Production of Phytoecdysteroids by Hairy Root Cultures of *Ajuga reptans* var. *atropurpurea*

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Over 20 'hairy root' clones of *Ajuga reptans* var. *atropurpurea* (Labiatae) were obtained from transformation with *Agrobacterium rhizogenes* MAFF 03-01724. Growth, opine synthesis and production of phytoecdysteroids in the clones have been examined. Mikimopine, a specific opine synthesized in the tissue transformed with strain MAFF 03-01724, was detected in most of the clones, and four phytoecdysteroids, 20-hydroxyecdysone, norcyasterone B, cyasterone and isocyasterone, were detected in all of the clones.

It was found that the production of phytoecdysteroids was closely related to the growth of hairy roots, by monitoring the changes in the culture. One of the rapidly growing and well branching hairy root lines, Ar-4, was selected and used for fermenter experiments. On an air-lift type culture of Ar-4 for 45 days, the weight of the root tissue was increased by 230 times and the content of 20-hydroxyecdysone reached 0.12% on a dry weight basis. This content is 4 times higher than those of the original roots.

The phytoecdysteroids are polyhydroxysteroids containing a 5β -H-7-ene-6-one system commonly hydroxylated at sites 2β , 3β , 14α , and usually $20R$, $22R$ (see structures 1–4 in Fig. 3). In the past two decades, a number of these steroids have been isolated from plants. Several hundred species of plants have been surveyed to date and the structures of more than 40 phytoecdysteroids are known. Among them, 20-hydroxyecdysone (β -ecdysone, 1), a principal physiological inducer of molting and metamorphosis in arthropoda, is common in these plants.¹⁾ Physiological properties of phytoecdysteroids in insects and in mammals have also been studied in many laboratories, and demonstrated the possibility of their practical use for the pest control,²⁾ chemotherapy³⁾ and silk production.⁴⁾

Despite the wide distribution of these steroids in the plant kingdom and their potential for the applications, little is known about the physiology of their production in plants. One of the reasons is the lack of suitable experimental systems for the study of their biosynthesis and accumulation in plants.

Cultured tissue or cells are sometimes a powerful tool to study secondary metabolism, but in case of undifferentiated cells they often produce components in low amounts compared to intact plants. Continuous cell selection to keep the genetic or epigenetic stability of callus tissue is also laborious. There were several tissue culture experiments with plants containing phytoecdysteroids,^{5,6)} but these problems are still unsolved.

On the other hand, the potential of 'hairy root' for the production of various secondary metabolites has recently been explored, showed that hairy roots have a high level of productivity in addition to genetic and biochemical stability.⁷⁾ Hairy roots are induced upon inoculation of *Agrobacterium rhizogenes* into the plants, and are cultured in hormone-free medium.

To study phytoecdysone production, hairy root lines have recently been established with several phytoecdysone-producing plants, such as *Ajuga reptans* var. *atropurpurea*, *Achyrantes fauriei*, *Pfaffia iresinoides* and *Vitex strickeri*.^{8,9)}

In this paper, we demonstrate the establishment of a rapidly growing and highly steroid-producing clone **Ar-4** obtained from *A. reptans* var. *atropurpurea* by the transformation with *A. rhizogenes* MAFF 03-01724. This report will also describe the relationship between growth of hairy roots and their steroid production, and the scale-up culture of **Ar-4** in an air-lift type fermenter.

Materials and Methods

Plant material. *Ajuga reptans* var. *atropurpurea* used in this study were greenhouse-grown plants. The original plant was collected in Himeji and kindly identified by Professor Shohei Kokawa, Faculty of Science, Osaka City University, Japan.

Callus culture. The callus of *A. reptans* var. *atropurpurea* was induced from leaf disks on MS medium containing 1 ppm of NAA. The callus have been subcultured at 4-week intervals and maintained over 3 years.

Bacterial strain. *Agrobacterium rhizogenes* MAFF 03-01724¹⁰⁾ was used for hairy root initiation. Bacteria were maintained at 4°C on 15 g/l of nutrient agar medium (Sigma Chemical Co., Ltd.) and cultured in 30 ml of Luria broth¹¹⁾ at 28°C on a rotary shaker one day before inoculation of plants.

