Hinokitiol Production in a Suspension Culture of Calocedrus formosana Florin

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A suspension culture of Calocedrus formosana Florin was studied as a material for efficient production of hinokitiol. Murashige-Skoog’s medium containing 3% sucrose and 1 mg/l 1-naphthylactic acid was most desirable for cell growth. Cell growth, expressed as fresh cell weight, showed a 20-fold increase after 4 weeks of culture in this medium. Adding potassium acetate or chitosan to the medium increased hinokitiol production. The highest hinokitiol yield, 1700 μg/g fresh cells, was obtained when cells were cultured in the growth medium with chitosan.

Key words: hinokitiol (β-thujaplicin); Calocedrus formosana Florin; suspension culture; chitosan

Hinokitiol (β-thujaplicin) was discovered in 1936 as an element of Chamaecyparis obtusa var. formosana and has since been found to be a strong antimicrobial compound, to which bacteria do not become resistant. Recently its suppression of ethylene synthesis and respiration in some fruits and vegetables has been reported, and it is used for food preservation. The spread of applications have caused an increase in the demand for hinokitiol.

In Japan, hinokitiol is extracted from the sawdust of Thuja plicata var. hondai Makino. However, each gram of sawdust only yields 200 μg of hinokitiol and the quantity of sawdust available is limited by regulated deforestation. Thus the supply of hinokitiol produced by conventional sawdust extraction cannot meet the increasing demand.

Fujii et al. have reported that the hinokitiol productivity in suspension-cultured cells of this plant was up to 10 times higher than that in the intact plant. Furthermore, when we investigated the hinokitiol productivity in cultured cells of several other plants belonging to the Cupressaceae family, it was found that the dedifferentiated cultured cells of Calocedrus formosana Florin produced appreciable quantities of hinokitiol, although hinokitiol was not identified from the intact plants.

For our study, we selected C. formosana Florin as a suitable cell material for hinokitiol production because the cells are not likely to cluster, they suspend easily in a liquid medium, and besides have high hinokitiol productivity. We therefore attempted to find a suitable medium for cell growth and efficient increasers of hinokitiol production.

Materials and Methods

Chemicals. Casamino acids was obtained from Difco Laboratories (Detroit, MI, USA). Unless otherwise indicated, all other chemicals were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan).

Cell culture. C. formosana leaves were obtained from the Kyoto herbal garden of Takeda Chemical Industries, Ltd. Callus was induced from these leaves on Murashige-Skoog’s (MS) medium containing 1% sucrose, 1 mg/l 1-naphthylactic acid (NAA), and 0.3% gellan gum, adjusted to pH 5.8 (solid MS medium). The culture was incubated at 25°C in darkness. The induced calluses were subcultured at 4-week intervals on solid MS medium.

A suspension culture was started by transferring the calluses to 40 ml of the liquid MS medium containing 3% sucrose and 1 mg/l NAA, adjusted to pH 5.8 (MS-1N medium), in a 300-ml Erlenmeyer flask. The suspension culture was agitated on a rotary shaker at 80 rpm while incubating at 25°C in darkness, and subcultured at 2-week intervals in the same medium freshly prepared.

Cell growth. After the liquid medium was removed by pipette, cells of approximately 0.3–0.5 g fresh weight (FW) were inoculated into 20 ml of liquid medium in Petri dishes (9 cm inner diameter), and the cultures were incubated at 25°C in darkness with 80 rpm agitation. After 2 weeks, each culture was transferred to fresh medium of the same composition, and after 4 weeks of culture, fresh cells were harvested and weighed. All experiments were done in triplicate.

Additives to increase hinokitiol production. The fol-
lowing additives were dissolved individually in 20 ml of MS-1N medium: potassium acetate, carboxymethyl cellulose, chitin, curdlan, amylose from corn, pectin from citrus fruit, dextran, cellulose (Merck, Darmstadt, Germany), polygalacturonic acid from oranges (Sigma Chemical Co., St. Louis, MO, USA), chitosan L (M, 1000±200), chitosan M (M, 4000±1000), chitosan H (M, 7000±1500) (Seikagaku Kogyo Co., Ltd., Tokyo, Japan), amylopectin (Nacalai Tesque Inc., Kyoto, Japan), and cyclodextrin (Takeda Chemical Ind., Osaka, Japan). The pH of each medium was adjusted to 5.8 before sterilization. Cells of approximately 0.5 g FW were then cultured in these media as described above. After 1 week, fresh cells were harvested and their hinokitiol was extracted and measured.

