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CATECHIN PRODUCTION IN CULTURED *POLYGONUM HYDROPIPER* CELLS

KANJI ONO,* MAYUMI NAKAO, MASAO TOYOTA,† YOSHIMI TERASHI, MASASHI YAMADA,‡ TETSUYA KOHNO‡ and YOSHINORI ASAKAWA†

Department of Biological Science, Faculty of Science, Kumamoto University, Kurokami 2-39-1, Kumamoto 860, Japan; †Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan; ‡Meiji Milk Products Company, Midori 1, Sumida-ku, Tokyo 130, Japan

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Key Word Index—*Polygonum hydropiper*; Polygonaceae; callus; suspension-cultured cells; (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate.

Abstract—Callus and suspension-cultured cells were induced from hypocotyls of *Polygonum hydropiper* seedlings. Both the callus and suspension-cultured cells produced mainly (+)-catechin accompanied by (−)-epicatechin and (−)-epicatechin-3-*O*-gallate. The (+)-catechin production of suspension-cultured cells increased with cell growth and reached the maximal value (29.0 mg g^{-1} dry wt) after 6 days from the start of subculture. This is the highest value of (+)-catechin content among reports on catechin production *in vitro* so far published. The amount of (−)-epicatechin was in the range of 1.1–7.7 mg g^{-1} dry wt and that of (−)-epicatechin-3-*O*-gallate was in the range of 2.6–6.4 mg g^{-1} dry wt for a culture period of 15 days, respectively. Comparing with plant parts in regard to (+)-catechin, the amount of suspension-cultured cells was about 1.5 times as much as callus cells, about 9 times that of leaves (3.2 mg g^{-1} dry wt) and about 7 times that of stems (4.0 mg g^{-1} dry wt). The maximal yield of total catechins in suspension-cultured cells was 4.3% dry wt.

INTRODUCTION

Catechins, a group of flavanols, were recently noted as antitumour agents [1, 2], and as antioxidant and radical scavengers [3]. The major catechins found in grape seeds (*Vitis vinifera*) were (+)-catechin, (−)-epicatechin and as minor components, procyanidin B1, B2, B3, B4 and C1 [4]. The major components detected in soluble instant tea (*Camellia sinensis*) were (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-gallate accompanied by a minor component (+)-catechin [5].

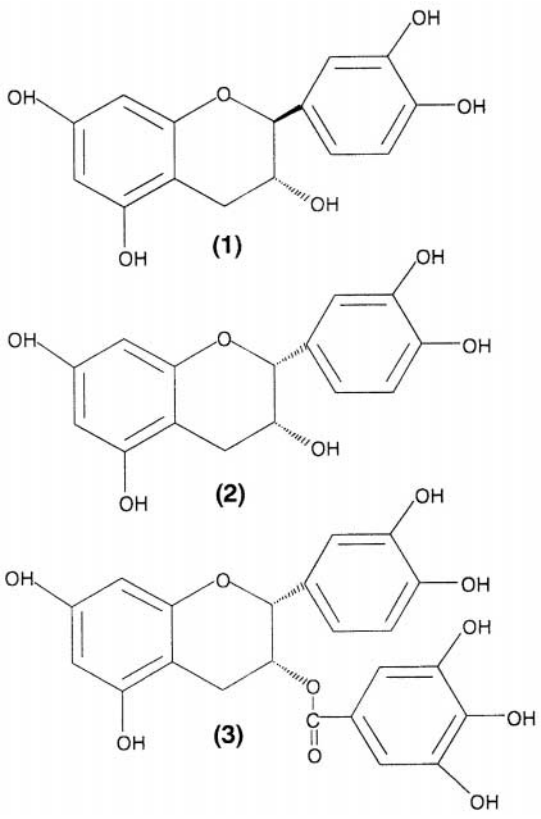
In general, dedifferentiated plant cells such as callus are known to reduce or stop the production of secondary metabolites which the parent plant produces *in vivo* [6]. Nevertheless, *in vitro* productions of (−)-epicatechin-3-*O*-gallate accompanied by (+)-catechin and (−)-epicatechin have been reported in *Fagopyrum esculentum* calli and cultured hairy roots [7, 8] and *Crataegus monogyna* calli [9].

