Growth and Alkaloid Production in *Duboisia myoporoides* and *D. leichhardtii* Root Cultures

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Root cultures of *Duboisia myoporoides* and *D. leichhardtii* have been established from granular tissues isolated from a culture line of callus. The granular tissues easily differentiated roots which vigorously grew in liquid Murashige-Skoog medium supplemented with indole-3-butyric acid (IBA) (2 mg/l) and gibberellic acid (1 mg/l).

These cultured roots produced atropine, scopolamine and nicotine. Furthermore, anabasine and nornicotine were detected in root cultures of *D. myoporoides*, and apoatropine in *D. leichhardtii* root cultures. A high concentration (7–10% (w/v)) of sucrose in the medium was effective in improving both root growth and tropane alkaloid production.

Keywords *Duboisia*; *D. myoporoides*; *D. leichhardtii*; granular tissue; root culture; indole-3-butyric acid; gibberellic acid; tropane alkaloid

Introduction

Tropane alkaloids are biosynthesized and decomposed in the roots of *Duboisia myoporoides*, although atropine esterase activity in adventitious roots from callus cultures was extremely low in comparison with that in roots of intact plants.¹⁾ The intact roots would decompose tropane alkaloids which were transported back from the leaf,²⁾ but since the alkaloid content in the intact roots was not so different from that in adventitious roots the cause of biochemical differences between cultured roots and intact roots with respect to tropane alkaloid decomposition is not clear.

Adventitious roots were infrequently differentiated from our callus cultures and their growth was very poor,³⁾ although Endo and Yamada⁴⁾ successfully cultured adventitious roots from callus cultures of three *Duboisia* species. Reportedly, hairy roots of *Duboisia* infected with *Agrobacterium rhizogenes* grew better than non-transformed cultured roots,^{5,6)} but the effect of the gene transfer on atropine esterase was not examined. We have established root cultures from granular tissues isolated from the callus of *D. myoporoides* and *D. leichhardtii* for the purposes of tropane alkaloid production and of investigation of atropine esterase activity in comparison with intact roots.⁷⁾

Materials and Methods

Plant Material and Culture Condition Calli were induced from stem segments of 2-month-old seedlings of *D. myoporoides* R. BR. and *D. leichhardtii* F. MUELL on a Murashige and Skoog basal (MS) agar medium⁸⁾ containing 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/l) and kinetin (0.01 mg/l) at 25 ± 1 °C in the dark. These calli were subcultured monthly under the same conditions. During 10 months of subculturing, *D. myoporoides* and *D. leichhardtii* calli were separated into 8 and 6 culture lines, respectively, according to morphological characteristics.

Granular tissues isolated from a callus line of *D. myoporoides* and *D. leichhardtii*, respectively, were cultured in a liquid MS medium (25 ml) containing 2,4-D (1 mg/l) in 100-ml flask on a rotatory shaker (120 rpm) for 4 weeks. These tissues were used for differentiation experiments. Indole-3-butyric acid (IBA), 2,4-D, 6-benzylaminopurine (BA) and gibberellic acid were used as plant hormones; MS, B5⁹⁾ and Modified White's¹⁰⁾ medium as basal media; and sucrose, glucose, fructose, mannose, maltose, xylose and rhamnose as carbon sources.

Histological Examination Granular tissues were fixed in formalin-acetic acid–50% ethanol (1 : 1 : 18), dehydrated in dist. ethanol, and embedded in paraffin. Cross sections (50 µm in thickness) were stained with Delafield's hematoxylin solution, followed by eosine solution, before examination.

Extraction and Identification of Alkaloids Extraction and determination of alkaloids were performed by the methods described elsewhere.¹¹⁾ Gas liquid chromatography-mass spectrometry (GC-MS) was also used for the identification of alkaloids. Commercially available atropine (Nakarai