*Extraction and analysis of hinokitiol.* Intracellular hinokitiol was extracted from the fresh cells with 10 volumes of 1% (v/v) HCl in methanol (conc. HCl was diluted 36-fold by methanol) by ultrasonic treatment for 2 h. Hinokitiol in the extract was measured by HPLC on an L-6200 system (Hitachi, Tokyo, Japan) with an Inertsil ODS-3 column (4.6 x 250 mm, GL Sciences Inc., Tokyo, Japan). As the mobile phase, 60% (v/v) methanol containing 0.1% (v/v) phosphoric acid was run at a flow rate of 1 ml/min. The column temperature was 50°C. Column effluents were monitored for absorbance at 254 nm by an L-4200 UV-VIS detector.

![Graphs A, B, C](image)

**Fig. 1.** Total Ion (A, B) and Ion m/z 164 (C) Gas Chromatogram. A, Hinokitiol standard; B, C, extract of cultured cells of *C. formosana.*
Hinokitiol was identified by GC-mass spectrometry and $^1$H-NMR. The extract was directly analyzed by a 5890 GC system (Hewlett Packard, Tokyo, Japan) with a CBP1-M25-025 column (0.25 mm i.d. × 25 m, Shimadzu, Kyoto, Japan) connected to a Jeol JMS-DX 303HF mass spectrometer (Jeol, Tokyo, Japan). The column temperature was programmed from 60°C to 220°C at 4°C/min. The injector temp was 275°C. Mass spectra were recorded at 70 eV.

For the $^1$H-NMR analysis, the extract was treated with a solution of 10% potassium ferrocyanide and 6 N HCl$^9$ to decompose hinokitiol-iron complexes. An $^1$H-NMR spectra was recorded at ambient temperature on a Jeol AL 400 spectrometer (Jeol, Tokyo, Japan) at 400 MHz and locked to the deuterium resonance of the acetone-D$_6$ solvent.

**Results**

**Hinokitiol identification**

When the extract from cultured *C. formosana* cells was analysed by HPLC, the UV spectrum of the peak with a $t_R$ identical to authentic hinokitiol corresponded with the authentic one (data not shown).

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**Fig. 2.** Mass Spectrum of Peak 5 of Cell Extract Shown in Fig. 1 B (A) and Hinokitiol Standard (B).
To confirm its chemical structure, the extract was directly analyzed by GC-mass spectrometry. Chromatograms are shown in Fig. 1. Peak 5 in the total ion chromatogram of the extract has the identical $t_R$ with the authentic hinokitiol. Only one peak was detected at this $t_R$ in 164 ion chromatogram. The mass spectrum of this peak gave a fragmentation pattern identical with the authentic hinokitiol (Fig. 2). Other peaks 1, 2, 3, and 4 in Fig. 1B were thought to be impurities from instruments because they were identified by mass spectrum as hexamethylcyclohexasiloxane, octamethylcyclotetrasiloxane, decamethylcyclopentasiloxane, and undecamethylcyclohexasiloxane, respectively.

Next, this extract was decomposed and analyzed by $^1$H-NMR. Figure 3 shows the $^1$H-NMR spectrum of the olefinic proton region. The chemical shifts of those olefinic protons were characteristic of hinokitiol ($\beta$-thujaplicin), not the other isomers, $\alpha$- and $\gamma$-thujaplicin. It was confirmed that the cultured C. formosana cells produced hinokitiol ($\beta$-thujaplicin) but no $\alpha$- or $\gamma$-thujaplicin.

**Cell growth**

Cell growth was compared among cultures grown in five standard media: MS, B5, White, Nagata-Takebe, and woody plant medium (Fig. 4). Cultures grown in MS medium had a more than 3 times higher growth rate than in the others, while they produced only a little hinokitiol. Thus, to optimize cell growth, the composition of MS medium was investigated further.

The effects of two inorganic nitrogen sources on cell growth and hinokitiol production were examined. The molar ratio of nitrate to ammonium was varied under a constant total nitrogen concentration of 60 mM (Fig. 5). The cells showed good growth when the ratio was between 48:12 and 36:24. The ratio of nitrate to ammonium in the MS medium (40:20) fell within this range. On the other hand, intracellular hinokitiol production was inhibited by the ammonium.