Polygonum hydropiper (Polygonaceae) is known to produce a potent pungent sesquiterpene dialdehyde polygodial [10] and its seedlings are used as a food spice in Japan. In the course of studies on the secondary metabolite production using cultured *P. hydropiper* cells, we found that cultured cells of this species produced a large amount of catechins, although it scarcely made polygodial. Here, we describe the identification of catechins produced by cultured *Polygonum* cells, their growth and catechin synthesis and a comparison of catechin production in cultured cells with that in intact plant tissues.

RESULTS AND DISCUSSION

Callus cultures of *P. hydropiper* were initiated from hypocotyls of seedlings by culturing explants on Murashige and Skoog's solid medium (MS) [11] containing 10^{-6} M 2,4-D, 10^{-6} M kinetin, 0.1% casamino acids, 3% sucrose and 0.8% agar and were maintained over a period of a year. The suspension cultures were established from calli by transferring them to a liquid medium of the same

*Author to whom correspondence should be addressed.



composition and maintained by subculturing them every week.

The MeOH or acetone extract from suspension culture was analyzed by TLC, GC-MS and ^{1}H NMR. (+)-catechin (1), (−)-epicatechin (2) and (−)-epicatechin-3-*O*-gallate (3) were identified as the major products. Different plant parts (leaves and stems of the field-collected plants and cotyledons and hypocotyls of seedlings) and cultured cells (14-day-old callus cells and 6-day-old suspension-cultured cells) were extracted with acetone; subsequently extracts were analyzed by HPLC and then individual catechin contents were compared (Fig. 1). In both plant parts and cultured cells, (+)-catechin was detected as the major product accompanied by (−)-epicatechin and (−)-epicatechin-3-*O*-gallate.

The highest content of (+)-catechin was found in suspension-cultured cells (29.0 mg g^{-1} dry wt) showing about 1.5 times as much as content of callus cells (18.6 mg g^{-1} dry wt), about 9 times that of leaves (3.2 mg g^{-1} dry wt), about 7 times that of stems (4.0 mg g^{-1} dry wt) and about 6 times that of cotyledons (4.3 mg g^{-1} dry wt). The (−)-epicatechin content was also highest in suspension-cultured cells (7.7 mg g^{-1} dry wt) and became lower in the order of callus cells (1.7 mg g^{-1} dry wt), cotyledons (0.8 mg g^{-1} dry wt), leaves (0.2 mg g^{-1} dry wt) and hycopotyls (0.1 mg g^{-1} dry wt), respectively. The (−)-epicatechin-3-*O*-gallate was found in suspension-cultured cells (6.4 mg g^{-1} dry wt) and callus cells (0.9 mg g^{-1} dry wt), although there is very little in the plant organs (stems, leaves, hypocotyls and cotyledons). In previous reports on catechin production *in vitro*, (−)-epicatechin was detected as the major product in callus culture of *Uncaria elliptica* [12], while (−)-epicatechin-3-*O*-gallate was found as the major product in callus and hairy root cultures of *Fagopyrum esculentum* [8].