Inoculation. Inoculation of plants was done as described previously with several improvements.¹²⁾ Leaves of *Ajuga* were soaked in a suitable concentration of commercially available detergent, and washed thoroughly with tap water. Then the leaves were sterilized by immersion in 10% sodium hypochlorite for 7 min, rinsed in an excess of sterile water for over 1 hr, and cut into 10 mm × 10 mm of leaf disks. These disks were soaked in the bacterial suspension ($1-10 \times 10^8$ /ml) for 5 min, wiped with filter paper, and then placed on water agar (1%) plates and incubated at 25°C under fluorescent light. Three days after inoculation, disks were transferred to MS medium, which was solidified by 0.2% Gellungum (San-ei Chemical Corp.) containing carbenicillin (500 µg/ml) and vancomycin (500 µg/ml), and subcultured every month.

Culture of hairy roots. After incubation for 10 days, initiated roots were cut 10 mm from the root tip, and transferred to the fresh MS medium as described above. At two-week intervals, elongated roots were transferred. After several transfers for the elimination of bacteria, hairy roots were cultured on the same medium without antibiotics at 25°C in the dark. Liquid culture was made as follows: 30–50 mg of fresh hairy roots were inoculated

in 100 ml flasks containing 50 ml of MS medium and cultured on a rotary shaker (150 rpm) at 25°C in the dark. The growth ratio was calculated as the ratio between inoculated weight and harvested weight on a fresh weight basis.

Opine assay. Hairy roots transformed by MAFF 03-01724 produced mikimopine,¹³⁾ which was stained with Pauly reagent. The hairy roots (50 mg) were homogenized in 50 µl of water with a glass rod and centrifuged at 12,000 rpm for 5 min. The supernatant (5 µl) was spotted on a filter disk (ϕ 5 mm), then put onto a filter paper (Whatman 3MM) and electrophoresed for 30 min. Formic acid–acetic acid–water (5:15:80), pH 1.8 or ammonium carbonate (5 g/l) adjusted to pH 9.8 with 0.5 N ammonia was used for the electrophoresis buffer, and Pauly reagent was used for staining. A quick opine assay,¹⁴⁾ the method of squeezing a root tip on a filter paper for electrophoresis, was useful for the selection of opine-containing clones from many root samples. Synthetic mikimopine as a standard was a kind gift from Dr. Akira Isogai (Department of Agricultural Chemistry, The University of Tokyo).¹⁵⁾

Isolation of phytoecdysteroids. Hairy roots and original plant roots were dried (50°C, 3 hr), then extracted by sonication in MeOH. After evaporation of the MeOH, the residue was redissolved in water and partitioned with *n*-butylacetate. The aqueous layer was then extracted with *n*-butanol. Four phytoecdysteroids were separated from the concentrated *n*-butanol extracts using preparative TLC (precoated silica gel plate: Merck Kieselgel 60 GF₂₅₄, CHCl₃:MeOH:H₂O = 13:7:4), and further purified by preparative HPLC (reversed phase column: Cosmosil 5C₁₈, CH₃CN:MeOH:H₂O = 18:2:80). Structures were identified spectroscopically [UV(EtOH), IR (CHCl₃), MS(FD), ¹H-NMR (270 MHz, C₅D₅N), ¹³C-NMR (50 MHz, C₅D₅N)] and confirmed by comparison with those of authentic samples.

Quantitative analysis of 20-hydroxyecdysone. Dried root samples (20 mg) were extracted and fractionated as described above. Liquid media were directly extracted with *n*-butanol. The *n*-butanol extracts were dissolved in MeOH (0.5 ml) and chromatographed by HPLC (Nucleosil 5C₁₈ column, CH₃CN:MeOH:H₂O = 18:2:80) and the UV absorption was monitored at 242 nm.

Results and Discussion

Establishment of hairy root culture

Hairy roots of *Ajuga reptans* var. *atropurpurea* were initiated from the petiole side of the vein of leaf disks 2 weeks after inoculation of *A. rhizogenes* MAFF 03-01724. Hairy roots cut from leaf disks grew slowly when they were

cultured on MS medium containing antibiotics and shoots were sometimes regenerated directly from the base side of hairy roots. After several transfers for elimination of bacteria, hairy roots grew rapidly with lateral branching on MS medium without antibiotics, and over 20 hairy-root clones were obtained.

