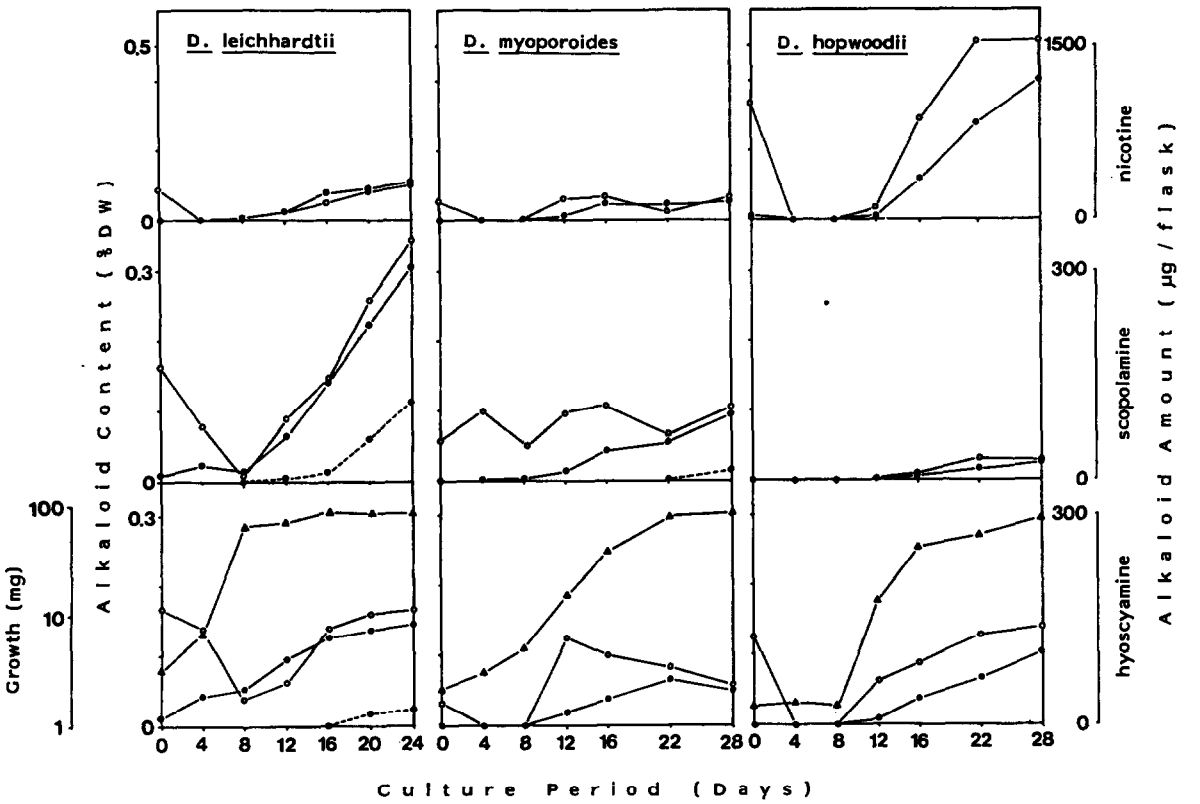

Fig. 1. Time course of growth and alkaloid production in cultured *Duboisia* roots. Symbols denote the alkaloid content (—○—, % dry wt), amount in roots (—●—, μg/flask), amount in medium (- -●- -, μg/flask), and growth (—▲—, mg dry wt).

Table 2. Effects of aeration on alkaloid-production in cultured *D. leichhardtii* roots

Culture vessel	Growth*	Roots above water surface	Hyoscyamine μg/g fr. wt	Hyoscyamine cell μg/vessel	Scopolamine μg/g fr. wt	Scopolamine cell μg/vessel	Nicotine μg/g fr. wt	Nicotine cell μg/vessel
300 ml flask	1.84	++	214	394	255	470	543	999
100 ml flask	3.90	++	201	620	289	893	304	939
50 ml flask	4.91	+	30.8	151	85.3	419	52.3	257
Test tube	0.69	—	31.9	22	53.6	37	—	n.d.

*g/culture vessel.
Average inoculum was 0.20 g/culture vessel. Medium volume was 25 ml.

production may be higher than that for growth. Tropane alkaloid production in the 300 ml flasks was considerably lower than that in the 100 ml flasks, which resulted from the low growth rate in the 300 ml flasks. In the 300 ml flasks, the growth may have been suppressed by mechanical injury caused by strong shaking.

As a potential material for production of tropane alkaloids, we believe that the cultured roots of *D. leichhardtii* are the most promising because of their rapid growth and high alkaloid content.

EXPERIMENTAL

Plant material. D. leichhardtii plants used in this study were plants regenerated from 2 to 3-month-old calluses [10]. The seeds of *D. myoporoides* were collected from plants cultivated in Barulega, Indonesia. The seeds of *D. hopwoodii* were collected from wild plants in Western Australia and stored in King's Park and Botanic Garden, Australia (*Duboisia hopwoodii* No. 5092). The *D. myoporoides* and *D. hopwoodii* seeds germinated in October 1981 and December 1981, respectively. Plants were

cultivated in a greenhouse (from April to November) and in a Phytotron at 15–25° (from December to March). The plant leaves and roots were harvested in November 1982 for an alkaloid assay.

Callus induction and subculture. Sterilized segments of leaves were incubated on Gamborg B5 medium [4] containing 5×10^{-5} M NAA (1-naphthaleneacetic acid), 5×10^{-6} M BA (6-benzyladenine), 3% sucrose and 0.9% agar under light (3000–5000 lx) at 25°. The calluses formed were maintained under the same conditions with 3 week transfers, but the hormone supplement was adjusted to 10^{-5} M NAA and 10^{-6} M BA.

GC and GC/MS. The alkaloid contents were measured by GC. Methods for alkaloid extraction and chromatographic conditions have been described elsewhere [10]. The mass spectra of hyoscyamine, scopolamine, nicotine and nornicotine were recorded by GC/MS. The chromatographic and spectrometric conditions for hyoscyamine, scopolamine and nicotine have been reported in our previous paper [10]. The conditions for nornicotine were a 2.5 m × 3.4 mm glass tube packed with 1% silicon OV-101, a column temp. of 100–180° (temp. programme 3°/min), He at a flow rate of 15 ml/min, TIM as the detecter, and an ionizing energy of 20 eV. Nornicotine: MS *m/z* (rel. int.): 148 $[M]^+$ (20), 118 (100), 146 (42), 70 (37). No distinction was made between *l*-hyoscyamine and atropine (*d*, *l*-hyoscyamine).

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