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# PRODUCTION OF CORNOSIDE IN *ABELIOPHYLLUM DISTICHUM* CELL SUSPENSION CULTURES

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**Key Word Index**—*Abeliophyllum distichum*; *Forsythia suspensa*; *F. viridissima*; *F. koreana*; Oleaceae; cell suspension cultures; phenylethanoid glycosides; benzoquinolethanoid glucoside; cornoside; production.

**Abstract**—The production of phenylethanoid derivatives in callus and cell suspension cultures of four oleaceous plants, *Abeliophyllum distichum*, *Forsythia suspensa*, *F. viridissima*, and *F. koreana* was investigated. Of two types of *A. distichum* cultured cells, the friable fine cells produced only the 4-hydroxyphenylethanoid-type glucoside, cornoside, whereas the small cell aggregates produced the 3,4-dihydroxyphenylethanoid-type glycosides (i.e. verbascoside) predominantly. In the cultured cells of *F. suspensa* and *F. viridissima*, the latter-type glycosides were produced predominantly and from those of *F. koreana*, lignan glucosides with 3,4-dioxygenated phenyl groups were isolated. 

## INTRODUCTION

Phenylethanoid glycosides are distributed in Oleaceae and most of the plant families belonging to Scrophulariales-Lamiales [1, 2]. Based on the oxidation pattern of the aromatic ring, phenylethanoids are classified into two groups, 4-hydroxyphenylethanol derivatives and 3,4-dihydroxyphenylethanol derivatives. The former are presumed to be further oxidized into benzoquinolethanoid derivatives such as cornoside [3, 4], and in turn the aromatic ring-reduced into unique cyclohexylethanol derivatives such as rengyosides [5]. The phenylethanoid moieties are thought to be biosynthesized from phenylpropanoids such as phenylpyruvic acid [5], but the details are still unknown. To clarify the biosynthetic mechanism of phenylethanoid glycosides formation, especially the benzoquinolethanoid glucoside, cornoside, which is presumed to be biosynthesized from *p*-hydroxyphenylethanol (tyrosol) via salidroside [6], and regarded as an important precursor of cyclohexylethanol derivatives in *Forsythia suspensa*, we established cell cultures from four oleaceous plants, *Abeliophyllum distichum*, *F. suspensa*, *F. viridissima*, and *F. koreana*. In this paper, we report on a comparative study of the chemical constituents of these cultured cells.

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## RESULTS AND DISCUSSION

### *Constituents in callus cultures*

White, friable callus tissues of *A. distichum*, *F. suspensa*, *F. viridissima*, and *F. koreana* were established on Murashige-Skoog gellan gum medium [7] containing 10 $\mu$M 1-naphthaleneacetic acid and 10 $\mu$M 6-benzylaminopurine, respectively. The MeOH extract of the fresh cells of *A. distichum* afforded four known compounds: the two 3,4-dihydroxyphenylethanol glycosides, verbascoside and arenarioside [8], a benzoquinolethanoid glucoside derived from 4-hydroxyphenylethanol, cornoside [4], and a cornoside-derived compound, halleridone [4]. From the MeOH extract of the fresh calli of *F. viridissima*, three lignan glucosides, arctiin, matairesinol 4′-*O*-glucoside and epipinoresinol 4′-*O*-glucoside [9], which have been also isolated from cultured cells of *F. intermedia* [10], were isolated besides verbascoside. Callus cultures of *F. suspensa* and *F. koreana* also produced all of above mentioned compounds, respectively, as determined by HPLC-photodiode array analysis.

The amounts of 3,4-dihydroxyphenylethanol glycosides, cornoside and lignan glucosides in cultured cells, in the dried leaves and in the dried stems of *A. distichum*, *F. suspensa*, *F. viridissima* and *F. koreana* are shown in Table 1. All compounds found in callus cultures were also detected in the parent plants used for the induction of callus cultures. In *A. distichum* and *F. suspensa* plants, 3,4-dihydroxyphenylethanol glycosides were found as the main constituents. *F.*

