Plant Cell Reports (1986) 5:239–242

Plant Cell Reports

Alkaloid production by hairy root cultures in *Atropa belladonna*

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Received January 14, 1986 / Revised version received June 10, 1986 – Communicated by F. Constabel

Abstract

Hairy roots were induced by inoculation of stems of sterile plants of Atropa belladonna with Agrobacterium rhizogenes. The axenic culture of the hairy roots isolated from the stems proliferated 60 fold as based on the initial fresh weight after one month of culture. The presence of atropine and scopolamine in hairy roots were examined by TLC and HPLC. Their amounts were analyzed by GLC. The results show that the amount of the two alkaloids in the axenic cultures was the same as or even higher than those of normal plants grown in the field.

Introduction

The large-scale production of secondary metabolites such as pigments and alkaloids by field grown plants has been limited due to the dependency of the metabolism on rate of propagation, cultivation of plants, and climate during the cultivation. Thus, numerous investigators have tried to utilize plant tissue culture techniques to minimize these disadvantages. In spite of these efforts, the large-scale production of only few substances such as shikonin has succeeded (Fujita et al. 1981). Of several alkaloids which are thought to be mainly produced in roots of plants, Szőke et al. (1982) reported the production of atropine and scopolamine by callus cultures. The production of these alkaloids by callus culture, however, is generally difficult (Bhandary et al. 1969, Hiraoka and Tabata, 1974, Hashimoto and Yamada, 1983). It seems that the biosynthesis of such alkaloids is correlated with the organization of cells as root.

Recently, it has been clearly demonstrated that the Ri plasmid present in Agrobacterium rhizogenes causes the transformation of plant cells by introducing T-DNA of the Ri plasmid into genomic DNA of plant cells and that on a hormone-free medium the transformed plant cells give rise to massive roots, so called hairy roots (White and Nester,1980, Chilton et al. 1982, Tepfer, 1984). The transformed plant cells can produce hairy roots even after Agrobacterium is eliminated.

In this report, we demonstrate the induction of hairy roots, establishment of the culture, and production of alkaloids by the hairy root culture in Atropa belladonna.

Materials and Methods

Induction of hairy roots

Sterile plants of Atropa belladonna L. were established by shoot tip culture of field-grown plants and maintained by repeated shoot culture on hormone-free Murashige and Skoog's (MS) medium under 25°C, 18h light/6h dark, 4,000 lux. Agrobacterium rhizogenes strain 15834 harboring Ri plasmid (pRi 15834) grown on YEB agar medium (Vervliet et al. 1975) was inoculated by a needle onto stems of the sterile plants.

Culture of the hairy roots

Two to five weeks after inoculation, hairy roots appeared on the inoculated sites. Segments of the hairy roots were cut off and cultured on hormone-free MS agar (1 %) medium containing antibiotic (carbenicillin, 1 mg/ml). After several passages onto MS medium, segments of the growing hairy roots were transferred and maintained on hormone-free MS agar medium without antibiotic. To examine the proliferation rate, segments of the hairy roots grown on the MS agar medium were transferred into hormone-free MS liquid medium and the fresh weight was measured before and after a four-week culture under 25 °C, continuous dark, 100 rpm.

Analysis of atropine and scopolamine

Extraction : Hairy roots harvested after 2-4 weeks of culture were lyophilized and ground. The powdered sample (40-460 mg) was

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weighed and subjected to the extraction of alkaloids. An appropriate volume of $CHCl_3$-MeOH-NH_4OH (15:5:1)(10 ml extraction solvent/100 mg dry sample) was added to the weighed sample, sonicated for 10 min, then kept at room temperature for 1 h (Kagei et al. 1978). After filtration, the residue was washed twice with 1 ml of $CHCl_3$. The pooled filtrate was evaporated to dryness. To the residue, 5 ml of $CHCl_3$ and 2 ml of 1N H_2SO_4 were added, then the solution was mixed well. The $CHCl_3$ phase was removed and the H_2SO_4 phase was adjusted to pH 10 with 28% NH_4OH in an ice-bath. From the solution, alkaloids were extracted once with 2 ml and twice with 1 ml of $CHCl_3$. The combined extracts were filtered after adding anhydrous Na_2SO_4 and then the residue was washed with 1 ml of $CHCl_3$. The combined filtrates were evaporated to dryness at 40°C.

