Variation of alkaloid productivity among several clones of hairy roots and regenerated plants of *Atropa belladonna* transformed with *Agrobacterium rhizogenes* 15834

Toshio Aoki 1,.*, Hideki Matsumoto 2, Youichi Asako 3, Yuji Matsunaga 4, and Koichiro Shimomura 1

1 Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences, 1 Hachimandai, Tsukuba, Ibaraki, 305 Japan
2 Research Institute for Molecular Genetics, Tsumura and Co., 3586 Yoshiwara, Ami-machi, Inashiki, Ibaraki, 300-11 Japan
3 Research Planning Division, Marine Biotechnology Inst. Ltd., 2-35-10, Hongo, Bunkyo-ku, Tokyo, 113 Japan
4 Biochemistry Lab., Kanebo Ltd., 5-3-28 Kotobuki-cho, Odawara, Kanagawa, 250 Japan

* Present address: Department of Applied Biological Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa, 252 Japan

Received 28 February 1996/Revised version received 10 August 1996 - Communicated by A. Komamine

**Abstract.** Hairy root cultures of *Atropabelladonna* were established by transformation with *Agrobacterium rhizogenes* 15834. Five clones of them were employed to study the production of hyoscyamine, the main constituent of the plant, together with other tropane alkaloids. The growth and alkaloid production of each clone were differently affected by basal liquid culture media tested. The transgenic plants regenerated from each clone of the hairy roots had different phenotypes and diverse alkaloid productivity both in the cultured condition and in hydroponics.

**Key words:** *Atropa belladonna* - *Agrobacterium rhizogenes* - Hairy root cultures - Tropane alkaloids

**Abbreviations:** ANOVA, analysis of variance; B5 medium, Gamborg B5 medium; BA, N6-benzyladenine; B.S., Balanced Solution; dw, dry weight; EC, electric conductivity; fw, fresh weight; GC/MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; MS medium, Murashige and Skoog medium; NAA, naphthalene-1-acetic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TMS, trimethylsilyl; WP medium, Woody Plant medium

**Introduction**

Root cultures established by transformation with *Agrobacterium rhizogenes* is regarded as advantageous resource of useful compounds because of the rapid growth in culture media without phytohormones and the relatively high productivity of secondary metabolites compared to undifferentiated calli or cell suspensions, or, in some cases, the roots of the mother plants. A number of reports have described the production of useful phytochemicals by the transformed roots, normally referred to as hairy roots (Verpoorte et al. 1991; Sauerwein et al. 1992 and Saito et al. 1992).

The transformation with T-DNAs is expected to produce genetically different adventitious roots. The productivity of secondary metabolites depends on the genetic character of the hairy roots as well as the culture conditions such as the composition of the culture medium. Mano et al. (1986) reported the various phenotypes of *Scoparia japonica* hairy roots and suggested the importance of clone selection when aiming for the high production of tropane alkaloids. However, compared to a number of studies demonstrating the significance of culture media, relatively limited attention has been paid to the variation of the hairy roots which were derived from the same plant and the same strain of *A. rhizogenes*.

*Belladonna* (*Atropa belladonna* L.) is one of well-known plants which produce tropane alkaloids. Alkaloid production by transformed and non-transformed root cultures of this plant have been investigated by several researchers (Kamada et al. 1986; Jung and Tepfer 1987; Ondrej and Protiva 1987; Sharp and Doran 1990; Walton et al. 1990; Kitamura et al. 1992; Hashimoto et al. 1993 and those cited therein). In this study, we investigated the characteristics of the hairy roots and the regenerated plants of *A. belladonna* transformed with *A. rhizogenes* ATCC 15834 with the aim of clarifying the variation among the clones. Tropane alkaloid production was examined with five clones of the hairy roots, which were cultured in five kinds of basal media.

**Material and Methods**

**Root culture.** *Agrobacterium rhizogenes* ATCC 15834 harboring pRI 15834 was directly inoculated by a needle to the stem of *Atropa belladonna* L. cultured on phytohormone-
free Murashige and Skoog (MS) (1962) medium of which macro inorganic elements were reduced to a half (1/2-macro MS). The induced hairy roots were excised and cultured on 1/2-macro MS medium supplemented with 1.5% sucrose, 0.2% Gelrite (Kelco), and 0.5 g l⁻¹ Claroan (Hoechst Japan Ltd.) to eliminate the bacteria. The axenic hairy roots thus obtained were subcultured at 4-week interval on the same solid medium without antibiotics.

To test the alkaloid production, hairy roots were precultured in 50 ml liquid culture medium (100 ml Erlenmeyer flask) supplemented with 3% sucrose at 25°C in the dark on a rotary shaker at 100 rpm for 3 weeks. Two root tips (ca 20 mg fw) excised from the conditioned hairy roots were inoculated into the same fresh medium and cultured under the same conditions.

**Plant regeneration.** Root segments (ca 1 cm long) were cultured on 1/2-macro MS solid medium supplemented with 1.5% sucrose, 0.5 μM NAA, 5 μM BA, and 0.2% Gelrite (Kelco) in a petri dish under 16 h day⁻¹ light (60 μmol m⁻²s⁻¹) at 25°C. After 4-6 weeks, the adventitious shoots regenerated from the root segments were isolated and transferred onto hormone-free 1/2-macro MS solid medium supplemented with 3% sucrose, 0.1% Gelrite, and 0.5% agar in a growth tube (i.d. 40 mm). The regenerated plants thus established were propagated by subculturing of nodal segments (ca 1.5 cm) on the same hormone-free medium at 6 to 8-week interval.

