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Production of Tropane Alkaloids by Hairy Root Cultures of *Scopolia japonica* *

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Abstract

Hairy root clones of *Scopolia japonica* were established by selection of a number of adventitious roots formed on the root segments inoculated with *Agrobacterium rhizogenes* strain 15834. Two highly alkaloid-productive clones S1 and S22 were examined for their growth rate and alkaloid productivity under various cultural conditions. When clone S1 was cultured in each optimum medium for 4 weeks at 25°C in the dark, the weight of the root tissue was increased by 79 times and the scopolamine content was 3 times higher than that of the original rhizome. Similarly, the weight of clone S22 was increased by 100 times and the hyoscyamine content was 8 times higher than that of the original rhizome. On two-stage culture method combined with both optimum media for growth and scopolamine production, clone S1 produced scopolamine at a level of 560μg per 50 ml culture for 4 weeks.

Keywords: Tropane alkaloids; *Agrobacterium rhizogenes*; Hairy root culture; Scopolamine; Hyoscyamine.

Abbreviations: A, Agropine; Hyos, Hyoscyamine; M, Mannopine; Scop, Scopolamine.

1. Introduction

Agrobacterium rhizogenes is the cause of the so-called "hairy root" disease of dicotyledonous plants and induces adventitious roots at the site of infection. This involves the integration of the root-inducing (Ri) plasmid into the plant genome (Chilton et al., 1982). Hairy roots can be cultured aseptically in phytohormone-free media and subcultured indefinitely (Tanaka et al., 1985).

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The tropane alkaloids scopolamine and hyoscyamine are medicinally important, having long been used as spasmolytics and anesthetics. These alkaloids are synthesized in the roots of Solanaceae plants such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia*. Studies on the production of tropane alkaloids in callus cultures of Solanaceae plants showed that alkaloid contents in the callus were generally much lower than those found in original plants, and synthesis of tropane alkaloids was increased significantly in the roots differentiated from the callus tissues (Hashimoto and Yamada, 1983). It was reported that hairy root clones of *Hyoscyamus muticus* produced about the same amount of alkaloids as normal roots (Flores and Filner, 1985). Mano et al. (1985) reported that hairy root clones of *Scopolia japonica* grew rapidly and produced fairly large amounts of scopolamine and hyoscyamine in liquid cultures.

Here we report on establishment of hairy root clones of *S. japonica* after inoculation of the root segments with *A. rhizogenes*, and on examination of medium conditions for tropane alkaloid production by cultures of hairy root clones.

2. Materials and Methods

Bacteria and plant materials

A. rhizogenes strain ATCC 15834 was used in the present study. *S. japonica* Maxim. (about 50 cm in height) was provided by Dr. H. Kimura of Kanazawa University, Kanazawa, Japan.

Culture media

The following basal media were used for culture of root segments and hairy root clones of *S. japonica*: (W) White, 1963; (H) Heller, 1953; (SH) Schenk-Hildebrandt, 1972 and (K) Knop, 1965. These basal media used were supplemented with 3% sucrose but no phytohormones, and adjusted to pH 5.8.

Establishment of hairy root clones

Inoculation of sterilized root segments of *S. japonica* with *A. rhizogenes* and selection of adventitious root formed on the root segments were carried out essentially as reported previously (Tanaka et al., 1985; Mano et al., 1985).

Cultures of hair root clones

About 60 mg fresh weight (ca 4.4 mg dry weight)of each of hairy root clones was inoculated into 50 ml of a liquid medium in a 100 ml beaker and incubated in a rotary shaker in the dark (100 rpm at 25°C).

Opine assay

Agropine and mannopine in hairy root clones were analyzed by paper electrophoresis (PE) and cellulose thin-layer chromatograpy (TLC), as reported previously (Tanaka et al., 1985).