Figure 2 shows the growth curve and catechin content with growth of *Polygonum* cells in suspension culture. Suspension-cultured cells entered an exponential growth phase without going through a lag phase just after transferring. Then, they reached a stationary phase after 7 days showing about a 9-fold increase over the initial value for dry wt. The (+)-catechin content of cells rose with cell growth and showed the maximum value of 29.0 mg g^{-1} dry wt in the late log phase (6 days after transferring) and then its amount dropped steeply in stationary phase. This is the highest value among (+)-catechin production from cultured cells previously reported in any plant material. The steep decrease of (+)-catechin amount in stationary phase seems to be due to the conversion of (+)-catechin into procyanidins, because an increase in procyanidin synthesis in stationary phase was confirmed by TLC analysis and secretion of catechins into culture medium was scarcely observed during a culture period (data not shown). In cell suspension cultures of *Cryptomeria japonica*, the amounts of catechin and procyanidin rapidly increased in log phase, but the content of catechin attained its maximum prior to that of procyanidin in stationary phase and then decreased rapidly [13]. The (−)-epicatechin and (−)-epicatechin-3-*O*-gallate were produced through a culture period (for 15 days) in the range of 1.1–7.7 mg g^{-1} dry wt in the former and 2.6–6.4 mg g^{-1} dry wt in the latter.

Our results show that suspension-cultured cells from *P. hydropiper* hypocotyls are characterized by significant production of catechins, particularly (+)-catechin, with the highest amount of (+)-catechin (maximum; 29 mg g^{-1} on a dry wt at 6 days) in vigorously dividing cells (log phase cells), and show that the maximal yield of total catechins in suspension-cultured cells is 4.3% dry wt. Therefore, *P. hydropiper* suspension-cultured cells are suitable for the mass production of catechins. We obtained the Japanese patent, Toku-Kai-Hei 9-224689 on our invention of catechin production from the cultured *Polygonum hydropiper* cells and are disclosing our patent in the Japanese Patent Official Gazette issued in September 2, 1997 [14].