The evaluation of the growth rate on each clone was first made by the observation of elongation and branching as shown in Fig. 1. The clones showing rapid elongation, **Ar-4, 9,**

Fig. 1. Growth Features of *A. reptans* var. *atropurpurea* Hairy Root Clones Induced by *A. rhizogenes* MAFF 03-01724 on Solid Medium.

A root tip (1 cm) of each clone was excised, placed, and cultured for 10 days at 25°C in the dark. Elongation (—), elongation of root per day (mm/day); branches (+), root with more than 1 branch per cm.

11 and **24**, grew 4–5 mm per day with extensive branching. The differences of growth rate among clones were also examined by liquid culture. As shown in Table I, clones **Ar-6** and **12** without branching did not grow well. In contrast, the clones with lateral branching grew well and the growth ratio of the fastest clone, **Ar-22**, was 240-fold after 4 weeks of culture in a 100 ml flask. The degree of elongation on solid medium (Fig. 1) had no correlation with the growth in the liquid medium by these limited data. After successive culture in the absence of phytohormones for over 6 months, six clones, **Ar-4, 8, 9, 11, 22** and **24**, which grow rapidly with lateral branching were selected. Cultured under light, the roots were slightly greenish, and their proliferation was rather inhibited. The selected hairy roots were subcultured on MS solid medium at 25°C in the dark.

Analysis of opine

When a homogenate of hairy roots transformed by MAFF 03-01724 was electrophoresed on filter paper under basic conditions (5 g/l ammonium carbonate, adjusted to pH 9.8 with ammonia) and then stained with Pauly reagent, a pink-red spot was detected. The pink-red spot from the hairy roots showed the same mobility as that of synthetic mikimopine. Mikimopine was detected in the six clones selected, and in about 70% of clones obtained from *Ajuga*

Table I. GROWTH AND 20-HYDROXYECDYSONE PRODUCTION IN HAIRY ROOTS CULTURED IN LIQUID MEDIUM

Clone No.	Branches	Growth ratio	Content in dry tissue (%)	Content in medium (μg/ml)
4	+	220	0.14	1.0
8	+	180	0.13	0.5
9	+	150	0.073	ND
22	+	240	0.066	ND
6	—	60	0.019	ND
12	—	14	0.019	0.35
Roots of original plant			0.03	

ND, not detected; +, root with more than 1 branch per cm; —, root with less than 1 branch per cm. Inoculated 30–50 mg of hairy roots into each 50 ml of MS liquid medium and cultured for 30 days. Original plant was grown in a greenhouse for a year after division to multiply.

Fig. 2. Paper Electrophoregram of Extracts from *Ajuga* Hairy Roots Induced by *A. rhizogenes* MAFF 03-01724.

A, basic conditions (5 g/l ammonium carbonate, pH 9.8); B, acidic conditions (formic acid–acetic acid–water=5:15:80, pH 1.8).

C, control roots; T, hairy roots; M, authentic mikimopine. The arrows mark the position of mikimopine spots.

leaves (Fig. 2). It should be noted that the pink-red spot of mikimopine was doubled if electrophoresis was done under acidic conditions (pH 1.8, formic acid–acetic acid–water=5:15:80). This behavior of the opine on paper electrophoresis is characteristic of mikimopine and its lactam.¹⁵⁾

Identification of phytoecdysteroids in *Ajuga reptans* var. *atropurpurea*

Although the chemical constituents of *Ajuga reptans* had been investigated,¹⁷⁾ that of *A. reptans* var. *atropurpurea* has not been reported.

A methanol extract of fresh roots (1.5 kg) was fractionated in the usual manner to give the steroid fraction. Four phytoecdysteroids, 20-hydroxyecdysone (**1**, 70 mg), norcyasterone (**2**, 32 mg), cyasterone (**3**, 22 mg) and isocyasterone (**4**, 8 mg), were obtained from the steroid fraction by preparative TLC and preparative HPLC.

All of their FD-MS showed peaks at m/z 363 and 345 due to the fission between C-20 and C-22, which were the most characteristic peaks of phytoecdysteroids. Side chain fragment ion peaks were also diagnostic.¹⁾ 20-Hydroxy-

Fig. 3. Structures of Phytoecdysteroids and HPLC Traces of Their Fraction from Callus (A), Hairy Roots (B) and Original Roots (C).