TLC analysis : The alkaloid fraction thus obtained was dissolved in an appropriate volume of MeOH, applied on Silica gel plate (60 F_{254}, Merk), and then analyzed with two different developing solvent systems, EtOAc-2PrOH-10%NH_4OH (9:7:3) and $CHCl_3$-acetone-MeOH-28%NH_4OH (75:10:15:2). The alkaloidal spots were detected with Dragendorff's reagent (Wagner et al. 1984).

HPLC analysis : The alkaloidal fraction was analyzed in the Trirotor V HPLC (Japan Spectroscopic Co., Ltd. Tokyo) equipped with a UV detector (model UVIDEC-100-V; Japan Spectroscopic Co., Ltd. Tokyo) and all data processing was done with SIC Chromatocorder 11(System Instruments Co., Ltd. Tokyo). An appropriate volume of the sample dissolved in MeOH was injected into a TSK gel ODS-120T (Toyo Soda Manufacturing Co., Ltd. Japan) column (5 μm, 25cm x 4.6 mm internal diameter) and eluted with a solution composed of 48 volumes of MeOH and 52 volumes of 10 mM sodium 1-heptanesulfonate in H_2O (pH 4.0 with acetic acid)(Yamada et al. 1984). The flow rate was 1.0 ml/min throughout the analysis. The effluent was monitored by measuring the absorbance at 215 nm.

GLC analysis : To the dried alkaloidal fraction, 50 μl of BSA (Wako Pure Chemical Industries, Ltd. Japan) was added and heated in a dry block heater at 42°C for 15 min. After a brief period at room temperature, the reaction mixture was diluted with an appropriate volume of hexane and, just prior to analysis, cocaine was added as an internal standard (25 μl/ml). Gas liquid chromatography (GLC) was performed on a Shimadzu GC-9A (Shimadzu Co., Japan) equipped with a flame thermoionic detector (FTD), using a glass column (3 mm x 2 m) packed with 5% OV-17 on Supelcoport (100-200 mesh: Supelco, Inc.). The GLC conditions were as follows; injection temperature, 230°C; detector temperature, 260°C; helium as carrier gas at a flow rate of 50 ml/min.

GC-MS analysis : Identification of alkaloids was done by mass spectrometry. The GC-MS conditions were as follows; a 2 m x 3 mm glass tube packed with 3 % silicon OV-101, column temperature of 180-240°C (temperature program, 5°C/min; isothermal at 240°C), helium as the carrier gas at a flow rate of 40 ml/min, TIM as the detector, and an ionizing energy of 20 eV. TMS-atropine; m/e (rel. int.) 361 [M+] (41), 140 (5), 124 (100), 95 (6), 94 (6), 83 (14), 82 (15), 73 (22). TMS-scopolamine; 375 [M+] (67), 193 (30), 154 (23), 138 (100), 128 (3), 108 (33), 103 (10), 94 (63), 73 (46).

Detection of opines

Extraction and detection of opines (agropine and mannopine) were performed by the method of Petit et al (1983). Hairy roots (ca. 50 mg fresh weight) were ground in an Eppendorf tube with 50 μl of distilled water using a glass rod. The extracts were centrifuged at 12,000 rpm for 2 min. Ten μl of the supernatant were spotted on a Whatman 3MM paper and then the paper was electrophoresed at the constant voltage of 20 V/cm. The buffer used for the electrophoresis was formic acid (99%), acetic acid (99.5%) and distilled water (1:3:16, V/V/V)(Otten and Schilperoort, 1978). After drying the paper, opines were visualized by alkaline silver nitrate reagent (Trevelyan et al. 1950).

Results and Discussion

The bacteria cultured on YEB agar medium for 3-7 days were inoculated on internodes of the sterile plants. After 2 to 4 weeks numerous hairy roots appeared on the inoculated site (Fig. 1A) and grew extensively. The elongating roots were cut off and put on hormone-free MS agar medium containing carbenicillin (1 mg/ml). After several days of culture, elongating root tips were cut off and transferred to hormone-free MS agar medium without carbenicillin. This procedure was repeated several times until no colony of bacteria appeared. After transfer of the axenic hairy roots into the MS liquid medium (70 ml) in 100 ml Erlenmeyer flasks or onto solid MS agar medium in petri dishes they grew rapidly and exhibited extensive lateral branching (Fig. 1B and 1C). As a control experiment, adventitious roots formed at the cut end of sterile plants were cultured on hormone-free MS agar medium. However, these roots grew slowly and no lateral branching appeared. Pieces of the established hairy root cultures were subcultured at 3-4 week intervals on liquid or agar-solidified MS medium without phytohormones for one year. The average weight increase of the hairy roots in liquid MS medium in five separate experiments was about 60 fold in one-month cultures.