Analysis of tropane alkaloids

Scopolamine and hyoscyamine in hairy root cultures (ca. 50 mg dry weight) were extracted, essentially according to Yamada et al., 1982, and identified by field-desorption mass spectrometry. Contents of scopolamine and hyoscyamine were each analyzed by high pressure liquid chromatography (HPLC) as reported previously (Mano et al., 1985).

3. Results

Establishment of hairy root clones of S. japonica

When aseptically prepared rhizomes of *S. japonica* were inoculated with *A. rhizogenes* strains 15834, numerous hairy roots emerged from the infection site after 4 weeks incubation at 25°C in the dark. Tips of hairy roots were individually excised and subcultured on 1% agar W medium every 4 weeks for 3 to 4 passages. About 10% of the total hairy roots excised continued to grow on the phytohormone-free medium. The hairy roots with no bacterial contaminations were designated as axenic hairy root clones.

Of a number of hairy root clones, two which were rapidly growing and highly alkaloid-productive were selected. The characteristics of both clones are listed in Table 1. When clones S1 and S22 were each cultured in H liquid medium at 25°C in the dark for 4 weeks, both clones grew very rapidly. The growth index (harvest dry weight per inoculum dry weight at 4 weeks) of clone S1 and S22 was 26 and 52, respectively. Scopolamine and hyoscyamine, the major tropane alkaloids of *S. japonica*, were produced by both clones.

Table 1. Characteristics of the selected hairy root clones S1 and S22 of *Scopolia japonica*.

Clone	Growth index	Alkaloid content	Alkaloid productivity	Opine production			
		Scop	Hyos	Scop	Hyos	A	M
S1	26	0.14	0.45	36	117	–	+
S22	56	0.01	0.46	6	258	+	+

Growth index (g harvest / g inoculum) and alkaloid content (% dry weight) were determined at 4 week cultures of each clone in H liquid medium. Alkaloid productivity (mg / g inoculum / 4 weeks) is defined in the text. Scop – scopolamine; Hyos – hyoscyamine; A – agropine; M – mannopine; + positive; – negative.

But the content and the pattern of the produced alkaloids were different between the two clones. Clone S1 produced a fair amount of scopolamine and a large amount of hyoscyamine. On the other hand, clone S22 produced a very large amount of hyoscyamine but a small amount of scopolamine. Alkaloid productivity was estimated by multiplying the alkaloid content by the growth index. The highly scopolamine-productive clone S1 produced only mannopine, and the highly hyoscyamine productive-clone S22 produced both agropine and mannopine. On subculture of clone S22 in SH-2 medium every 4 weeks, the growth rate, tropane alkaloid production and opine production of the clone were stably maintained during 6 successive subcultures, as shown in Fig. 1.

Tropane alkaloid production by cultures of hairy root clones

In order to maximize culture conditions for growth and tropane alkaloid production, hairy root clones S1 and S22 were each cultured in basal media under various culture conditions. Optimum temperature (22°C), shaking speed (100 rpm) and light-irradiation (dark) conditions were established for both clones in H medium. The optimum basal media for growth were H and SH for clones S1 and S22, respectively. When H medium was supplemented with 0.078 mM of FeNaEDTA (ethylenediaminetetraacetic acid sodium iron) instead of $FeCl_3$, the weight of clone S1 was increased 40 fold during 4 weeks, as indicated with HF in Table 2. On the other hand, when the pH of SH medium was adjusted to 6.4, the growth index of clone S22 was increased 102 fold, as indicated with SH-1. Removal of NH_4 + ion from SH-1 medium (see SH-2), stabilized the rapid growth, with lateral branches, of the hairy root clones and reduced the callus formation during cultivation, although