Table 1. Phenol glucosides in leaves, stems and callus cultures of *A. distichum* and three *Forsythia* plants

| | 3,4-Dihydroxy-phenylethyl alcohol glucosides* | Cornoside | Lignan glucosides† |
|---|---|---|---|
| *A. distichum* | | | |
| Leaves (mg/g dry wt) | 38.08 | 0.23 | 3.46 |
| Stems (mg/g dry wt) | 9.62 | 0.92 | 5.39 |
| Callus cultures (mg/g fr. wt) | 7.15 | 0.16 | 0.00 |
| *F. suspensa* | | | |
| Leaves (mg/g dry wt) | 32.31 | 0.58 | 0.00 |
| Stems (mg/g dry wt) | 1.35 | 0.04 | 0.00 |
| Callus cultures (mg/g fr. wt) | 0.21 | 0.03 | 0.00 |
| *F. viridissima* | | | |
| Leaves (mg/g dry wt) | 3.08 | 0.71 | 1.15 |
| Stems (mg/g dry wt) | 6.73 | 1.50 | 11.35 |
| Callus cultures (mg/g fr. wt) | 0.50 | 0.02 | 0.09 |
| *F. koreana* | | | |
| Leaves (mg/g dry wt) | 13.27 | 0.00 | 2.69 |
| Stems (mg/g dry wt) | 4.23 | 0.12 | 6.54 |
| Callus cultures (mg/g fr. wt) | 1.19 | 0.00 | 1.53 |

* Verbascoside and arenarioside.

† Arctiin, matairesinol 4′-*O*-glucoside and epipinoresinol 4′-*O*-glucoside.

*viridissima* plant mainly contained cornoside, arctiin, matairesinol 4′-*O*-glucoside and epipinoresinol 4′-*O*-glucoside. In the *F. koreana* plant, the total content of phenolics was relatively low when compared with the two other *Forsythia* plants, and 3,4-dihydroxyphenylethanol glycosides were the main constituents. In all the plants we examined, 3,4-dihydroxyphenylethanol glycosides were mainly distributed in the leaves, whereas the amounts of both cornoside and lignan glucosides were higher in the stems than in the leaves. Compared with the original plants, the amount of each glycoside in the callus cultures was low. In *A. distichum* callus cultures, lignan glucosides were not detected and 3,4-dihydroxyphenylethanol glycosides were present in greater amounts than cornoside. In *F. koreana* callus cultures, cornoside was not detected whereas both 3,4-dihydroxyphenylethanol glycosides and lignan glucosides were detected.

### *Cell suspension cultures of* A. distichum

To investigate the mechanism for the production of phenylethanoid derivatives, we tried to establish cell suspension cultures of *A. distichum*. On transferring *A. distichum* callus cultures to liquid medium containing the same phytohormone as the solid media, large grayish cell clusters (ca 2–5 cm in diameter) were formed. These clusters consisted of two types of cells, white fine cells and grayish small cell aggregates (ca 0.5 cm diameter). Separate subcultivation of these cells in liquid medium established three cell lines, i.e. fine cell cultures, small cell aggregates and the original large cell clusters. The amounts of cornoside and 3,4-dihydroxyphenylethanol glycosides in these three cell lines are shown in Table 2. Small cell aggregates contained 3,4-dihydroxyphenylethanol glycosides as the main constituents, together with a small amount of cornoside, whereas fine cell cultures did not contain 3,4-dihydroxyphenylethanol glycosides but fairly large amounts of cornoside. Large cell clusters contained both 3,4-dihydroxyphenylethanol glycosides and cornoside in almost the same amounts as in the callus cultures. As the difference in the amounts of the two types of phenylethanoid glycosides between small cell aggregates and fine cell cultures may have been due to cell growth stages of these cultures, a comparative study was made of the time-courses of cell growth and

Table 2. Phenylethanoid glucosides in three cell lines of *A. distichum* cell suspension cultures

| Cell line | 3,4-Dihydroxy-phenylethyl alcohol glucosides (mg/g fr. wt) | Cornoside (mg/g fr. wt) |
|---|---|---|
| Small cell aggregates | 2.67 | 0.13 |
| Fine cell cultures | 0.00 | 0.84 |
| Large cell clusters | 1.63 | 0.30 |