Agropine and mannopine are well known opines detectable in hairy roots transformed with Ri plasmid (Petit et al. 1983). To examine whether transformation with the Ri plasmid had occurred, presence of agropine and mannopine in extracts of the hairy roots was examined by paper electrophoresis, followed by alkaline silver nitrate staining. Both agropine and mannopine were detected in the extracts of the freshly isolated hairy roots, but not in those of the hairy roots after a prolonged culture (Fig. 2). This tendency of disappearance of agropine and mannopine during long term culture of hairy roots has also been reported in tobacco and carrot, even when full length of the T-DNA of Ri plasmid can be detected by Southern-blot hybridization in genomic DNA of the hairy roots after prolonged culture (Tepfer, 1984). Although agropine and mannopine were not detected after prolonged culture of hairy

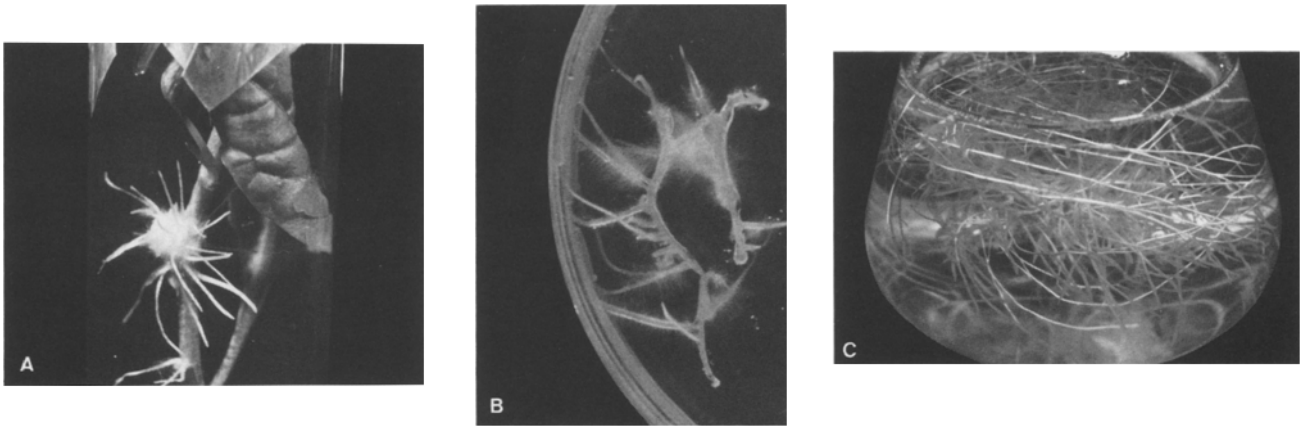

Fig. 1. A:Hairy roots on stem segments. B:Hairy roots grown on hormone-free MS agar medium. C:Hairy roots grown in hormone-free MS liquid medium.

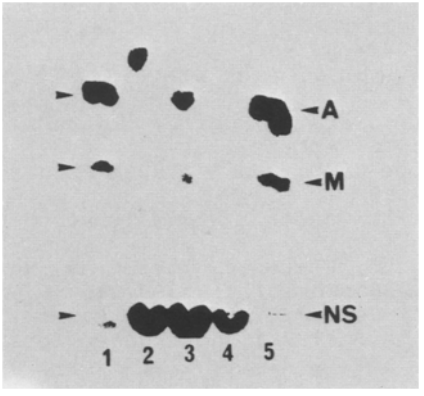

Fig. 2. Detection of agropine and mannopine in the extracts of the hairy roots. A;agropine, M;mannopine, NS;neutral sugar. Lanes 1 and 5;Standard marker, Lane 2;Non-transformed control roots, Lane 3;Freshly isolated hairy roots, Lane 4;Hairy roots after repeated sub-cultures (one year).

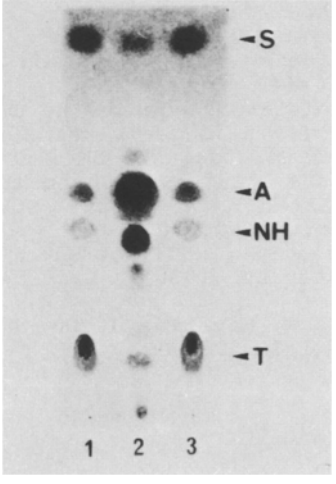

Fig. 3. TLC pattern of the alkaloidal fraction. S;scopolamine, A;atropine, NH;norhyoscyamine, T;tropine. Lanes 1 and 3;authentic compounds, Lane 2;extracts of the hairy roots.