Next, the total inorganic nitrogen concentration was varied (0, 30, and 60 mM) under a constant molar ratio of nitrate to ammonium of 40:20, with combined casamino acids as organic nitrogen at the concentrations of 0–5 g/l (Fig. 6). When casamino acids, which was reported to increase the cell growth of T. dolabrata var. hondai, was added to the medium that contained inor-

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![Graph](image-url)

**Fig. 4.** Cell Growth and Hinokitiol Production of C. formosana Suspension Culture in Standard Media. NT, Nagata-Takebe; WP, woody plant medium. Approximately 0.5 g FW cells were inoculated into 20 ml of medium containing 1 mg/l NAA and 3% sucrose. Cells were cultured for 4 weeks. Values of cell weight are means of triplicate measurements (± S.E.), and hinokitiol content are single measurements.

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![Graph](image-url)

**Fig. 3.** $^1$H-NMR Spectra of the Olefinic Proton Region of Hinokitiol Standard (A) and Extract of Cultured Cells of C. formosan after Decomposing Hinokitiol-iron Complex (B).
Fig. 5. Effects of Molar Ratio of NO$_3^-$ to NH$_4^+$ on Cell Growth and Hinokitiol Production of _C. formosana_ Suspension Culture

NO$_3^-$ and NH$_4^+$ concentrations were each varied under constant total nitrogen concentration at 60 mM. Each medium contained 1 mg/1 NAA and 3% sucrose. Other culture conditions were the same in Fig. 4. Values are means of triplicate measurements (±S.E.).

Fig. 6. Effects of Inorganic Nitrogen Concentrations and Combination of Casamino Acids on Cell Growth of _C. formosana_ Suspension Culture.

Total inorganic nitrogen concentration was varied under a constant ratio of NO$_3^-$/NH$_4^+$ = 40/20. Approximately 0.3 g FW cells were inoculated into 20 ml of medium contained 1 mg/1 NAA and 3% sucrose. Other culture conditions were the same as in Fig. 4. Values are means of triplicate measurements.

Fig. 7. Effects of Sucrose Concentration on Cell Growth and Hinokitiol Production of _C. formosana_ Suspension Culture.

MS medium containing 1 mg/1 NAA was used as a basal medium. Other culture conditions were the same as in Fig. 4. Values are means of triplicate measurements (±S.E.).

Inorganic nitrogen (30 and 60 mM), it had no beneficial effect on cell growth of this culture. Thus, inorganic nitrogen was suitable for the cell growth and the optimum concentration was 30 mM (equal in concentration to the original MS medium).

The effects of other components in the MS medium were also tested by varying their concentration. The omission of each component resulted in lower cell growth, indicating that all components contribute to cell growth. However, doubling the concentration of each component showed no marked improvement in cell growth (data not shown).

As a carbon source, sucrose was tested by varying its concentrations (Fig. 7). Maximum cell growth was obtained at the concentrations of 2–3%, and hinokitiol contents were small in this range. It was found that sucrose had been converted into Glc and Fru within a few days after the start of culture, and then only Glc was used by the cells. However, when Glc was tested as carbon source to compared with sucrose, the growth was not better than with sucrose (data not shown).

As 1 mg/1 NAA was a suitable auxin for the cell growth, four cytokinins (thiazuron, benzyladenine, kinetin, and zeatin) were combined with this. However, only the medium that contained no cytokinins had normal cell growth, indicating that cytokinin addition is not necessary for the growth and may retard it (data not shown).

Based on these results, MS medium containing 3% sucrose and 1 mg/1 NAA (MS-1N medium) was selected as the most suitable medium for cell growth. In this medium, cell growth reached stationary phase at about 2 weeks of culture. After continued culture for 2 more weeks in fresh medium, the cell growth, expressed as fresh cell weight, showed a 20-fold increase (Fig. 6). When 0.5 g FW was inoculated, it was confirmed that the final cell weight reached to 10 g FW/20 ml in medium (data not shown).