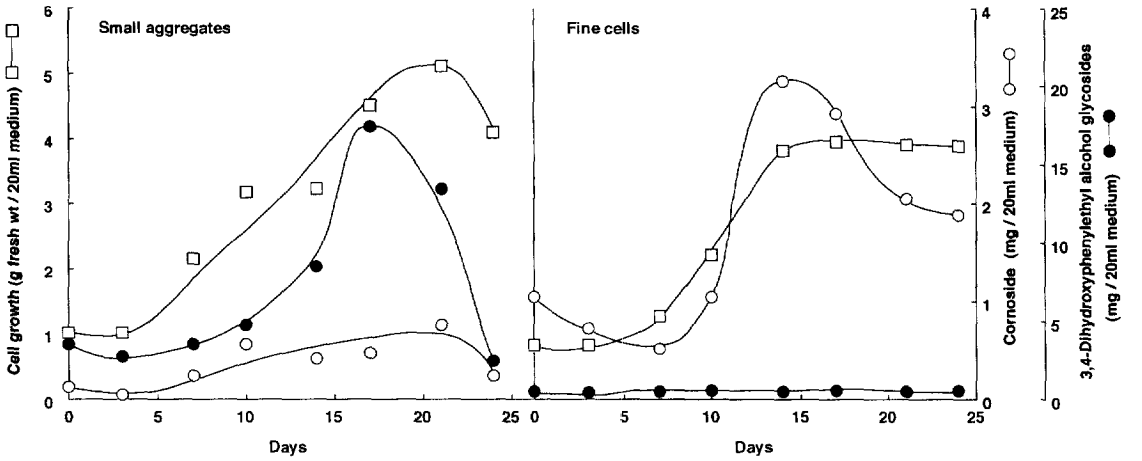



Fig. 1. Time-courses of cell growth and phenylethanoid glucosides production in small cell aggregates and fine cell cultures of *A. distichum* cell suspension cultures.

phenylethanoid glycoside production both in small aggregates (Fig. 1, left) and in fine cell cultures (Fig. 1, right). The small cell aggregates began to grow 3 days after inoculation, then grew almost linearly until day 21. The content of 3,4-dihydroxyphenylethanol glycosides was almost stable for the first week after inoculation, then sharply increased for the late growth stage. Maximal production (4 mg/g fr. wt) was observed at day 17. By contrast, the content of cornoside showed only a slight increase between day 3 and day 21 (0.15 mg/g fr. wt). In fine cell cultures, cell growth was observed between day 3 and day 14, and cornoside was produced between day 7 and day 14, being slightly delayed for cell growth. At day 14, the content of cornoside in the fine cell cultures reached 0.9 mg/g fr. wt, which was six times higher than that in the small cell aggregates. In this cell line, the content of 3,4-dihydroxyphenylethanol glycosides was very low and remained almost unchanged during the culture period.

These results indicated that the difference of phenylethanoid distribution pattern between small cell aggregates and fine cell cultures was not due to the growth stages of the cell lines but might depend on the degree of cell aggregation. In Table 3, the amounts of phenolic compounds bound to cell walls of small cell aggregates and fine cell cultures are shown. Of the five phenolic acids for which the extracts were analysed, 4-coumaric acid, ferulic acid and 4-hydroxylbenzoic acid were detected. The total amount of phenolic acids in the cell wall of small cell aggregates was three times higher than that in fine cell cultures, and ferulic acid was predominant in small cell aggregates. In fine cell cultures, 4-hydroxybenzoic acid, which was not present in small cell aggregates, was detectable, and the total content of 4-hydroxyphenyl-type compounds was relatively higher (35% of the total of phenolics) than that in small cell aggregates. In small cell aggregates, the productivity of 3,4-dihydroxyphenylpropanoids related to the formation of cell-to-cell linkage was high, which might result in the formation of cell aggregates and the increasing production of 3,4-dihydroxyphenylethanol derivatives such as verbascoside and arenarioside. In fine cell cultures, the relatively low levels of 3,4-dihydroxyphenylpropanoids might result in the formation of the fine cells and 4-hydroxyphenylpropanoids excessively produced might be metabolized to cornoside.

Table 3. Cell wall-bound phenolic acids in small cell aggregates and fine cell cultures of *A.distichum* cultured for 14 days

| Phenolic acid | Small cell aggregates (μg/g fr. wt) | Fine cell cultures (μg/g fr. wt) |
|---|---|---|
| *t*-Cinnamic acid | — | — |
| 4-Coumaric acid | 8.55 | 5.73 |
| Caffeic acid | — | — |
| Ferulic acid | 99.49 | 24.14 |
| 4-Hydroxybenzoic acid | — | 7.09 |
| Total amount | 108.04 | 36.96 |

—, Not detected.