Table 1. Alkaloid content of hairy roots

	alkaloid content (% dry wt. ± SD) atropine	scopolamine
NR-field[1)]	0.340 ± 0.031	0.008 ± 0.005
NR[2)]	0.031 ± 0.004	trace
HR[3)]	0.371 ± 0.013	0.024 ± 0.010

1) Normal roots from one-year old plants grown in the field (harvested in autumn).
2) Normal roots from plantlets cultured in vitro.
3) Hairy roots cultured for 4 weeks in liquid medium.
Each value was obtained from five independent experiments.

roots in this report, other phenotypic features of the hairy roots, such as rapid growth and extensive lateral branching, were maintained. When the hairy roots of Atropa belladonna were cultured on MS medium containing phytohormones, plantlets regenerated and exhibited characteristic features, such as wrinkled leaves, shortened internodal length, and active adventitious root formation (data not shown here). These phenotypic features observed in the regenerated plants have also been reported for tobacco, carrot and other plant species (Tepfer, 1984, Ooms et al. 1985). From these facts, it seems likely that the Ri T-DNA is conserved during prolonged culture of Atropa hairy roots, though agropine and mannopine could not be detected.

By using the established hairy root cultures, production of tropane alkaloids, such as atropine and scopolamine, was examined. By TLC analysis, two spots corresponding to scopolamine and atropine were detected on the TLC plate (Fig. 3). The results of TLC analysis indicated that the hairy roots produced alkaloids resembling those of normal plants grown in the field. The alkaloids produced in the hairy roots

were further analyzed by HPLC. The identification of these alkaloids as scopolamine and atropine was performed by co-chromatography with standard compounds. Two peaks separated by HPLC were isolated and confirmed as atropine and scopolamine by TLC analysis. Atropine and scopolamine in hairy roots were also identified with GC-MS. Next, the contents of scopolamine and atropine were examined by GLC. As shown in Table 1, GLC analysis of the root extract also demonstrated the presence of scopolamine and atropine. Although the roots of the normal plantlets in vitro contained these alkaloids, the concentrations were lower than those of the normal plants grown in a field. In contrast we found that the hairy roots contained scopolamine and atropine at concentrations of 0.024 %dW and 0.371 %dW, respectively. The concentration of atropine in the hairy roots was comparable to that of the normal plants grown in a field (Table 1). The production of tropane alkaloids, such as atropine and scopolamine, has been examined in callus cultures of various plant species, but large-scale production has been unsuccessful in almost all cases (Deus-Neuman and Zenk, 1984, Hiraoka and Tabata, 1974, Hashimoto and Yamada, 1983). Recently, successfull production was reported for cell lines which originated from roots (Szőke et al. 1982) and for cell lines resulting from selection (Deus-Neuman and Zenk, 1984). However, these selected cell lines produced the alkaloids in high amounts only during the selection period, and the amounts became lower after several sub-cultures under non-selective condition. As compared with these experiments, the hairy root culture system established in this report constantly produced atropine and scopolamine under non-selective conditions during a prolonged period (at least one year and possibly for several years) in the same amount as or slightly higher amounts than those in roots of plants grown in the field. It was reported that in root cultures grown in medium containing a low concentration of auxin tropane alkaloids were produced in comparable amounts to those of the intact plants (West et al. 1957, Bhandary et al. 1969, Hashimoto et al. 1983, Tabata et al. 1972). These results indicate that the synthesis of tropane alkaloids seems to be closely correlated to morphological differentiation as root. Thus, hairy root cultures may be a useful system for large - scale production of these alkaloids. In our recent work, this system has been applied successfully to other plant species, such as Scopolia, Datura, and Hyoscyamus (data not shown). We are now examining the optimum conditions necessary for rapid growth of hairy roots and high production of the alkaloids.

Note added in proof : We learned that some comparable results in Hyoscyamus muticus are being obtained by Drs. H. E. Flores and P. Filner at Louisiana State University after the preparation of this manuscript.

Acknowledgment

We thank Mr. T. Tanaka for critical reading of this manuscript. Utilization of cocaine during GLC analysis was done under control of Dr. H. Shiomi of Fukuyama Univeristy.

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