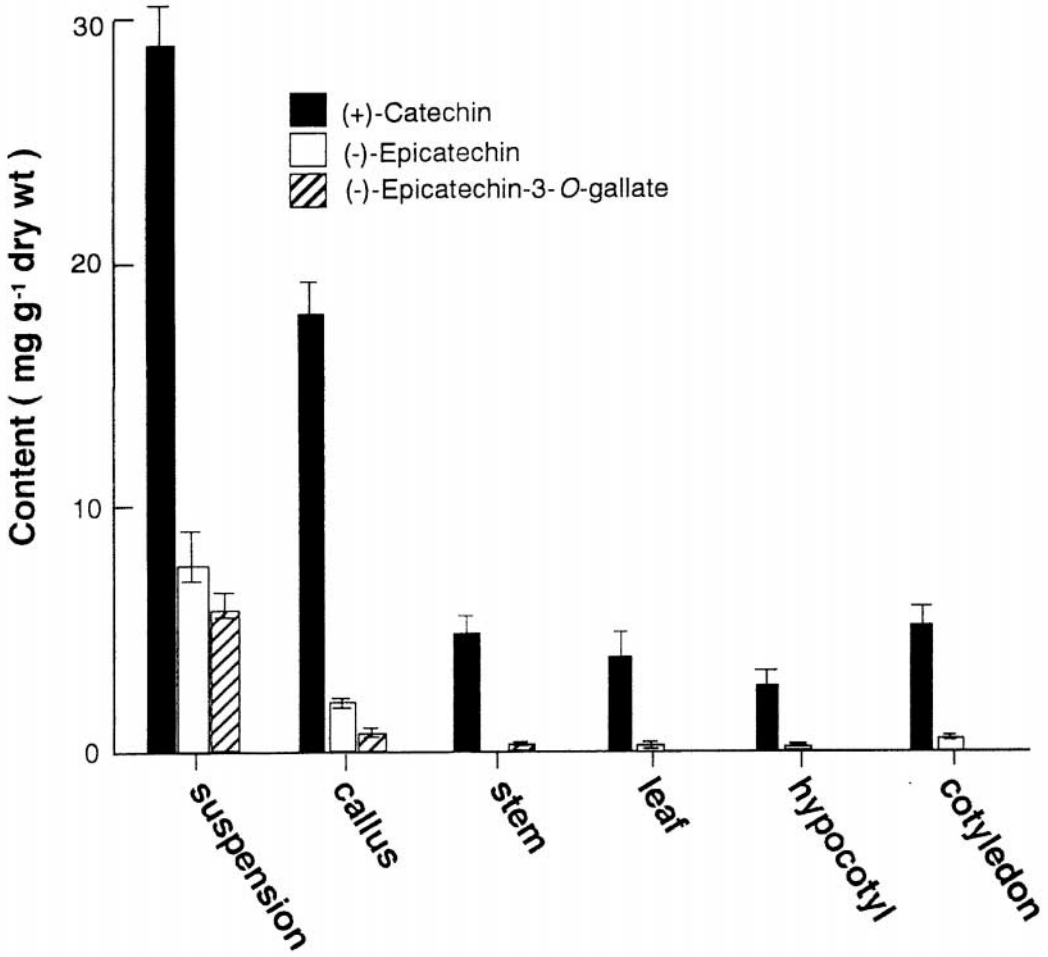


Fig. 1. Amounts of catechins (dark area: (+)-catechin, white area: (−)-epicatechin, diagonally striped area: (−)-epicatechin-3-*O*-gallate) in cultured cells *in vitro* and different tissues of *Polygonum hydropiper* L. Acetone extracts were obtained from 6-day-old cells in suspension culture and 14-day-old cells in calli. Each amount was expressed as mg g^{-1} dry wt of cells. Average + SE of three independent experiments are shown.

EXPERIMENTAL

General

TLC and GC-MS were carried out as previously reported [15, 16].

Plant material

Polygonum hydropiper L. and its seeds were collected at the campus of Kumamoto University, Kumamoto-city, Japan, in 1995. The voucher specimens are deposited in the Department of Biological Science, Faculty of Science, Kumamoto University.

Tissue culture

Hycopotyls of seedlings were excised and surface-sterilized with 1% sodium hypochlorite sln for 10 min followed by washing 3 times with sterilized H_2O and then used as explants to initiate callus formation. Calli were induced by culturing axenic explants on solid Murashige and Skoog's medium (MS) containing 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D), 10^{-6} M kinetin, 0.1% casamino acids, 3% sucrose and 0.8% agar. Calli were grown in the light (0.6 W m^{-2}) and subcultured every a month. After three subcultures, suspension cultures were started from these calli with an inoculum of 0.5 g fresh wt in a 50-ml flask containing 20-ml of a liquid medium of the same composition as that of callus culture on a gyratory shaker at 120 rpm at 25° in the light (0.6 W m^{-2}). The pH of media was adjusted to 5.7 before autoclaving. Growth was determined as dry wt every 3 days for a period of 15 days. For dry wt determination, all cells in suspension culture in each flask were harvested by filtration through Miracloth disc, and the wt of each of cell pellets was measured after desiccation at 80° overnight.

TLC analysis

Week-old-cells (1 g) were extracted with MeOH (5 ml × 3). The extract was separated by adding Et_2O (×3). The ether phase was evaporated, col-