**Hydroponic cultivation.** The regenerated plants were cultivated hydroponically with a hydroponic apparatus placed in a growth chamber (Hitachi, Japan) and a hydroponic medium named Balanced Solution (B.S.) composed of 8 mM K⁺, 5.2 mM Ca²⁺, 2 mM Mg²⁺, 0.7 mM NH₄⁺, 12.6 mM NO₃⁻, 2.1 mM SO₄²⁻, 2 mM PO₄³⁻, 59 μM Fe³⁺, 10 μM Mn²⁺, 0.7 μM Zn²⁺, 0.1 μM Mo⁷⁺, 0.2 μM Cu²⁺, 49 μM BO₃⁻, and 1 μM 1' (Shimizu 1977). The total ion strength of the medium was adjusted with water by monitoring the electric conductivity (EC). In vitro plantlets (4-6 weeks after the subculture, the aerial parts were 5-10 cm long with 4-9 leaves), a half number of leaves removed, were transferred to the hydroponics and acclimatized with B.S. adjusted to pH 6.0 and EC of 0.05 Ω⁻¹m⁻¹ under 11 h day⁻¹ light (150-180 μmol m⁻²s⁻¹) at 20°C for 7 days. For the first 3 days, each plant was covered with a transparent plastic cap. The relative humidity was adjusted to more than 70%. The acclimatized plants were cultivated under B.S. adjusted to pH 6.0 and EC of 0.05 Ω⁻¹m⁻¹ or 0.24 Ω⁻¹m⁻¹ and the temperature was 25°C.

**Opine assay.** Agropine and manнopine were detected by paper electrophoresis as described by Petit et al. (1983).** PCR amplification.** The primers for TL-DNA were 5'-ATGGAGATTAGCCCGGACTAACGCO-3' and 5'-ATGGAGATCCEAAATTGTGCTTGCACGTGCAAg-3', which are complementary to 5' coding sequence of rol A and 3' coding sequence of rol B, respectively, and those for TR-DNA were 5'-CGGAAAAATTTGCTGTTGCAGGCGCCGAAGTT-3' and 5'-AATCGTTCCAGAGGCGCGAGGC-3', complementary to 5' and 3' flanking sequences of ags gene, respectively (Slightom 1986). Template DNA for polymerase chain reaction (PCR) was extracted from 10-20 mg of fresh hairy roots or non-transformed roots according to Edwards et al. (1991). For the positive control of TL and TR-DNAs, the relevant cosmids pLJ 1 and pLJ 85 (Jouanin 1984) were used as the templates, respectively. The initial denaturation was carried out at 94°C for 6 min, followed by 35 cycles of amplification (denaturation at 90°C for one min, annealing at 55°C for two min, and extension at 72°C for three min) and the final extension at 72°C for 7 min. The PCR reaction mixture was electrophoresed on 0.8% agarose gel and visualized by ethidium bromide staining and UV light (312 nm).

**Culture media.** The basal liquid media used for the investigation of alkaloid production were as follows: MS, a half strength MS medium, which consisted of a half concentration of inorganic elements (1/2 MS), Gamborg B5 (Gamborg et al. 1991) (B5), Woody Plant (Lloyd and McCown 1980) (WP), and Balanced Solution supplemented with the same minor organic elements as MS medium (BL). The culture media, supplemented with 3% sucrose, were adjusted to pH 5.7 and autoclaved at 121° C for 15 min.

**GC/MS analysis of tropane alkaloids.** Dried alkaloid fraction prepared as described previously (Kamada et al. 1986) was mixed with 10 μl bis (trimethylsilyl) trifluoroacetamide and heated at 60°C for one hour. The TMS-derivatized sample was dissolved in dichloromethane and applied to GC/MS equipped with a DB-1 column (0.25 mm x 30 m). The column temperature was isothermal for 2 min at 70°C, increased to 300°C at 10°C/min, and held at 300°C for 5 min. Littorine (retention time, 20.46 min; m/z 361, 124, 94, 82, 72, 41) was identified in comparison with the authentic sample (Sauerwein et al. 1991).

**Determination of tropane alkaloids by HPLC.** About 50 mg of lyophilized sample was extracted with 5 ml of CHCl₃ - CH₃OH - 28% NH₄OH (15 : 5 : 1, by vol.) (Kamada et al. 1986). An appropriate volume of the alkaloid fraction dissolved in CH₃OH was injected into ODS 120A column (4.6 x 250 mm, TOSOH, Japan) and eluted with CH₃CN - 10 mM SDS solution (adjusted to pH 3.3 with phosphoric acid) (2 : 3) (Shimomura et al. 1991). 6β-Hydroxyhyoscyamine (1), scopoline (2), hyoscyamine (3) and littorine (4) were simultaneously quantified (retention time, 14.1, 14.8, 18.7, 21.9 min, respectively). Significant effect of the culture medium was tested by analysis of variance (ANOVA) at 5% level of significance.

**Results and Discussion.**

**Establishment of hairy root culture.**

Direct infection of Agrobacterium rhizogenes and antibiotic treatment yielded 35 clones of axenic adventitious roots, of which transformation were proved by the detection of opines. Further experiments were carried out with five clones, #6, #14, #16, #20, and #33. Clone #6 was classified in the group of rapid growth (elongation more than 3.5 mm day⁻¹) on 1/2-macro MS solid