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

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Hairy root clones of *Scopolia japonica* were established by selection of adventitious roots formed on the root segments inoculated with *Agrobacterium rhizogenes* strain 15834. Twenty-nine isolated hairy root clones displayed various phenotypes characterized by growth rate, opine production and tropane alkaloid production. Of these, two highly alkaloid productive clones S1 and S22 were examined for their growth rate and alkaloid productivity under various cultural conditions. When the most scopolamine-productive clone S1 was cultured for 4 weeks at 25°C in the dark, the weight of the root tissue was increased by 40 times and the content of scopolamine reached a level of 0.5% on a dry weight basis in each optimum medium. On culture of the most hyoscyamine-productive clone S22 under the same conditions as with S1, the weight was increased by 102 times and the content of hyoscyamine was 1.3% on a dry weight basis in each optimum medium.

The tropane alkaloids scopolamine and hyoscyamine are medicinally important, having long been used as spasmolytics and anesthetics. Commercial sources of these alkaloids are Solanaceae plants such as Atropa, Datura, Duboisia, *Hyoscyamus* and *Scopolia*, in which alkaloids are synthesized in the roots, followed by transport and accumulation in the leaves.1) There were some attempts to produce tropane alkaloids by culture of cells and root tissues of the Solanaceae. However the contents of these alkaloids in undifferentiated calli or cell suspensions were much lower than those found in original plants,2~4) although cultured roots and redifferentiated roots produced the alkaloids at levels comparable to those of natural roots.5,6)

*Agrobacterium rhizogenes*, which is the cause of the so-called "hairy root" disease of dicotyledonous plants, induced adventitious roots at the inoculation sites.7) This involved the integration of the root-inducing (Ri) plasmid into the plant genome.8~10) Hairy roots arising at the site of infection were cultured aseptically in phytohormone-free media.11,12) The isolated roots were subcultured indefinitely on basal media. Recently, Flores and Filner13) reported that hairy root clones of *Hyoscyamus muticus* produced about the same amount of alkaloids as normal roots, and the alkaloid pattern was similar to that of original plants.

Here we report on the establishment of hairy root clones of *Scopolia japonica* Maxim. after inoculation of root segments with *A. rhizogenes*, the characterization of hairy root clones, and tropane alkaloid production in the culture of hairy root clones.

**MATERIALS AND METHODS**

Chemicals. Scopolamine hydrobromide and L-hyoscyamine were purchased from Sigma Chemical Co., Ltd. (St. Louis, U.S.A.). Agropine and mannopine were each synthesized according to Tate et al.14) The structure of both compounds was confirmed by field-desorption mass spectrometry as reported previously.12) Agar purified, nutrient broth and casamino acids (vitamin free)
were purchased from Difco Laboratories (Detroit, U.S.A.). Ethylenediaminetetraacetic acid sodium iron(III) salt (FeNaEDTA) was obtained from Nakarai Chemicals (Kyoto, Japan).

**Bacteria and plant materials.** *A. rhizogenes* strains ATCC 15834, A4, NCPPB 1855 and NCPPB 2659 were used in the present study. These strains were each cultured in nutrient broth at 27°C. *S. japonica* Maxim. (about 50 cm in height) was provided by Dr. H. Kimura of Kanazawa University, Kanazawa, Japan.

**Culture media.** The following 9 basal media were used for the culture of root segments and hairy root clones of *S. japonica;* White (W),\(^{15}\) Linsmaier-Skoog (LS),\(^ {16}\) Heller (H),\(^ {17}\) Gamborg (G),\(^ {18}\) Nitsch (N),\(^ {19}\) Nitsch and Nitsch (NN),\(^ {20}\) Schenk–Hildebrandt (SH),\(^ {21}\) Kohlenbach–Schmidt (KS),\(^ {22}\) and Knop (K).\(^ {23}\) These basal media used were supplemented with 3% sucrose but no phytohormones, and adjusted to pH 5.8. For optimization of the culture conditions, the components and pH of these media were modified as follows. HF medium-supplement of H medium with 0.078 mM FeNaEDTA instead of FeCl\(_3\); HC medium-supplement of H medium with 1% casamino acids instead of NaNO\(_3\) (pH 7.0); SH-1 medium-adjustment of the pH of SH medium to 7.0.

**Establishment of hairy root clones.** Inoculation of sterilized root segments of *S. japonica* with *A. rhizogenes* and selection of adventitious roots formed on the root segments were carried out essentially as reported previously.\(^ {12}\) After 2 to 3 weeks of incubation of the inoculated root segments at 25°C in the dark, the numerous adventitious roots formed were removed individually and placed on 1% agar W medium. After 1 to 3 weeks of incubation at 25°C in the dark, root tips (0.5 cm long) of rapidly growing roots were excised and placed on 1% agar W medium. Rapidly growing roots without bacterial and fungal contaminations were transferred to a fresh medium every 4 weeks.