**Effects of potassium acetate on hinokitiol production**

In previous studies, the addition of potassium acetate to medium was found to increase the production of hinokitiol in suspension-cultured cells of _T. dolabrata_ var. _hondai_. The effect of potassium acetate on suspension-cultured _C. formosana_ cells was examined using the growth medium, MS-1N medium. More hinokitiol was produced when cells were cultured with potassium acetate than without it. The maximum hinokitiol production was found at the addition of 2.2–4.3 mM potassium acetate (Fig. 8). The maximum amount of hinokitiol attained from 1 g FW of cells was 1300 µg, 8 days after addition of 4.3 mM potassium acetate (Fig. 9).

**Effects of polysaccharides on hinokitiol production**

Twelve polysaccharides commonly found in plants or microorganisms were added to the growth medium to discover if any of them increased hinokitiol production. The Table shows the hinokitiol production with these polysaccharides, compared with the effect of 4.3 mM potassium acetate addition. When 250 µg/ml chitosan was added, it increased hinokitiol production as effectively as 4.3 mM potassium acetate and it was the most
Fig. 8. Relation between Potassium Acetate (AcK) Concentration and Hinokitiol Production in C. formosana Suspension Culture. MS-1N medium was used as a basal medium. Approximately 0.5 g FW cells were inoculated into 20 ml of each medium, and cells were harvested after 7 days of culture. Values are means of triplicate measurements (±S.E.).

Fig. 9. Course of Hinokitiol Production in C. formosana Suspension Culture in the Presence (closed circle) and Absence (open circle) of 4.3 mM Potassium Acetate.
Culture conditions were the same in Fig. 8. Values are means of triplicate measurements (±S.E.).

effective among these 12 polysaccharides.

To confirm the increased production caused by chitosan, the effects of chitosan and potassium acetate on the hinokitiol production were investigated in four cell lines, which derived from the same callus and show different levels of hinokitiol production in response to the addition of potassium acetate (Fig. 10). The phenomenon that the addition of chitosan caused higher hinokitiol production than the addition of potassium acetate was reproduced in three cell lines (Fig. 10, No.1-No.3). But in a cell line No.4, which did not respond to potassium acetate addition, chitosan did not increase hinokitiol production either.

This stimulation by chitosan was further investigated using chitosan of three different molecular weights (Fig. 11). It was observed that as chitosan molecular weight decreased, hinokitiol production increased. The maximum amount of hinokitiol attained from 1 g FW of cells was 1700 μg, from adding 1000 μg/ml chitosan of 1000±200 molecular weight to MS-1N medium.

Discussion

In this study hinokitiol was identified for the first time in cultured C. formosana cells. It is noteworthy that this dedifferentiated callus culture accumulated hinokitiol because there is no report of its presence in the intact plant.

Potassium acetate increased hinokitiol production about 7-fold (13 mg/g dry weight (DW)) than that in suspension-cultured T. dolabrata var. hondai cells in a similar way (2.1 mg/g DW). Using this plant as cell material is considered promising for hinokitiol production. Further, using chitosan raised hinokitiol production about 1.3 times still higher (17 mg/g DW) than using potassium acetate.

Table. Effects of Polysaccharides on Hinokitiol Production in C. formosana Suspension Culture

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Structure</th>
<th>Hinokitiol production (μg/g FW)</th>
<th>Improvement [%]Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Improving activity [%])</td>
<td>added polysaccharide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25 μg/ml 250 μg/ml</td>
</tr>
<tr>
<td>Cellulose</td>
<td>β-1,4-D-Glc</td>
<td>0(0)</td>
<td>699(5.8)</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>α-1,4-D-Glc</td>
<td>30(2.3)</td>
<td>850(65.3)</td>
</tr>
<tr>
<td>Chitin</td>
<td>β-1,4-D-Glc-NAc</td>
<td>547(42.1)</td>
<td>902(69.4)</td>
</tr>
<tr>
<td>Chitosan</td>
<td>β-1,4-D-Glc-NH₂</td>
<td>569(43.8)</td>
<td>1348(103.7)</td>
</tr>
<tr>
<td>(M, 4000±1000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curdlan</td>
<td>α-1,3-D-Glc</td>
<td>80(6.6)</td>
<td>222(17.1)</td>
</tr>
<tr>
<td>Amylose</td>
<td>α-1,4-D-Glc</td>
<td>147(11.3)</td>
<td>516(39.7)</td>
</tr>
<tr>
<td>Amylopenticyclosin</td>
<td>α-1,4-D-Glc-α-1,6-D-Glc</td>
<td>80(6.6)</td>
<td>10(0.8)</td>
</tr>
<tr>
<td>β-Cyclodextrin</td>
<td>α-1,4-D-Glc</td>
<td>98(7.5)</td>
<td>343(26.4)</td>
</tr>
<tr>
<td>Pectin</td>
<td>α-1,4-D-Gal(Met)-UA</td>
<td>7(0.5)</td>
<td>783(60.2)</td>
</tr>
<tr>
<td>Polygalactaritonic acid</td>
<td>α-1,4-D-Gal-UA</td>
<td>60(0.5)</td>
<td>636(48.9)</td>
</tr>
<tr>
<td>α-D-Galactaritonic acid</td>
<td>α-D-Gal</td>
<td>5(0.4)</td>
<td>7(0.5)</td>
</tr>
<tr>
<td>Dextran</td>
<td>α-1,6-D-Glc</td>
<td>12(0.9)</td>
<td>48(3.7)</td>
</tr>
<tr>
<td>(M, 6000±9000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3 mM AcK</td>
<td></td>
<td>1300(100.0)</td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td></td>
<td>60(0.5)</td>
<td></td>
</tr>
</tbody>
</table>

