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Tropane Alkaloids in Auxin-Independent Root Cultures of *Physochlaina physaloides*

Koichiro Shimomura [*1], Makoto Hirose [*2], Shinsaku Natori [*3], Motoyoshi Satake [*4], Kayo Yoshimatsu[#] and Kanji Ishimaru [*5]

Adventitious and hairy root cultures of *Physochlaina physaloides* were established. These roots grew well and produced high amounts of tropane alkaloids (particularly hyoscyamine and 6β-hydroxyhyoscyamine) in auxin-free culture medium. The effects of basal media and temperature on the growth and alkaloid production of these roots were investigated. Both root cultures produced highest amount of tropane alkaloids in B5 medium though the optimum temperature for hairy roots were lower than that for adventitious roots.

Keywords: *Physochlaina physaloides*, Solanaceae, root culture, hairy root, tropane alkaloid

Introduction

Physochlaina physaloides G. Don, a small solanaceous plant, grows in the north and northeast parts of China, Inner Mongolia and Russian countries. The plant, containing tropane alkaloids such as scopolamine (**1**) and hyoscyamine (**2**)(Fig. 1), has been used as a medicine in China for its antitussive and sedative properties[1]. We established the adventitious root cultures of this plant and investigated the effect of auxins on the growth and alkaloid production[2]. Although this plant is regarded to be rich in biosynthetically important tropane alkaloid 6β-hydroxyhyoscyamine (**3**)[2] (an intermediate in the conversion of **2** to **1**), detailed research on the production of the alkaloids and the tissue culture experiments have not been done. In the present study, we established auxin-independent root cultures, non-transformed and transformed root cultures, of this plant and determined their growth and secondary metabolites (tropane alkaloid production) in different culture conditions.

Materials and Methods

General experimental procedures

The volume of the liquid medium for root cultures of *Physochlaina physaloides* was 50 ml / 100 ml Erlenmeyer flask. The cultures were maintained on a rotary shaker at 100

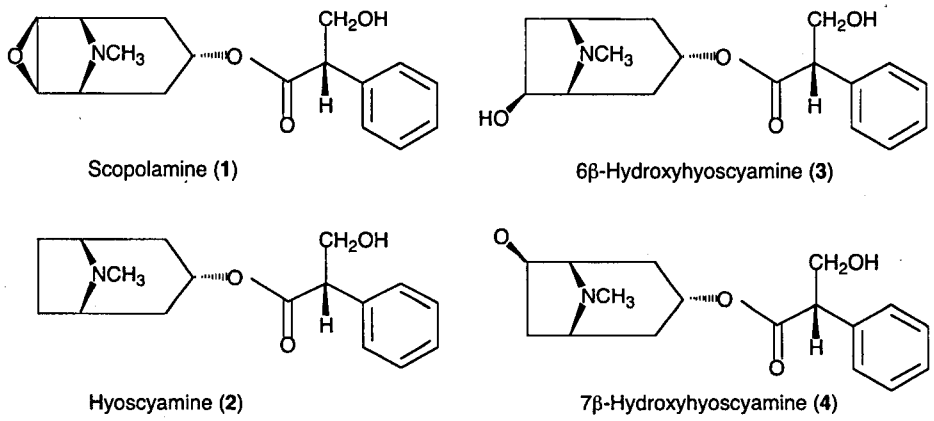

Fig. 1 Tropane alkaloids analyzed by HPLC in this study

[*1] Faculty of Life Science, Toyo University, 1-1-1 Izumino, Itakura-machi, Oura-gun, Gunma, 374-0193 Japan

[*2] Health Sciences Division, Minister's Secretariat, Ministry of Health, Labor and Welfare, 1-2-2 Kasumigaseki, Chiyoda-ku, Tokyo, 100-8916 Japan

[*3] 2-5-2-401 Otsuka, Bunkyo-ku, Tokyo, 112-0012 Japan

[*4] 2-2-24 Mizuhiki, Atsugi, Kanagawa, 243-0004 Japan

[*5] Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, 1 Honjo, Saga, 840-8502 Japan

[#] To whom correspondence should be addressed: Kayo Yoshimatsu; 1 Hachimandai, Tsukuba, Ibaraki, 305-0843 Japan; Tel: 0298-38-0573; Fax: 0298-38-0575; E-mail: yoshimat@nihs.go.jp

rpm in the dark at 25 ℃ (except for the experiment for the effects of temperature). All the media [a half strength Murashige-Skoog (1/2 MS)[3], Gamborg B5 (B5)[4] and Woody Plant (WP)[5] media] were adjusted to pH 5.7 before autoclaving at 121 ℃ for 15 min. Solidified medium was obtained by adding 0.2 % Gelrite.