In an attempt to increase the production of cornoside in fine cell cultures, we examined the effects of phytohormones such as 2,4-dichlorophenoxyacetic acid and indoleacetic acid, concentration of basal medium, yeast extract [11] and cork pieces [12], but none of them stimulated benzoquinolethanoid glucoside production (data not shown). Our attempts to detect cyclohexylethanoid derivatives in *A. distichum* cultured cells by TLC (visualized by $I_2$ vapour) also were unsuccessful (lower than 0.2 μg/g fr. wt). The amounts of cyclohexylethanoids in cultured cells

might be quite low or they might not be produced in the cultured cells.

## EXPERIMENTAL

### *Plant material and culture method*

For induction of callus cultures, segments of surface-sterilized stems of *Abeliophyllum distichum*, *Forsythia suspensa*, *F. viridissima*, and *F. koreana* were placed on MS medium [7] containing 10 $\mu$M NAA and 10 $\mu$M 6-benzylaminopurine, solidified with 0.3% gellan gum (Wako pure chemical, Japan). Callus tissues produced by this treatment were subcultured at 23° in the dark at intervals of 1 month for over 1 year. Cell suspension cultures were initiated by transferring callus tissue (1.0 g) to 20 ml of MS liquid medium containing 10 $\mu$M NAA and 10 $\mu$M 6-benzylaminopurine in 100 ml Erlenmeyer flasks. These cultures were agitated on a rotary shaker at a speed of 100 rpm at 23° in the dark and subcultured every 2–3 weeks for 6 months. To establish large cell clusters, small cell aggregates and fine cell cultures of *A. distichum*, the large cell clusters (ca 2–5 cm in diameter) formed were crushed with a spoon, and relatively large clusters (ca 0.5–1 cm diameter), small clusters (0.2–0.4 cm diameter) and fine cells scrubbed from the inside of the cell aggregates were transferred to fresh medium, respectively, and subcultured every 2 weeks.

### *Isolation and identification of glycosides*

2500 g fr. wt. of *A. distichum* cultured cells were extracted with EtOH for 24 h. The EtOH extract was evaporated *in vacuo* to dryness, and the residue dissolved in $H_2O$ was extracted with $Et_2O$, EtOAc and *n*-BuOH, successively. The $H_2O$ fraction was passed through SEPABEADS HP-207 (Mitsubishi Chemical, Japan), and absorbed compounds were eluted with MeOH. One g of the MeOH fraction was separated by Sephadex LH-20 (aq. 50% MeOH) CC followed by ODS (Chromatorex DM1020T, Fuji Silysia Chem., Japan, $H_2O$ as eluent) CC to give 15 mg cornoside [4]. Six g of the *n*-BuOH fraction (35 g) was separated by silica gel (EtOAc–MeOH–$H_2O$, 100:16:5), Sephadex LH-20 ($H_2O$) and ODS (aq. 15% MeCN) CC successively, to give verbascoside (110 mg, [8]) and arenarioside (94 mg, [8]). The EtOAc fraction (2.9 g) was separated by silica gel ($CHCl_3$–MeOH, 10:1) and ODS (MeCN/$H_2O$) CC to afford halleridone (17 mg, [4]).