Chemicals), scopolamine-HBr (Nakarai Chemicals) and nicotine (Tokyo Kasei Co.) were used as standard samples. Anabasine and nornicotine were isolated from leaves of *D. myoporoides*.¹²⁾ Apoatropine was synthesized in our laboratory according to Maeda's method.¹³⁾ Chromatographic conditions for the analysis of atropine, scopolamine and apoatropine were as follows: a glass column (1 m × 3 mm) packed with 2% Silicon OV-1 coated with acid-washed and silanized Chromosorb W, column temperature 190 °C, injector temperature 240 °C, helium as the carrier gas at a flow rate of 30 ml/min, total ion chromatogram (TIC) detector and an ionizing energy 70 eV. For the analysis of nicotine, nornicotine and anabasine, a glass column (2 m × 3 mm) packed with 10% Silicon DC-550 was used at a column temperature of 180 °C and an injector temperature of 230 °C. Atropine *m/z* (rel. int.): 289 [M⁺] (23), 140 (9), 124 (100), 94 (17), 83 (25), 82 (24). Scopolamine: 303 [M⁺] (55), 154 (42), 138 (86), 136 (34), 108 (49), 94 (100). Apoatropine: 271 [M⁺] (31), 124 (100), 103 (15), 96 (35), 83 (30), 82 (34). Nicotine: 162 [M⁺] (23), 161 (14), 133 (22), 119 (6), 85 (6), 84 (100). Nornicotine: 148 [M⁺] (39), 147 (36), 120 (36), 119 (100), 80 (32), 70 (82). Anabasine: 162 [M⁺] (53), 133 (49), 119 (36), 106 (43), 105 (53), 84 (100).

Results

Isolation of Granular Tissue Calli induced from stem segments of a *D. myoporoides* seedling and subcultured on a solid MS medium containing 2,4-D (1 mg/l) and kinetin (0.01 mg/l) were not uniform in appearance. Therefore, calli were separated into various culture lines during subculture according to such morphological characters as color, shape, and texture. After 10 months, 8 culture lines were established. A culture line having hard and compact callus produced granular tissues. When the callus was transferred to a liquid MS medium containing 2,4-D (1 mg/l) and cultured for 4 weeks on a shaker (80 rpm), granular tissues were separated from suspension cells, aggregating in the bottom of culture flasks since they were hard and heavy. About 2 g of callus tissues produced 15–20 granular tissues (1–3 mm in diameter) which existed as round to oval single bodies generally, although occasionally there were several in the medium (Fig. 1a). Granular tissues of *D. leichhardtii* were formed same as those of *D. myoporoides*. These granular tissues never differentiated roots as they were subcultured in the MS medium containing 1 mg/l 2,4-D.

Histological examination of the granular tissues showed that they were meristematic and formed a root apex (Fig. 1b).

Differentiation of Granular Tissue In a previous paper, we showed that the callus of *D. myoporoides* regenerated shoots and roots on a MS medium supplemented with either BA (1–10 mg/l) or IBA (1–5 mg/l).³⁾ Accordingly, the effect of BA and IBA on morphogenesis in granular tissues

Fig. 1a. Granular Tissues Separated from Cell Suspension Cultures of *D. myoporoides* ($\times 2$)1b. Cross Section of a Granular Tissue of *D. myoporoides* ($\times 40$)
Strong black parts are meristematic tissue and an arrow shows a root apex. Fig. 2a. Roots Differentiated from Granular Tissues of *D. myoporoides* Cultured in a Medium Containing Gibberellic Acid for 2 Weeks ($\times 3$)2b. Roots Differentiated from the Granular Tissues Cultured in a Medium Containing Gibberellic Acid and IBA for 4 Weeks ($\times 1.5$)2c. Roots Subcultured under the Same Condition for 4 Weeks ($\times 1$)

TABLE I. Effect of IBA and BA on Organ Formation in Granular Tissue

Morphogenesis	Plant growth hormones (mg/l) added to medium					
	IBA		BA		IBA + BA	None
	0.5	2	0.5	2	0.5+0.5	
No. of roots	60/19	68/20	21/19	0/15	35/19	23/17
/No. of G.T.						
No. of buds	0/19	0/20	46/19	145/15	0/19	0/17
/No. of G.T.						

Granular tissues (G.T.) were cultured for 6 weeks in MS medium containing hormone and 3% sucrose.

was examined (Table I). Granular tissues formed adventitious roots (1–5 roots/granular tissue) on media containing no BA other than IBA (0–2 mg/l); the roots had a lot of hairs, but did not elongate (<0.5 mm in length). Buds were formed only when BA alone was added to the medium. Both roots and buds were formed on a medium containing 0.5 mg/l BA. Adventitious roots also differentiated when granular tissues were treated with 2,4-D or gibberellic acid where root formation was poor (1–2/granular tissue) but root elongation was good (Table II) (Fig. 2a). Gibberellic acid at a concentration of 1 mg/l was most suitable for root growth.