Plant materials

The seeds of *P. physaloides* were sterilized [2% NaOCl with Tween 20 (1 drop in 50 ml)] for 10 min and germinated aseptically on 0.5% agar medium containing 0.5% sucrose under 16 h light/day (70 $\mu E \cdot m^{-2} \cdot S^{-1}$) at 25 ℃. The axenic plants were transferred to 1/2 MS solid medium containing 2% sucrose and subcultured monthly.

Induction of adventitious roots

The adventitious roots were induced with some calli from leaf segments (5 x 5 mm) of the axenic plants cultured on MS solid medium containing 1 mg/l indole-3-acetic acid (IAA) in the dark for 3 weeks. The roots were cut off and maintained in the same liquid medium. One clone was selected by its rapid growth and high production of tropane alkaloids (quantified by HPLC) and transferred into phytohormone-free 1/2 MS liquid medium. The roots could be maintained as a phytohormone-independent culture and used for this experiment.

Induction of the hairy roots

Agrobacterium rhizogenes A4 strain harboring Ri plasmid was inoculated onto the stem of the axenic plant by a needle. The hairy roots, appeared at the infected sites, were cut off and cultured on phytohormone-free 1/2 MS solid medium containing the antibiotic (0.5 g/l Claforan) to eliminate the bacteria. After elimination of the bacteria, the axenic hairy roots (10 clones) were transferred and maintained on phytohormone-free 1/2 MS liquid medium. Among them, one clone that showed rapid growth was selected and used for this experiment. Opines (agropine and mannopine) of the hairy roots were extracted and detected using paper electrophoresis[6].

HPLC analysis

The sample preparation and the method of HPLC analysis for tropane alkaloids were done by the same method as mentioned in our previous paper[7].

Study of culture conditions

The adventitious [40 - 90 mg, fresh weight (fw)] and hairy (10 - 20 mg, fw) roots were inoculated in a liquid medium and cultured under the conditions indicated in the Tables 1 and 2. The growth and alkaloid production in these roots were determined.

Periodical study of growth and alkaloid production

The adventitious (90 mg fw) and the hairy roots (10 mg fw) were inoculated in phytohormone-free MS or B5 liquid medium. The growth and alkaloid production were determined periodically (1 to 7 weeks).

Results and discussion

The growth and contents of four tropane alkaloids, **1**-**3** and related compound 7 β-hydroxyhyoscyamine (**4**)[8] (Fig. 1) in the adventitious and hairy roots cultured in four phytohormone-free liquid media (MS, 1/2 MS, B5 and WP) are shown in Table 1. The adventitious roots grew well in phytohormone-free liquid medium (37- to 80-fold increase of fw after 3-week culture), especially in B5 medium (*ca.* 80-fold increase of fw). In this medium, the highest amount of **2** (926.15 μg / flask) was obtained, however, the accumulation of **1** was much less than those cultured in 1/2 MS or WP medium. Taking into account the relatively high yield of **3** (73.16 μg / flask) and **4** (27.67 μg / flask) in B5 medium, the conversion of **3** (or **4**) to **1** in the roots seemed to be suppressed. In the case of the hairy roots, the clear difference of the growth was not observed in these four media tested. Despite the vigorous growth of the hairy roots (approximately 300 fold increase of fw after 3-week culture), their alkaloid production was much less than those in the adventitious roots. The highest amount of **2** in the hairy roots was obtained in MS medium. Therefore we selected MS and B5 medium for further experiments.