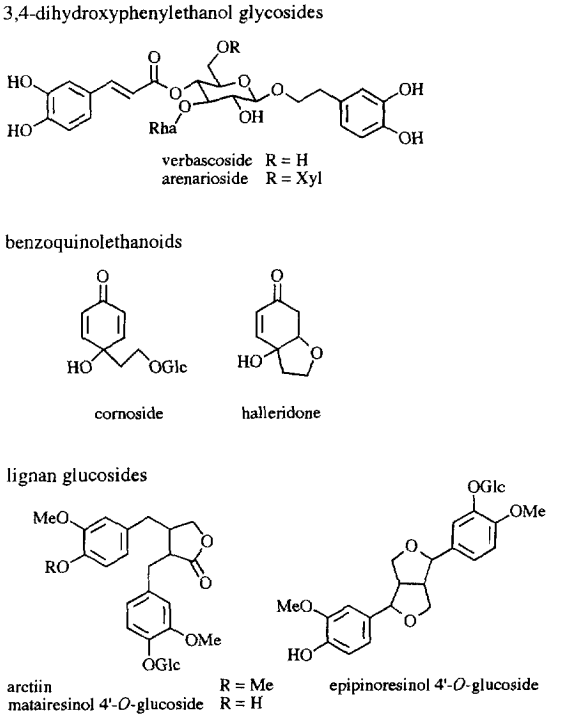
The EtOH extract from 260 g fr. wt of *F. viridissima* cultured cells was separated into $Et_2O$, EtOAc and *n*-BuOH fractions, successively. The EtOAc fraction (6.4 g) was chromatographed on silica gel ($CHCl_3$/MeOH), and the $CHCl_3$–MeOH, 8:1 eluate subjected to Sephadex LH-20 (MeOH) CC to give arctiin (56 mg, [9]). Epipinoresinol 4′-*O*-glucoside (31 mg, [9]) and matairesinol 4′-*O*-glucoside (105 mg, [9]) were isolated from the $CHCl_3$–MeOH (7:1) fraction by ODS CC (aq. 20% MeCN). Verbascoside (452 mg) was also isolated from the $CHCl_3$–MeOH (3:1) fraction. All of compounds were identified by comparison of their $^1$H NMR data with those reported previously.

### *Quantitative analysis of glycosides*

Fresh cells (3.0 g) were extracted with heating with 7 ml MeOH (50°, 4 h). The extracts were subjected to RP-HPLC and the quantities of the glycosides estimated on the basis of the area of their respective absorption peaks at 240 nm [Chromatopac C-R4A (Shimadzu, Japan)] using benzyl alcohol (15 mg) as an int. standard. The HPLC conditions were as follows: column: Hikarisil C18 (4.6 × 250 mm, Asahi Chemical, Japan), solvent: MeCN–$H_2O$ linear gradient of 0% MeCN to 30% MeCN in 60 min, flow rate: 1 ml/min, oven temp.: 40°.

### *Determination of phenolic acids bound to cell walls*

Preparation of cell walls was carried out according to the method of Hartly *et al.* [13] with a slight modification. Two-week-old cultured small cell aggregates and fine cell cultures of *A. distichum* (150 g fr. wt each) were extracted with hot MeOH (three times), respectively. Each residue was dried *in vacuo*, and resuspended in 150 ml of an aq. soln containing neutral detergent [(sodium laurylsulfate (30 g), disodium dihydrogen ethylenediaminethetraacetic dihydrate (18.61 g), sodium borate decahydrate (6.81 g),




Scheme 1. Structures of phenylethaniod derivatives.

$NaH_2PO_4$ (4.56 g), ethylene glycol monomethyl ether (10 ml) in $H_2O$ (1 l), pH 6.9–7.1] and strongly shaken at 25° for 24 h. The solid collected by the filtration with a glass filter was washed successively with $H_2O$ (1.5 l), boiling $H_2O$ (1.5 l), MeOH (600 ml), $Me_2CO$ (700 ml) and $Et_2O$ (300 ml), and the cell walls dried over $P_2O_5$ *in vacuo*. 500 mg of each cell wall preparation was hydrolyzed with 50 ml 1N NaOH at 20° for 48 h under $N_2$ gas. The reaction mixture was filtered to remove a precipitate, and the filtrate was acidified (pH 2.5) with dil HCl. The acidic soln was extracted with EtOAc. The EtOAc extract was dried over $MgSO_4$, and concentrated *in vacuo* to give the residue which was dissolved in MeOH (1 ml) and analyzed by RP-HPLC. HPLC conditions as follows: column, Capcellpak 5C18 AG-120 (4.6 × 250 mm, Shiseido, Japan); solvent: 1% AcOH containing MeCN–$H_2O$ linear gradient of 0% MeCN to 60% MeCN in 60 min; flow rate: 0.9 ml/min, oven temperature: 40°; detection, 280 nm. Identification of phenolic acids was carried out by direct comparison of authentic samples using a photodiode-array SPDM-6A system (Shimadzu, Japan).

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