These results showed that treatment with IBA caused an

TABLE II. Effect of 2,4-D and Gibberellic Acid on Root Formation in Granular Tissue

Root formation	Plant growth hormones (mg/l) added to medium						
	None	2,4-D			Gibberellic acid		
		0.001	0.01	0.1 < ^{a)}	0.1	1	10
No. of roots /G.T.	0-2	1-2	1-2	0	1-2	1-2	1-2
Length (mm)	20-30	20-30	30-40	—	50-60	50-60	30-40
Diameter (mm)	0.3-0.5	0.3-0.5	0.3-0.5	—	0.8-1.0	0.8-1.0	0.8-1.0

Granular tissues (G.T.) were cultured for 6 weeks in MS medium containing hormone and 3% sucrose. a) No root formation was observed.

increase in root number, whereas gibberellic acid contributed to root elongation. Accordingly, a combination of gibberellic acid (1 mg/l) and IBA (2 mg/l) was added to the culture medium. The treated roots grew well for 8 weeks, forming a mass from which thin roots extended in every direction from the center (Fig. 2b, c). Root cultures from granular tissues of *D. leichhardtii* have been also established with a medium containing gibberellic acid and IBA. They could be subcultured in the same medium for more than 3 years.

TABLE III. Effect of Basal Medium on Root Growth and Alkaloid Production

Basal medium	Increase in weight (final/initial)		Alkaloid production ($\mu\text{g}/\text{flask}$)	
	Fresh wt.	Dry wt.	Atropine	Scopolamine
MS	10.4 ± 1.1	4.8 ± 0.4	35 ± 10	93 ± 6
B5	8.3 ± 0.9	3.9 ± 0.7	33 ± 10	44 ± 12
White	4.8 ± 3.6	2.0 ± 0.5	25 ± 18	30 ± 5

Average of three determinations (mean $\pm$ S.E.). Initial fresh wt. and dry wt. of *D. leichhardtii* roots were 150 and 17 mg, respectively. The roots were cultured for 3 weeks in the basal medium containing IBA (2 mg/l), gibberellic acid (1 mg/l) and 3% sucrose.

Fig. 3. Effect of Sucrose Concentration on the Root Growth of *D. leichhardtii*

Initial fresh weight, 150 mg; initial dry weight, 17 mg; culture periods, 4 weeks. $n=3$. ▨, fresh wt.; ■, dry wt.

Alkaloid Production by Cultured Roots Alkaloids in the cultured roots of *D. leichhardtii* and *D. myoporoides* were separated by TLC and confirmed by GC-MS. Both roots contained tropane and pyridine alkaloids. Atropine, scopolamine, and nicotine were the main alkaloids common to both. Apoatropine was found in the roots of *D. leichhardtii*, whereas anabasine and nornicotine were detected in the roots of *D. myoporoides* as minor alkaloids.

Effects of Culture Conditions on Root Growth and Tropane Alkaloid Production In *D. leichhardtii* root cultures, the total tropane alkaloid content was higher than that of *D. myoporoides*. Therefore, *D. leichhardtii* roots were used in the following experiments.

(a) Basal Medium Among MS, B5, and modified White's basal media tested, MS basal medium was more suitable for both root growth and scopolamine production (Table III).

Regarding the effect of the comparative strength of the MS basal medium ($\times 1/3$, $\times 1/2$, $\times 1$ and $\times 2$) on root growth and alkaloid production, no remarkable difference was found (data not shown). However, a high concentration of mineral salts tended to inhibit alkaloid production in cultured roots.

(b) Carbon Source Sucrose was most suitable for root growth among 7 carbon sources tested, followed by fructose, maltose, and glucose. Mannose, xylose or rhamnose failed to support root growth. Cultured roots grew better at higher

Fig. 4. Relationship between Yields of *D. leichhardtii* Roots Cultured with Various Sucrose Concentrations and Their Atropine Production
 $n=18$, $r=0.778$. Fig. 5. Relationship between Yields of *D. leichhardtii* Roots Cultured with Various Sucrose Concentrations and Their Scopolamine Production
 $n=18$, $r=0.603$.

sucrose concentrations (5–10%) (Fig. 3). The yields of atropine and scopolamine tended to increase with increasing root growth (Figs. 4 and 5), showing a good correlation with growth ($r=0.778$ for atropine; $r=0.603$ for scopolamine).