Δ To calculate improving activity (%), the produced hinokitiol amount with 4.3 mM AcK (1300 μg/g FW) was taken as 100.0%.

Glc, glucose; Gal, galactose; Man, mannose; CM, carboxymethyl; Ac, acetyl; NH₂, amine; Met, methoxy; UA, uralonic acid; Gal, galucose

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Fig. 10. Comparison of Effects of Potassium Acetate (AcK) and Chitosan on Hinokitiol Production among Several Cell Lines.
AcK, 4.3 mM potassium acetate; chitosan, 200 μg/ml chitosan M. Culture conditions were the same in Fig. 8. Values are means of duplicate measurements.
Because the medium selected in this study is capable of supporting 20-fold cell growth and these cells contain 90% water, the final dry cell density is calculated to be 5%. Assuming the theoretical limit of plant cell density in suspension culture to be approximately 4–5%, the cell growth obtained here was regarded as maximum and the medium developed was regarded as the composition most suitable for cell growth.

During long-term continuous subculturing, the cells became either yellowish white or brown. The yellowish white cells had a higher growth rate and produced little hinokitiol, but the brown cells had slow growth and produced more hinokitiol. So it is considered that yellowish white cells would be in the growth step, and brown cells in the production step. Hinokitiol accumulated primarily within the brown cells, with only a small quantity leaking into the culture medium. The presence of hinokitiol in the medium, even at concentrations as low as 0.1 μg/ml, inhibited cell growth strongly (data not shown). From these observations and Figs. 4, 5, and 7, cell growth appeared to conflict with hinokitiol production, and it seemed difficult to select fast-growing cells with high resistance to the growth inhibition by hinokitiol.

The effectiveness as elicitor of polysaccharides with β-1,4 linkage, such as cellulose, carboxymethyl cellulose, chitin, and chitosan, were higher than those with β-1,3, α-1,4, and α-1,6 linkages. Among polysaccharides with β-1,4 linkage, the functional groups were observed to increase the hinokitiol production to various degrees; amine groups produced a greater increase than acetate groups and carboxymethyl groups produced the least increase (Table).

In this study, the chitosan was most effective for hinokitiol production. Because hinokitiol is an antimicrobial substance and would be involved in a plant’s defense mechanism against microbial infection, chitosan, one component of a fungal cell wall and a very efficient chemical elicitor in the production of phytoalexins, might be expected to stimulate the biosynthesis of hinokitiol. On the other hand, potassium acetate increased the hinokitiol production, because acetate is probably the basic precursor for the synthesis of hinokitiol. A combination of the elicitor and the precursor is expected to have some further beneficial effects on the hinokitiol production.

The source of nitrogen affects the formation of secondary metabolites. In this study, nitrate was a suitable nitrogen source and ammonium inhibited hinokitiol production in C. formosana cells (Fig. 5). However, in T. dolabra var. hondai cells, ammonium increased hinokitiol production. We cannot explain why these sources of nitrogen would cause a discrepancy in the biosynthesis of the same secondary metabolite, hinokitiol, between two species belonging to the same family, Cupressaceae.

References