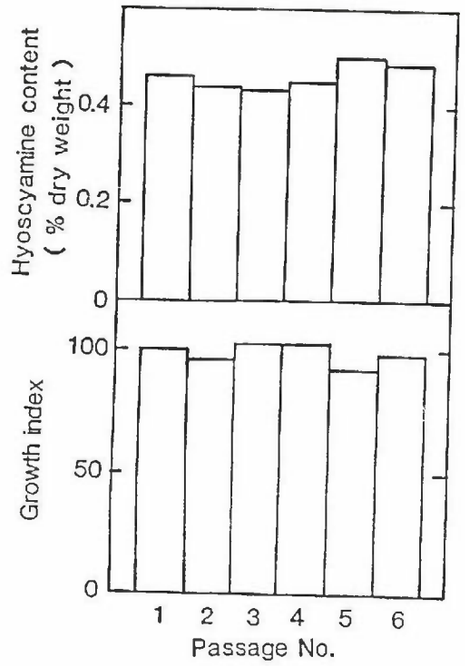

Figure 1. Stability of clone S22 in growth and hyoscyamine content during 6 successive subcultures. Clone S22 was subcultured every 4 weeks in SH-2 medium. Growth index and hyoscyamine content were determined at 4 weeks culture.

the growth rate was not remarkably increased. The weight of clones S1 and S22 was increased by 79 times and 100 times, respectively, after 4 weeks cultivation in SH-2 medium.

Addition of 1% casamino acids to H medium instead of $NaNO_3$ increased the scopolamine content of clone S1 by 3 times as indicated with HC in Table 2. Also, the optimum pH for scopolamine production of clone S1 in HC medium was 7.0. The scopolamine content of clone S1 in HC medium reached a level of 0.5% on a dry weight basis for 4 weeks culture. This content was 3 times higher than that of the original rhizome. For hyoscyamine production of clone S22, K was optimum among the tested basal media. Although the effects of nitrogen source, pH and component concentration in K medium was examined, almost no increment was found in the hyoscyamine content

Table 2. Medium conditions for growth and tropane alkaloid production of hairy root clones S1 and S22

Clone	Medium	Growth index	Alkaloid content		Alkaloid productivity	
			Scop	Hyos	Scop	Hyos
S1	H	26	0.14	0.45	36	117
	HF	41	0.08	0.40	32	164
	SH-2	79	0.08	0.55	63	435
	HC	3	0.50	0.36	2	11
S22	SH	64	–	0.42	–	269
	SH-1	102	–	0.45	–	459
	SH-2	100	–	0.48	–	480
	K	2	–	1.30	–	26

Growth index and alkaloid content were determined at 4 weeks culture of clones S1 and S22. Contents of scopolamine and hyoscyamine in the original rhizome was 0.1% and 0.16% (dry weight), respectively. – not detected; abbreviations as above.

of clone S22 (data not shown). The hyoscyamine content of clone S22 in K medium reached a level of 1.3% on a dry weight basis for 4 weeks. This content was 8 times higher than that found in the original rhizome. In order to increase scopolamine production in clone S1, a two-stage cultivation method was examined. After 3 weeks culture of clone S1 in SH-2 medium, the hairy root was transferred to HC medium and cultured for one more week (Fig. 2). The scopolamine content in clone S1 reached a level of 560μg per 50 ml culture by the two-stage method. This level was 5 times higher than that of the 4 weeks culture in SH-2 medium.

4. Discussion

Hairy root clones of *S. japonica* were established by selection of adventitious roots formed on the root segments inoculated with *A. rhizogenes* strain 15834. By screening of a number of hairy root clones, the highly scopolamine-productive clone S1 and the highly hyoscyamine-productive clone S22 were selected. The characteristics of *S. japonica* hairy root clones were stably maintained for at least 6 successive subcultures.

The productivity of the established clones S1 and S22 for scopolamine and hyoscyamine, respectively, was increased by maximizing medium conditions. The growth of hairy root clones was significantly increased by addition of FeNaEDTA and change of pH in the medium. Also, the content of scopolamine in clone S1 was increased by supplement of H medium with casamino acids, which may act as precursors of tropane alkaloid synthesis. The growth of hairy root clones was repressed in the optimum media for tropane alkaloid production. This suggested that the alkaloid synthesis resumes when growth slows down. Therefore, the scopolamine productivity of clone S1 was increased by the two-stage cultivation method combined with both optimum media for growth and scopolamine production. These results indicated that the hairy root culture of *S. japonica* is useful for production of tropane alkaloids.