(c) Shaking Speed Since aeration is known to be important for root growth and alkaloid production,⁴⁾ the effect of shaking speed on root growth was determined. Cultures were performed at 3 different shaking speeds (80, 100 and 120 rpm), but no significant difference was found among them.

Discussion

We have established root cultures of *D. myoporoides* and *D. leichhardtii* from granular tissues isolated from callus cultures. There had been no previous report on isolation of such a granular tissue having meristematic structures in *Duboisia*. These granular tissues were found in a culture line of callus subcultured for more than 10 months, and we were able to maintain the ability to differentiate among these roots for the following 15 months. Simultaneous addition of IBA and gibberellic acid were effective in promoting not only root differentiation from the granular tissue, but also subsequent growth. This combination was

also found to be useful in promoting cultured roots of other tropane alkaloid-producing plants, *Datura*, *Atropa*, *Scopolia* and *Hyoscyamus* (data not shown).

Root cultures of *D. myoporoides* and *D. leichhardtii* in the present study produced tropane and pyridine alkaloids, as reported by Endo and Yamada.⁴⁾ *D. leichhardtii* root cultures produced not only atropine and scopolamine but also apoatropine, which was detected in *Duboisia* cultured roots for the first time. Both growth and alkaloid production in the *D. leichhardtii* root could be improved in a MS medium containing a higher concentration (7–10%) of sucrose. Changes in the nitrogen source and its concentration did not affect the increase of tropane alkaloid production (data not shown).

Leaves of *D. leichhardtii* plants contain *ca.* 0.1% scopolamine and 0.3% atropine, whereas the roots cultured with 7% sucrose produce *ca.* 0.1% scopolamine and only 0.08% atropine. Cultured roots of *D. leichhardtii* established by Endo and Yamada produced *ca.* 0.9 mg scopolamine and 0.6 mg atropine with a 25 ml medium during 4 weeks when 0.20 g roots were used as the initial fresh weight. Since our cultured roots produced *ca.* 0.5 mg scopolamine and 0.4 mg atropine under the same conditions, but with 0.15 g roots at first, the alkaloid productivity of our roots is at a similar level to theirs. To increase the alkaloid productivity of our cultured roots, careful selection of the mother plant,

granular tissue, and cultured roots is required.

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References

- 1) Y. Kitamura, H. Miura and M. Sugii, *Chem. Pharm. Bull.*, **33**, 5445 (1985).
- 2) Y. Kitamura, H. Miura and M. Sugii, *J. Plant Physiol.*, **133**, 316 (1988).
- 3) Y. Kitamura, H. Miura and M. Sugii, *Shoyakugaku Zasshi*, **34**, 65 (1980).
- 4) T. Endo and Y. Yamada, *Phytochemistry*, **24**, 1233 (1985).
- 5) H. Deno, H. Yamagata, T. Emoto, T. Yoshioka, Y. Yamada and Y. Fujita, *J. Plant Physiol.*, **131**, 315 (1987).
- 6) P. Bachmann and F. C. Czygan, Braunschweig Symposium on Applied Plant Molecular Biology, 1988, Braunschweig, Germany.
- 7) Y. Kitamura, M. Sato and H. Miura, Abstracts of Papers, the 111th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, March 1991. (in preparation).
- 8) T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
- 9) O. L. Gamborg, R. A. Miller and K. Ojima, *Exp. Cell Res.*, **50**, 151 (1968).
- 10) F. C. Steward, Advanced Laboratory Course in Plant Physiology, Cornell University, New York, 1964.
- 11) Y. Kitamura, H. Miura and M. Sugii, *Planta Medica*, **489** (1985).
- 12) Y. Kitamura, H. Miura and M. Sugii, *Shoyakugaku Zasshi*, **34**, 117 (1980).
- 13) S. Maeda, *Yakugaku Zasshi*, **56**, 736 (1936).