The effects of temperature (15 ℃ - 25 ℃) on the growth and alkaloid production in the adventitious and hairy roots were determined (Table 2). The growth of the roots (both adventitious and hairy roots) cultured at 15 ℃ was inferior to those at 20 ℃ or 25 ℃. Highest yield of **2** by adventitious roots was obtained in B5 medium at 25 ℃. On the other hand, hairy roots produced highest amount of **2** in B5 medium at 20 ℃. It was noteworthy that for the production of **1** (conversion of **2** to **1**), low temperature (20 or 15 ℃) was suitable in both adventitious and hairy root cultures.

Because the adventitious and hairy roots showed the rapid growth in B5 liquid medium at 25 ℃, we examined the amount of **1**-**4** and the growth of these roots periodically (Fig. 2). In this medium the adventitious roots grew gradually and the fresh weight reached *ca.* 5 g at week 7 (Fig. 2A). On the other hand, the growth of the hairy roots rapidly increased

Table 1 Effects of various basal liquid media on the growth and alkaloid production (μg / flask) in adventitious and hairy root cultures of *Physochlaina physaloides*

material	medium	fw (g)	GI	dw (mg)	1	2	3	4
Adventitious roots	MS	1.99	36.9	170.1	0.61	519.86	68.25	trace
	1/2 MS	3.08	57.0	210.2	4.44	567.84	67.59	trace
	B5	4.31	79.8	266.3	0.84	926.15	73.16	27.67
	WP	2.31	42.8	189.6	2.43	702.33	41.16	trace
Hairy roots	MS	3.09	309	208.1	trace	378.11	7.74	trace
	1/2 MS	3.07	307	181.1	trace	258.53	trace	trace
	B5	3.18	318	200.4	trace	234.58	trace	trace
	WP	3.46	285	285.4	trace	363.54	10.12	trace

The roots were cultured for 3 weeks at 25℃. fw, fresh weight; dw, dry weight; GI, growth index: final fw / inoculum fw (*ca.* 50 mg for adventitious roots and *ca.* 10 mg for hairy roots).
1, scopolamine; **2**, hyoscyamine; **3**, 6β-hydroxyhyoscyamine; **4**, 7β-hydroxyhyoscyamine.

Table 2 Effect of temperature on the growth and alkaloid production (μg / flask) in adventitious and hairy root cultures of *Physochlaina physaloides*

material	medium	temp. (℃)	fw (g)	GI	dw (mg)	1	2	3
Adventitious roots	MS	15	0.60	14.6	39.1	5.25	40.23	10.31
		20	2.01	49.0	138.8	2.01	420.95	39.68
		25	3.81	92.9	273.2	0.91	572.43	92.23
	B5	15	0.99	24.1	55.9	3.37	124.59	12.54
		20	4.62	112.7	243.8	2.22	684.55	48.20
		25	4.52	110.2	300.5	trace	983.32	71.41
Hairy roots	MS	15	0.69	43.1	46.8	1.41	32.79	3.76
		20	5.30	331.3	307.0	20.38	509.92	144.63
		25	6.09	380.6	357.3	4.09	579.71	106.87
	B5	15	1.92	120.0	110.0	2.42	158.53	13.20
		20	4.93	308.1	319.6	0.85	1252.17	140.33
		25	6.02	376.3	361.4	trace	1014.73	89.49

The roots were cultured for 4 weeks. fw, fresh weight; dw, dry weight; GI, growth index: final fw / inoculum fw (*ca.* 40 mg for adventitious roots and *ca.* 20 mg for hairy roots).
1, scopolamine; **2**, hyoscyamine; **3**, 6β-hydroxyhyoscyamine.

through the entire culture period until it reached to *ca.* 17 g (fw) at the end of culture (Fig. 2B). In both cultures (adventitious and hairy roots), the amount of **2** increased at the early stage of the culture, reached the maximum level at week 4 and began to decrease. The amount of **3** was slightly enhanced at the last stage (week 6 to week 7) and conversion of **2** to **3** became active after week 3 when the level of **2** approached the maximum level in both cultures. No clear difference in the accumulation of tropane alkaloids (**1-4**) between hairy and adventitious root cultures was observed. We also determined the periodical accumulation of **1-4** and the growth of adventitious and hairy roots in MS liquid medium. The patterns of the growth and alkaloid production were similar to those observed in B5 liquid medium, however, the amount of the alkaloids was almost half the level in B5 medium (data not shown).

