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Production of (-)-Epicatechin 3-*O*-gallate in Cell Suspension Cultures of *Saxifraga stolonifera* Meerb.

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Abstract

Cell suspension cultures of *Saxifraga stolonifera* Meerb. were established in MS liquid medium containing 1 μM NAA by using the callus induced from adventitious roots of cultured plantlets, and the production of (-)-epicatechin 3-*O*-gallate (ECG) was confirmed (0.6% of dry weight). The effects of the concentration of major inorganic salts and plant growth regulators in the medium on ECG production were examined. Reduction of KH_2PO_4 or $MgSO_4$ concentration in MS medium enhanced ECG production. Maximum ECG contents, 1.6% and 1.2%, were obtained at half the original concentration for KH_2PO_4 and at one-eighth that for $MgSO_4$, respectively. In the modified MS medium, where KH_2PO_4 and $MgSO_4$ concentration were reduced, increased NAA levels suppressed ECG production, while the addition of BAP stimulated it. Maximum ECG production (2.5%) was achieved in the modified MS medium containing 1 μM NAA and 1 μM BAP.

1. Introduction

Flavan-3-ol derivatives, which are the structual elements of condensed tannins, occur widely in the plant kingdom (Nonaka *et al.*, 1983, Ricardo da Silva *et al.*, 1991, Lee *et al.*, 1992). They are well known to possess a variety of biological properties, such as anti-HIV activity (Mahmood *et al.*, 1993), antioxidant activity (Hong *et al.*, 1994, Salah *et al.*, 1995), antitumor effects (Kashiwada *et al.*, 1992), improvement of renal function (Yokozawa *et al.*, 1993, Yokozawa *et al.*, 1997, Fujitsuka *et al.*, 1997), antibacterial activity (Ahn *et al.*, 1991), and so on. For these activities, the gallate esters, such as (-)-epicatechin 3-*O*-gallate (ECG, **Fig. 1**) or (-)-epigallocatechin 3-*O*-gallate (EGCG), seem to be more effective than (+)-catechin, (-)-epicatechin, or (-)-epigallocatechin in many cases (Hong *et al.*, 1994, Salah *et al.*, 1995, Kashiwada *et al.*, 1992, Yokozawa *et al.*, 1997, Ahn *et al.*, 1991).

There have so far been only a few reports on *in vitro* production of flavan-3-ol gallate esters.

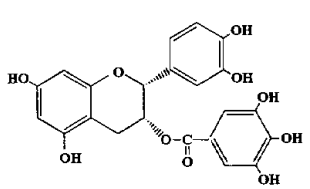

Fig. 1 A structure of (-)-epicatechin 3-*O*-gallate (ECG).

Ishimaru *et al.*, reported the production of ECG or EGCG in adventitious root, hairy root, shoot, and crown gall cultures of *Phyllanthus niruri* (1992a) and in callus cultures of *Liquidambar styraciflua* (1992b). In order to provide a system capable of producing flavan-3-ol gallate esters efficiently, we undertook a study on their production by plant cell suspension cultures, and recently found ECG production in suspension cultures of *Saxifraga stolonifera* Meerb. (Saxifragaceae), whose fresh leaves had been used to treat burn, frostbite, and whooping cough in Japanese folk medicine. In this paper, we report on the establishment of cell suspension cultures of *S. stolonifera* and ECG production in these cultures. In addition, we investigate the effects of the concentration of major inorganic salts and plant growth regulators in the medium on cell growth and ECG production.

2. Materials and Methods

2.1 Callus and cell suspension cultures

Surface-sterilized leaf segments (3×3 mm) of *S. stolonifera* were placed on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) which contained 5 μM 2,4-dichlorophenoxy-acetic acid (2,4-D) and 30 $g l^{-1}$ sucrose, and was solidified with 0.2% gellan gum. On the surface of the induced callus, many plantlets were regenerated. They were subsequently grown in MS liquid medium without plant growth regulators at 25°C in light (16-hour photoperiod, 2,000 lux) on a gyratory shaker (90 rpm). Callus

used for cell suspension cultures was again induced by inoculating adventitious roots of these plantlets on MS solid medium containing 1 μM 1-naphthaleneacetic acid (NAA). The obtained callus was subcultured at 25°C in the dark every two months. Cell suspension cultures were initiated by transferring this callus to the same liquid medium in which the callus had been induced. Thereafter, these cultures were subcultured by inoculating 20 m*l* suspension cultures into the same liquid medium (40 m*l*/200 m*l* flask) every two weeks. Cell suspension cultures were grown at 25°C in the dark on a gyratory shaker (120 rpm).