**Cultures of hairy root clones.** About 60 mg fresh weight (ca. 4.4 mg dry weight) of each of the 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 chromatography (TLC), as reported previously.\(^ {12}\)

**Analysis of tropane alkaloids.** Scopolamine and hyoscyamine in hairy root cultures (ca. 50 mg dry weight) were extracted with a 10 ml mixture of ethanol and 28% NH\(_4\)OH (19:1), essentially according to Yamada et al.\(^ {4}\) Extracts were applied onto a 5 cm Extralut-3® column (1.4 cm diameter, E. Merck A. G., West Germany) and eluted with 15 ml of CHCl\(_3\). The CHCl\(_3\) eluants were evaporated to dryness by a rotary evaporator at 40°C and the dry residues were dissolved in 0.2 ml of 70% ethanol. Contents of scopolamine and hyoscyamine in the ethanol solution were analyzed by high pressure liquid chromatography (HPLC) on a Resolve C18® stainless steel column (3.9 mm diameter × 15 cm long, Waters) at 40°C. After injection of 2.5 μl of a sample solution, the column was eluted with a mixture of 1% triethylamine-formic acid (pH 3.5) and ethanol (9:1) at a flow rate of 1 ml/min. The alkaloids were detected by measuring the absorbance at 254 nm. In this method, the lower limits of detection were 2.5 ng of scopolamine and 5 ng of hyoscyamine, respectively.

**RESULTS**

**Establishment of hairy root clones of *S. japonica***

*A. rhizogenes* strains 15834, A4, 1855 and 2659 were tested for their ability to induce hairy roots on a rhizome of *S. japonica* Maxim. After 7 to 10 days of incubation of the root segments treated with each of the strains, calli were slightly formed on the root segments. After 4 weeks, hairy roots emerged from the calli and grew extensively, as shown in Fig. 1. The strain 15834 was most active in induction of hairy roots among the tested strains. Therefore, this strain was used for further experiments. After 4 weeks, hairy roots emerged from the calli and grew extensively, as shown in Fig. 1. The strain 15834 was most active in induction of hairy roots among the tested strains. Therefore, this strain was used for further experiments. The number of hairy roots induced by the least active strain 2659 was 2 to 3% of that of strain 15834. No hairy roots were induced on uninoculated root segments during 4 weeks under the same incubation conditions as with the treated one. Tips of 1500 hairy roots induced by strain 15834 were each excised and cultured on 1% agar W medium for 3 to 4 passages every 4 weeks. About 10% of the total hairy roots tested continued to grow on the phytohormone-free medium. The growth of the other 90% of the roots was arrested on the same medium, although some roots grew slowly at the first transfer. In order to examine for the asepsis of the isolated hairy roots, the homogenate of each of the hairy roots in sterile distilled water was placed onto nutrient agar and incubated for a week at 27°C. The hairy roots which formed no bacterial col-
Fig. 1. Hairy Root Induction on Root Segments of *S. japonica* Inoculated with *A. rhizogenes*.
Root segments of *S. japonica* Maxim. were inoculated with *A. rhizogenes* strain 15834. The photograph was taken after 4 weeks incubation at 25°C in the dark.

Fig. 2. Selection of Hairy Root Clones of *S. japonica*.
Root tips (0.5 cm long) were excised from hairy roots formed on *S. japonica* root segments and cultured on 1% agar W medium for 3 weeks at 25°C in the dark.

Onies on the agar plates were designated as axenic hairy root clones.

**Characterization of hairy root clones**
A total of 125 hairy root clones were individually placed on 1% agar W medium and cultured for 4 weeks at 25°C in the dark. About 30% of the clones grew rapidly with extensive lateral branches, although the other 70% grew slowly with a few lateral branches, as shown in Fig. 2. Rapidly growing clones were selected and each of them was transferred to a fresh medium every 4 weeks. Their properties, such as rapid growth and lateral branching, were stably maintained during successive transfers.

Finally, 29 hairy root clones were established and each was cultured in H liquid medium, which was the most suitable one for the growth of clone S1 among the 9 basal media tested, and then assayed for opine production by PE and cellulose TLC. Figure 3 shows typical chromatograms of the opine assay. The results with 29 clones are listed in Table I. Both agropine and mannopine were found in 14 clones and mannopine but no agropine was detected in 9 clones. Six clones produced neither agropine nor mannopine. The pattern of opine production in these clones was stably maintained in subcultures for at least 6 months.