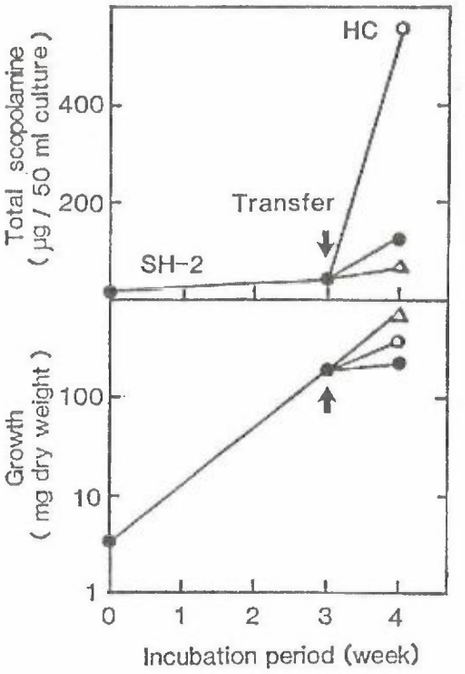

Figure 2. Two-stage cultivation of clone S1. Clone S1 was cultured in SH-2 medium for 3 weeks and then transferred to the fresh HC (○) or SH-2 (△) medium. Total scopolamine in 50 ml culture was determined after one more week cultivation. (●); Continuous cultures in SH-2 medium for 4 weeks.

5. Conclusions

Hairy root clones of *S. japonica* were established by selection of a number of hairy roots formed on the root segments inoculated with *A. rhizogenes*. Of these clones, two highly alkaloid-productive clones S1 and S22 were selected and examined for growth and tropane alkaloid productivity under various medium conditions. The tropane alkaloid production of these clones was increased by maximizing medium conditions.

REFERENCES

Chilton, M.D., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F., and Tempe, J. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host-plant root cells. *Nature* **295**: 432–434.

Flores, H.E. and Filner, P. 1985. Metabolic relationships of putrescine, GABA and alkaloids in cell and root cultures of Solanaceae. In: *Primary and Secondary Metabolism in Plant Cell Cultures*. K.H. Neumann, ed. Springer-Verlag, Berlin, pp. 174–185.

Hashimoto, T. and Yamada, Y. 1983. Scopolamine production in suspension cultures and redifferentiated roots of *Hyoscyamus niger*. *Planta Med.* **47**: 195–199.

Heller, R. 1953. Recherches sur la nutrition minérale des tissus végétaux cultivés *in vitro*. *Ann. Sci. Nat. Bot. et Bio. Veg.* **14**: 1–223.

Knop, W. 1965. Quantitative Untersuchungen über die Ernährungsprozesse der Pflanen. *Landw. Versta.* **7**: 93.

Mano, Y., Nabeshima, S., Ohkawa, H., and Matsui, C. 1985. Characteristics of hairy roots of *Scopolia japonica* induced by *Agrobacterium rhizogenes*. In: *Abstracts of Annual Meeting of the Agricultural Chemical Society of Japan*.

Schenk, R.U. and Hildebrandt, A.C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* **50**: 199–204.

Tanaka, N., Hayakawa, M., Mano, Y., Ohkawa, H., and Matsui, C. 1985. Infection of turnip and radish storage roots with *Agrobacterium rhizogenes*. *Plant Cell Rep.* **4**: 74–77.

White, P.R. 1963. *The Cultivation of Animal and Plant Cells*. Ronald Press, New York, N.Y.

Yamada, Y., and Hashimoto, T. 1982. Production of tropane alkaloids in cultured cells of *Hyoscyamus niger*. *Plant Cell Rep.* **1**: 101–103.