2.2 Time course study in cell suspension cultures

After the cells subcultured for 14 days were harvested by nylon cloth (37μm), 1.5 g fresh weight of cells was inoculated to 30 m*l* MS liquid medium containing 1 μM NAA (100 m*l* flask). Cells in more than three flasks were harvested every three days throughout the growth cycle and then lyophilized. After cell dry weight was measured, ECG content of the cells was determined by HPLC.

2.3 Experiments on the medium optimization

In order to investigate the effects of the concentration of major inorganic salts in MS medium on cell growth and ECG production, cells subcultured for 14 days (1.5 g fresh weight) were inoculated in 30 m*l* liquid medium containing 1 μM NAA, in which the concentration of NH_4NO_3 and KNO_3 (in combination and in keeping their proportion in the MS medium), $CaCl_2$, KH_2PO_4, or $MgSO_4$ were varied from one-eighth to twice that of MS medium without changing any other components. For the experiments on the effects of plant growth regulators in the medium, cells (1.5 g fresh weight) were transferred to 30 m*l* modified MS liquid medium, supplemented with NAA (1, 10, and 100 μM) and 6-benzylaminopurine (BAP) (0, 1, and 10 μM) in nine combinations, where KH_2PO_4 and $MgSO_4$ concentrations were reduced to a half and one-eighth that of the original medium, respectively. These experiments were all carried out for 18 days in triplicate.

2.4 Quantitative analysis of ECG by HPLC

Lyophilized cells (200 mg) were extracted with MeOH (50 m*l*) for 1 hour by sonicator at room temperature. The MeOH extract was filtered and then analyzed on a Jasco LC-800 HPLC system under the following conditions: column, TOSO 80-Tm ODS (6 mm×150 mm); mobile phase, CH_3CN-THF-H_2O-H_3PO_4 (120 m*l*-20 m*l*-860 m*l*-0.5 m*l*); flow rate, 1.0 m*l*/min; column oven temperature, 40°C; detection, 280 nm.

2.5 Isolation and identification of ECG

Cells subcultured for 14 days were harvested and then lyophilized. These cells (35 g dry weight) were refluxed with 80% aqueous acetone (400 m*l*) for 2 hours (×2). The extract obtained was concentrated under reduced pressure and applied to a column of HP-20 (30-60% aq. MeOH), MCI gel CHP-20P (30-40% aq. MeOH), and Sephadex LH-20 (EtOH) to give ECG (94.5 mg). The identification of ECG was carried out by direct comparison ($[\alpha]_D$, IR, ^{1}H-NMR, FAB-MS, HPLC, TLC) with an authentic sample.

3. Results and Discussion

3.1 Callus and cell suspension cultures

Although callus was first induced from leaf segments of *S. stolonifera*, the obtained callus ceased cell proliferation in the first or second passage of subculture and could not be maintained any longer. So, callus was again induced from adventitious roots of cultured plantlets, which were regenerated from the callus mentioned above. This callus was pale yellow and soft, and could be maintained easily.

Cell suspension cultures were established by transferring the callus derived from adventitious roots to the same MS liquid medium in which callus had been induced. The cells cultured in liquid medium were found to produce ECG, but not EGCG, by HPLC analysis. Moreover, in order to confirm the ECG production in *S. stolonifera* suspension cultures, this compound was isolated from cultured cells and identified.