The hairy root clones cultured in H liquid medium were also examined for their growth rates and alkaloid contents as shown in Fig. 4. Marked differences were observed in the growth rate among the 29 clones. Of these, clone S22 grew most rapidly, followed in order by clones S44, S11 and S1. The growth index (harvest dry weight per inoculum dry weight at 4 weeks) of clone S22 was 52. On the other hand, the index of clones S31 and S52 was about 5. Thus the growth rate in
clone S22 was 10 times greater than that in S31 and S52.

The contents of scopolamine and hyoscyamine, which are major tropane alkaloids of *S. japonica*, were analyzed after the hairy root clones were cultured for 6 weeks in H liquid medium. Both alkaloids were found in all hairy root clones tested. Marked differences in the content of these alkaloids were found among the hairy root clones analyzed. Clone S1 contained the highest amount of scopolamine and the content was slightly higher than that of natural roots. The scopolamine content of the lowest clone S44 was about 5% of that of clone S1. On the other hand, clone S21 contained the highest amount of hyoscyamine and the content was about 4 times higher than that of natural roots. The hyoscyamine content of the lowest clone S32 was about 35% of that of natural roots. Alkaloid productivity was estimated by multiplying the alkaloid content by the biomass yield of hairy root clones. Clone S1 was the highest scopolamine-productive clone. On the other hand, clone S22 was the highest hyoscyamine-productive clone. The pattern of alkaloid content also varied among the hairy root clones examined, and mostly differed from that of natural roots.

**Tropane alkaloid production in cultures of hairy root clones**

When both clones S1 and S22 were cultured in H liquid medium, time-course changes of growth and tropane alkaloid contents were examined, as shown in Fig. 5. The growth of both clones reached a stationary phase at around 4 weeks. The growth rate of clone S22 was 2.5 times higher than that of clone S1. Clone S1 produced both scopolamine and hyoscyamine, and the content of hyoscyamine was higher than that of scopolamine in every growth phase. On the other hand, clone S22 produced a fairly large amount of hyoscyamine, but showed a very low level of scopolamine even in a late logarithmic growth phase.

To optimize culture conditions for growth and tropane alkaloid synthesis, clones S1 and S22 were each cultured in the 9 basal media

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### Table I. Opine Assay of Hairy Root Clones

<table>
<thead>
<tr>
<th>Hairy root clone</th>
<th>Opine detected</th>
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<tbody>
<tr>
<td></td>
<td>Agropine</td>
</tr>
<tr>
<td>S19, S21, S22, S32, S34, S35, S36, S40, S41, S44, S45, S46, S48, S50</td>
<td>+</td>
</tr>
<tr>
<td>S1, S11, S17, S31, S33, S47, S49, S51, S52</td>
<td>-</td>
</tr>
<tr>
<td>S2, S4, S14, S16, S20, S30</td>
<td>-</td>
</tr>
</tbody>
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+, positive; -, negative.
Tropane Alkaloids in Hairy Roots of *S. japonica*  

**Fig. 4.** Growth Rate and Alkaloid Production of *S. japonica* Hairy Root Clones.  
Twenty-nine hairy root clones were each cultured in H liquid medium for 6 weeks at 25°C in the dark. Growth index represents harvest dry weight (4 weeks) per inoculum dry weight. Productivity represents the amount of an alkaloid produced in root tissues for the incubation period per inoculum dry weight. Triangles show the content of scopolamine (▲) and hyoscyamine (△) in the rhizome of the original plant. ■, growth index; ▲, scopolamine; △, hyoscyamine; DW, dry weight.

**Fig. 5.** Time Course of Growth and Alkaloid Content of Hairy Root Clones S1 and S22.  
Clones S1 and S22 were cultured in H liquid medium for 8 weeks at 25°C in the dark. (a), growth of clones S1 (●) and S22 (○); (b), alkaloid content of clone S1; (c) alkaloid content of clone S22; (●), scopolamine; (○), hyoscyamine; DW, dry weight.
under various culture conditions. Optimum temperature (25°C), shaking speed (100 rpm) and light-irradiation (dark) conditions were established for both clones in H medium as described in Materials and Methods. The optimum basal medium for growth was H and SH for clones S1 and S22, respectively. When H medium was supplemented with 0.078 mM of FeNaEDTA instead of FeCl₃, the growth rate of clone S1 was remarkably increased, as indicated by HF in Fig. 6 (left column). On the other hand, when the pH of SH medium was adjusted to 7.0, the growth of clone S22 was increased, as indicated by SH-1. After 4 weeks culture, the weight of clone S1 was increased by 40 times on a dry weight basis in HF medium and that of clone S22 was increased by 102 times on a dry weight basis in SH-1 medium, respectively. These results indicated that the optimum medium for the growth of clones S1 and S22 was HF and SH-1, respectively.