We established two kinds of auxin-independent root culture, adventitious and hairy root cultures. Although the intact roots were reported to contain **3** as the main alkaloid [**1**, 0.06 % dry weight (dw); **2**, 0.21 % dw; **3**, 0.47 % dw] [1], both root cultures accumulated **2** as the main alkaloid in all the culture periods (except week 7 in hairy roots) and under all the culture conditions tested. This might be one of the differences between field-grown roots and cultured roots that previously reported [9,10]. Yields of **3** increased according to the decrease of **2** in the adventitious and the hairy root cultures (Fig. 2). Therefore the expression of genes responsible for the conversion of **2** to **3** might be more

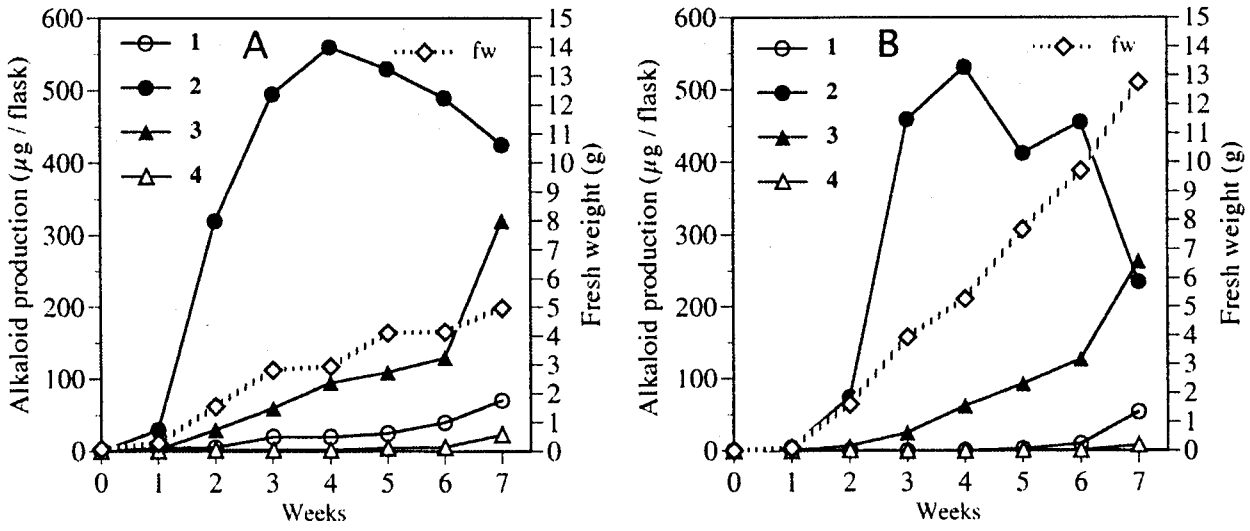

Fig. 2 Periodical growth and alkaloid production of *Physochlaina physaloides* adventitious (A) and hairy (B) roots cultured in phytohormone-free B5 liquid medium at 25℃

1 (○), scopolamine; **2** (●), hyoscyamine; 3 (▲), 6β-hydroxyhyoscyamine; **4** (△), 7β-hydroxyhyoscyamine; fw (◇), fresh weight (g).

obvious in the intact plants than cultured roots. With the optimization of culture conditions rather than auxin, even adventitious root cultures produced high amounts of tropane alkaloids (983.32 μg / flask of **2** and 71.41 μg / flask of **3** in Table 2) in phytohormone-free B5 medium. In the case of hairy roots, results in Table 1 (highest yield of 2 in MS medium) seems to be contrary to the results in Table 2 (higher yield of 2 in B5 medium at 25℃). However the results of Table 1 was obtained after three weeks of culture while after 4 weeks in Table 2. In addition productivity of alkaloids by hairy root cultures was much higher in B5 medium in the time course study. Therefore the optimum culture medium for the growth and the alkaloid accumulation in hairy roots might be B5 medium. These plant materials (adventitious and hairy root cultures) could be one of the most useful systems for biosynthetic experiments of tropane alkaloids, especially the pathway in the conversion of **2** to **3**, because they do not require phytohormones (especially auxins) for their growth and alkaloid production.

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