The time course experiments were carried out to investigate the relationship between the cell growth and ECG production in *S. stolonifera* suspension cultures. The results are shown in **Fig. 2**. Cell yield increased up to 18 days after inoculation without a lag phase, and then declined rapidly. On the other hand, ECG content increased up to 1.1% of dry weight three

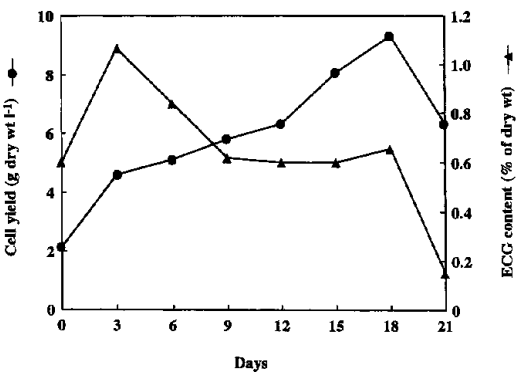

Fig. 2 Time course of cell growth and ECG production in *S. stolonifera* suspension cultures. Cells (1.5 g fresh weight) were inoculated in 30 m*l* MS liquid medium containing 1 μM NAA.

days after inoculation, and decreased to 0.6% of dry weight on the sixth day. Thereafter, the content kept a constant value, and decreased again over 18 days when cell yield declined.

3.2 *Effects of major inorganic salts on cell growth and ECG production*

In order to optimize the medium components in *S. stolonifera* suspension cultures, the effects of the concentration of major inorganic salts in MS medium on cell growth and ECG production were investigated. The changes of NH_4NO_3 and KNO_3, or $CaCl_2$ concentration in the medium did not exert obvious effects on either cell growth or ECG production, except for the decrease of ECG content at twice the original concentration of NH_4NO_3 and KNO_3 (data not shown). On the other hand, the reduction of KH_2PO_4 concentration in the medium suppressed cell growth but enhanced ECG production, as shown in **Fig. 3**. Maximum ECG production (1.6% of dry weight) was achieved at half the original concentration, in which cell growth did not show the significant decrease. Reducing $MgSO_4$ concentration also stimulated ECG production, while it did not affect cell growth (**Fig. 4**). For $MgSO_4$, maximum ECG production (1.2% of dry weight) was obtained at one-eighth the original concentration.

A number of studies have shown that the reduction of nitrogen or phosphate in the medium led to an increase in phenolic production. For *in vitro* production of flavan-3-ol derivatives, Phillips and Henshaw (1977) reported that the addition of urea as a nitrogen source to stationary phase cultures of sycamore inhibited the production of phenolics, which included condensed tannins composed of (+)-chatechin, (-)-epichatechin, and leucocyanin. Ishimaru *et al.*, (1992b) showed that the production of flavan-3-ol derivatives which contained ECG in *Liquidambar styraciflus* callus cultures was enhanced ten times in the medium from which NH_4NO_3 was removed. For ECG production in *S. stolonifera* suspension cultures, the limitation of NH_4NO_3 and KNO_3, or $CaCl_2$ in the medium had no effects, whereas limiting KH_2PO_4 or $MgSO_4$ were effective. The enhancement of ECG production by reducing KH_2PO_4 concentration might be due to antagonistic regulation of primary and secondary metabolism discussed by Phillips and Henshaw (1977), since it was accompanied with the decrease in cell growth. However, it is not clear how reducing the $MgSO_4$ level in the medium stimulated ECG production.

3.3 *Effects of plant growth regulators on cell growth and ECG production*

In the modified MS medium, where KH_2PO_4 and

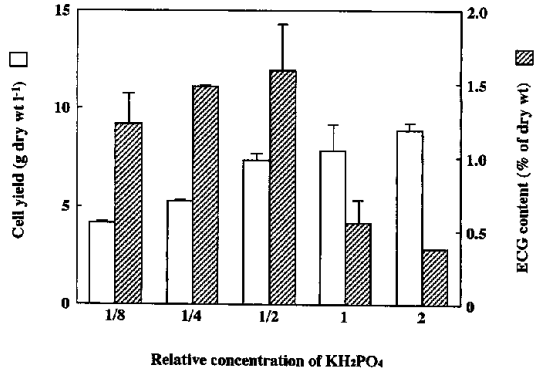

Fig. 3 Effects of KH_2PO_4 concentration in MS medium on cell growth and ECG production in *S. stolonifera* suspension cultures.
Cells (1.5g fresh weight) were inoculated in 30ml liquid medium containing 1μM NAA, and cultured for 18 days. KH_2PO_4 concentration in MS medium (1 fold): 1.25mM. The results are represented as means of three experiments. Bars indicate SD.