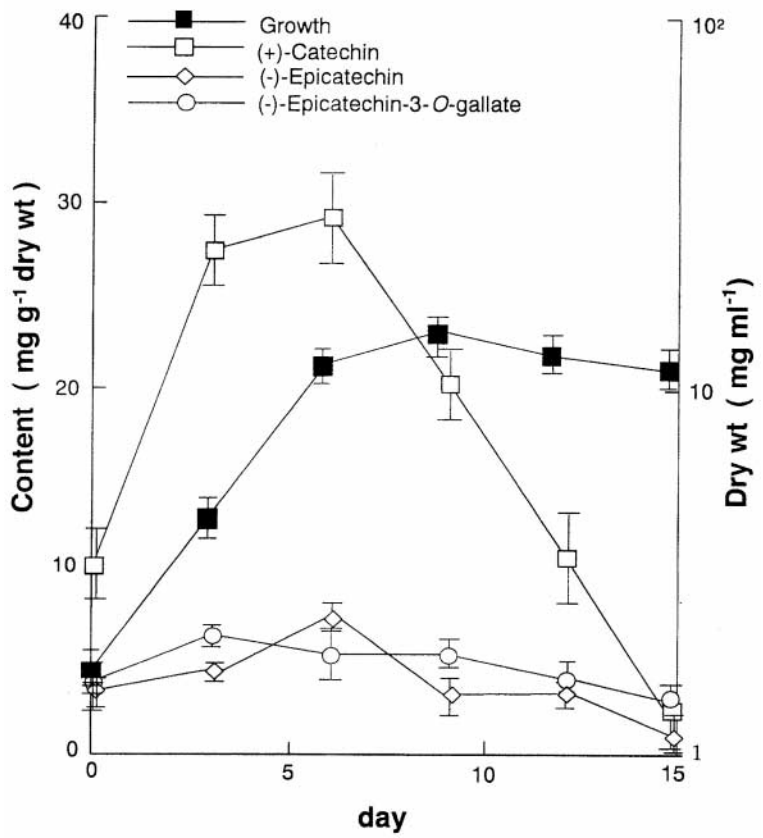


Fig. 2. Growth and changes of amounts of catechins (dark area: (+)-catechin, white area: (−)-epicatechin, diagonally striped area: (−)-epicatechin-3-*O*-gallate) in *P. hydropiper* cells in suspension culture. Each amount was expressed as mg g^{-1} dry wt of cells, except for mg dry wt ml^{-1} of cell suspension in the expression of growth. Averages ± SE of three independent experiments are shown.

lected and dissolved in 1 ml 50% MeOH after concentration and dryness and used for a TLC sample. TLC was in the following systems: cellulose plate, FUNACELL SF, Funacoshi Co. with *n*-BuOH:HOAc:H_2O (4:1:5, v/v) in 1D-TLC and with 6% HOAc in 2D-TLC. Catechins were visualized by spraying with 1% slns of diphenylborinic acid and ethanolamine ester (Aldrich).

HPLC analysis

Each (1 g) of the materials of calli, cell suspensions, leaves, stems hypocotyls and cotyledons was ground mechanically with liquid nitrogen and extracted with 2 ml acetone (2 ml × 3). Each extract was, after filtration through a filter paper (Advantec No. 3), dissolved in 2 ml H_2O after evaporation and dryness at 30°C and their lower phases were collected after separation by addition of 2 ml *n*-hexane (2 ml × 3). Then, the EtOAc phases were separated and collected by adding 2 ml EtOAc into the lower phases and dissolved in 1 ml MeOH:0.1% H_3PO_4 = 15:85 after concentration and dryness (2 ml × 3) and were analyzed by HPLC after filtration through a Millipore filter (0.45 μm). HPLC system; Shimadzu LC-6A, liquid-pump; LC6A, UV detection; SPD-6AV, column oven; GTO 6A, column; Fluofix 120N ϕ 4.6 mm × 25 cm (NEOS Co.), moving bed; MeOH: 0.1% H_3PO_4 = 15:85, column temperature; 40°, detection of wavelength; 280 nm, velocity of flow; 0.5 ml/min, injection volume; 5 μl, chart-speed; 10 mm/min. Authentic samples of (+)-catechin (Nakarai), (−)-epicatechin (Aldrich) and (−)-epicatechin-*O*-gallate (Sigma) were used for quantitation.

Spectral data

NMR spectra were recorded at 100 or 50 Mhz for ^{13}C and 400 or 200 Mhz for ^{1}H. The temperature programming of GC-MS analysis was performed from 50° isothermal for 3 min^{-1}, then 50–250° at 5° min^{-1} and finally isothermal at 250° for 15 min. The injection temp. was 250°. A fused silica column coated with DB-17 (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used.

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