On culture of clone S1 in H medium, the scopolamine content was affected by both the nitrogen source and pH of the medium. Addition of 1% casamino acids to the medium instead of NaNO₃ increased the scopolamine content by 3 times as indicated by HC in Fig. 6 (left column). Also, the optimum pH for scopolamine synthesis of clone S1 in HC medium was 7.0. From these results, the optimum medium for scopolamine synthesis of clone S1 was HC, in which the scopolamine content reached a level of 0.5% on a dry weight basis for 4 weeks culture. This content was 3 times higher than that of original roots (rhizome). For hyoscyamine synthesis of clone S22, K was optimum among the 9 basal media tested. Although the effect of the nitrogen source, pH and component concentration in K medium was examined, almost no increment was found in the hyoscyamine content of clone S22 (data not shown). In 4 weeks culture of clone S22 in K medium, the hyoscyamine content reached a
Tropane Alkaloids in Hairy Roots of *S. japonica*

level of 1.3% on a dry weight basis. This content was 8 times higher than that found in original roots. The alkaloid productivity was estimated by multiplying the growth index by the alkaloid content. The scopolamine productivity of clone S1 was highest in H medium and the hyoscyamine productivity of S22 was highest in SH-1 medium, as shown in Fig. 6 (right column).

DISCUSSION

Hairy root clones of *S. japonica* were established by selection of adventitious roots formed on root segments inoculated with *A. rhizogenes* strain 15834. The characterization of the established hairy root clones revealed that the clones showed marked differences from each other in growth rate, opine production and tropane alkaloid production. With respect to opine production, the established hairy root clones were classified into three types; clones producing both agropine and mannopine, clones producing mannopine but no agropine and clones producing no opine, although the hairy roots of *Kalanchoë tubiflora* induced by *A. rhizogenes* strain 15834 were reported to produce both agropine and mannopine. In the growth rate, there was a difference of 10 times between the most rapidly growing and the slowest growing clones. In addition, tropane alkaloid production was significantly different among the individual hairy root clones. By the screening of a number of hairy root clones, the highly scopolamine-productive clone S1 and the highly hyoscyamine-productive clone S22 were established. The pattern of alkaloid production in the hairy root clones was also varied, and different from that of original roots, although Flores and Filter reported that hairy root clones of *H. muticus* showed an alkaloid pattern similar to that of the original plants. These characteristics of *S. japonica* hairy root clones were stably maintained during 6 successive subcultures. The variances in alkaloid content and growth rate of each subculture were less than 10% (date not shown).

Byrne *et al.* reported that the transformed hairy roots of carrot contained multiple copies with various length of T-DNAs of Ri plasmid derived from *A. rhizogenes* strain 8196. In addition, tobacco plants regenerated from the hairy roots induced by *A. rhizogenes* strain A4 showed various morphological changes in leaves and flowers. Furthermore, Taylor *et al.* reported that an abnormal phenotype of the regenerated *Nicotiana* was correlated with the presence of TL-DNA integrated into the plant genome. Therefore, the various phenotypes of the hairy root clones of *S. japonica* may be attributable to differences in the length and copy number of T-DNAs integrated into the plant genome. In addition, expression of the integrated T-DNAs and/or their related genes may affect the growth and the synthesis of opine and alkaloids in the hairy root clones. However, an obvious correlation was not observed between the pattern of opine production and the growth rate or the production of tropane alkaloids in *S. japonica* hairy root clones.

Comparison of the characteristics of *S. japonica* hairy root clones revealed that the selection of a number of the hairy roots can be a useful method for the establishment of highly productive root clones for tropane alkaloid production. The productivity of the established clones S1 and S22 for scopolamine and hyoscyamine, respectively, was increased by optimizing the medium conditions. The supplement of H medium with FeNaEDTA increased the growth of clone S1. Also, the content of scopolamine in clone S1 was increased by supplementing H medium with casamino acids, which may act as precursors of tropane alkaloid synthesis, since L-phenylalanine and L-ornithine are precursors of these alkaloids. From these results, the optimum media for growth and scopolamine synthesis in clone S1 were HF and HC, respectively. Similarly, the optimum media for growth and hyoscyamine synthesis in clone S22 were SH-1 and K, respectively. Based on these efforts, it is reasonable to presume that a two-stage cultivation method combined
with both optimum media for growth and alkaloid synthesis may increase the productivity of scopalamine and hyoscyamine by cultures of clones S1 and S22, respectively.

REFERENCES

20) C. Nitsch and J. P. Nitsch, Planta, 72, 355 (1967).