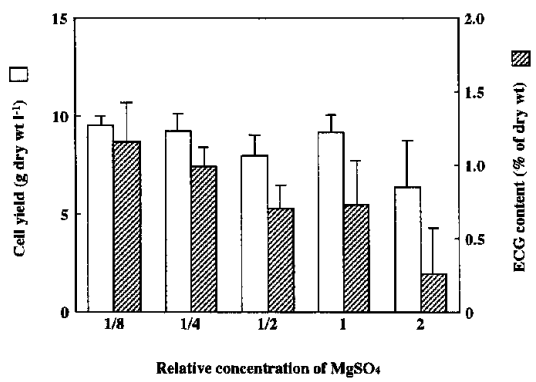

Fig. 4 Effects of $MgSO_4$ concentration in MS medium on cell growth and ECG production in *S. stolonifera* suspension cultures.
Cells (1.5g fresh weight) were inoculated in 30ml liquid medium containing 1μM NAA, and cultured for 18 days. $MgSO_4$ concentration in MS medium (1 fold): 1.5mM. The results are represented as means of three experiments. Bars indicate SD.

$MgSO_4$ concentrations were reduced to a half and one-eighth that of the original medium, respectively, nine combinations of NAA (1, 10, and 100μM) and BAP (0, 1, and 10μM) were examined for cell growth and ECG production in *S. stolonifera* suspension cultures. The results are shown in **Fig. 5**. The increase in NAA concentration in the medium caused the decline of both cell growth and ECG production. However, the extent of inhibition in ECG production was much larger than the inhibition in cell growth. Especially, cells cultured in the medium supplemented with 100 μM NAA could hardly produce ECG (0.08% of dry weight). On the other hand, the addition of BAP in the medium stimulated cell growth at 1μM, and enhanced ECG production at 1 and 10μM. Most

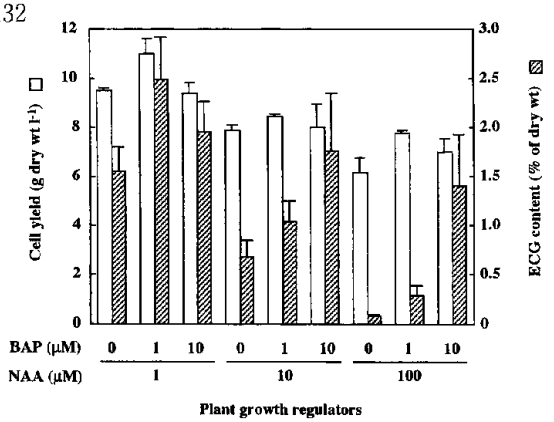

Fig. 5 Effects of plant growth regulators on cell growth and ECG production in *S. stolonifera* suspension cultures.
Cells (1.5 g fresh wt) were inoculated in 30 m*l* modified MS liquid medium, where KH_2PO_4 and $MgSO_4$ concentration were reduced to a half and one-eighth that of the original medium, respectively, and cultured for 18 days. The results are represented as means of three experiments. Bars indicate SD.

notably, the loss of ECG production caused by increasing the NAA level was completely recovered by the addition of 10 μM BAP. Maximum cell growth (11.0 g dry weight) and maximum ECG production (2.5% of dry weight) were both achieved in the modified MS medium supplemented with 1 μM NAA and 1 μM BAP.

Phillips and Henshaw (1977) reported that the administration of 2,4-D to stationary phase cultures of sycamore suppressed the accumulation of phenolics, which included flavan-3-ol derivatives. Ozeki and Komamine (1981) showed in carrot suspension cultures that 2,4-D inhibited the synthesis of anthocyanin, while zeatin promoted it in the medium lacking 2,4-D. Moreover, they (1985) suggested that chalcone synthase (CHS) might be a key enzyme in anthocyanin synthesis regulated by 2,4-D in this culture system. In *S. stolonifera* suspension cultures, also, elevated auxin levels in the medium suppressed ECG production, and the addition of BAP stimulated it. These results suggest that ECG synthesis in *S. stolonifera* suspension cultures might be controlled through the regulation of CHS by NAA or BAP.

In this study, cell suspension cultures of *S. stolonifera* were established, and the production of ECG was confirmed. Moreover, through the optimization of the concentration of major inorganic salts and plant growth regulators in the medium, the ECG content of cultured cells increased from 0.6% to 2.5% of dry weight (4.2 fold). Further study is in progress to increase ECG productiion in *S. stolonifera* suspension cultures.

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