Column, RP-C₁₈; mobile phase, CH₃CN:MeOH:H₂O=2:18:80; flow rate, 1 ml/min; detection, 242 nm. *n*-Butanol extract dissolved in MeOH (10 μ l) was used.

1, 20-hydroxyecdysone; 2, norcyasterone; 3, cyasterone; 4, isocyasterone.

ecdysone (1), a C₂₇ steroid with three OH groups, gave peaks at m/z 161 (C-17/C-20) and 117 (C-20/C-22). The side chain mass fragments of C₂₉ steroids were as follows: norcyasterone (2); m/z 187 (C-17/C-20), 143 (C-20/C-22), cyasterone (3) and isocyasterone (4); m/z 201 (C-17/C-20), 157 (C-20/C-22). Other spectral data (IR, UV, ¹H-NMR and ¹³C-NMR) of steroids we isolated were identical with those reported previously.^{17,18)}

Phytoecdysteroid production by hairy roots

Four phytoecdysteroids, 1–4, were detected in all of hairy root clones obtained, and the relative proportions of these steroids in the clones were similar to that of original roots as shown in Fig. 3. In contrast, phytoecdysteroids in the callus tissue induced from same original plant were only traces. Among these four phytoecdysteroids, 20-hydroxyecdysone (1) was most abundant. For the quantitative evaluation of phytoecdysteroid production in

Fig. 4. The Growth and Accumulation of 20-Hydroxyecdysone in Ar-4.

Ar-4 was cultured in 50 ml of MS medium at 25°C in the dark. (●), growth ratio; (■), total weight of 20-hydroxyecdysone.

hairy roots, we have analyzed the contents of 20-hydroxyecdysone. Table I showed the contents in original roots and in some clones of one-month-old cultures in liquid media. The 20-hydroxyecdysone was retained almost entirely in the root tissue, less than 1 $\mu\text{g}/\text{ml}$ of it being released in the medium. The content in clone **Ar-4** reached 0.14%, which is more than 4 times that of original roots. The clones that grow fast have contained large amounts of steroid compared to the slow-growing clones, suggesting that steroid production is related to the growth of roots. We therefore examined the time course of growth and steroid production using clone **Ar-4**.

About 50 mg of fresh roots (4–6 mg dry wt) cultured in liquid MS medium were inoculated into 14 of 100 ml flasks with 50 ml of the same medium, and cultured at 25°C in the dark using a rotary shaker for 30 days. At 5-days intervals, flask cultures were harvested in duplicate, and growth ratio and steroid content were measured on a dry weight basis (Fig. 4). From an initial inoculum, the fresh weight of roots increased more than 250 times in about 30 days and entered a stationary phase. These data indicate that the production of 20-hydroxyecdysone is apparently related to the growth of roots. It is noteworthy that when hairy roots were cultured under light, growth was inhibited and steroid content was also decreased to one-third to -fourth. In phytoecdysteroid producing plants, the fluctuation of phytoecdysteroid levels during development of various organs have recently been reported.¹⁹⁾ It is also said that phytoecdysteroid production is involved in a dynamic process during development and that production may be tissue- and organ-specific. Although the reasons of growth-associated steroid production observed in this study remain unclear, this experimental system using hairy root clones will be an advantageous tool for such studies.

Scale-up culture

Although hairy root clones once established could preserve their genetic stability in growth and in product formation, it is possible to

Fig. 5. Hairy Roots Cultured in an Air-lift Type Fermenter.

Cultured hairy roots were supported with polyurethane foam, and cultured in a 1.5-l fermenter for 45 days at 25°C in the dark.

enhance the productivity of valuable substances by various fermentation techniques. We have tried an preliminary scale-up experiment using an air-lift type fermenter. An inoculum was prepared from the liquid culture in 50 ml of MS medium for 30 days, then 0.9 g of fresh roots were transferred to the air-lift type fermenter containing 1500 ml of MS medium. The hairy roots were supported by polyurethane foam.²⁰⁾ The fermenter was aerated from an inlet port at the base of the vessel, and the medium was supplied by a peristaltic pump. After 45 days of culture, the fresh weight of the final biomass was 210 g (Fig. 5). The growth ratio was 230-fold, which is comparable to that of a flask culture. The content of 20-hydroxyecdysone in the final biomass was 0.12% by dry weight basis. To enhance the steroid productivity, more suitable physio-

logical conditions are now being investigated.

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