Rotenoid Biosynthesis by Tissue Culture of  
*Derris elliptica*

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Callus induction was carried out by tissue culture using leaves of *Derris elliptica* to obtain rotenoids. A small amount of rotenoids (2.9 µg/gDW) was detected in the callus tissue thus induced and subcultured for 4 months. Rotenoid biosynthesis, however, decreased with frequent subcultures of callus tissue, and was finally lost. Then, a callus with imperfectly differentiated rootlets was induced from the leaves or stems of derris by regulating plant hormones. This root-like organ was found to contain rotenoids which were identified as rotenone and deguelin by GC-MS. The content was 160 µg/gDW as rotenone.

*Derris elliptica* Bentham (Leguminosae) has been cultivated in the countries of Southeast Asia, and its root has been used as a fish poison or an insecticide. Rotenoids is known to be the active principle of derris root. Uddin and Khanna reported the production of rotenoids by tissue culture of *Crotalaria burhia*,¹ but there is no report appeared concerning the formation of rotenoids in cultured derris tissue.

We tried to induce the calluses of derris and to subculture them for the purpose of producing rotenoids, because callus culture or cell suspension culture is suitable for the production of metabolites of plant cells. However, content of the rotenoids in the callus tissues was very low and the ability of rotenoid biosynthesis was unstable in this callus. Among the many cell lines of derris calluses we obtained, some were able to synthesize rotenoids and the others were not. It was difficult to find a marker in calluses to distinguish immediately whether they could synthesize the rotenoids or not. On the other hand, we can easily distinguish the differentiation stage of rootlets that may indicate the ability of synthesizing secondary metabolites. So we tried to induce the imperfectly differentiated root-like organ which would produce rotenoids.

In this report, we describe the biosynthesis of rotenoids in calluses and imperfectly differentiated rootlets of derris.

MATERIALS AND METHODS

**Callus tissue.** *Derris elliptica* (Viny derris) was collected at Tokyo Metropolitan Medicinal Plants Garden. Calluses were induced from leaves of derris on the agar medium (10 g agar/liter) containing the inorganic elements reported by Murashige and Skoog² with addition of 1.0 mg/liter thiamine·HCl, 0.3% yeast extract, 100 mg/liter myo-inositol, 3% sucrose, 2.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/liter kinetin. The induced calluses were maintained by transferring at 2~3 week intervals at 30°C in the dark. Cell suspension culture was carried out in a liquid medium containing the same components as above except for yeast extract. Cultivation was performed on a reciprocal shaker (300 rpm, 2 cm) in test tube (21 mmφ).

**Callus with imperfectly differentiated rootlets.** Callus with imperfectly differentiated rootlets were induced also from leaves or stems of derris on the agar medium. The medium contained the same inorganic elements as those used for callus culture with addition of 1.0 mg/liter thiamine·HCl, 100 mg/liter myo-inositol, 3% sucrose, 15% coconut milk, 10~50 mg/liter indole-3-acetic acid (IAA), 0.5 mg/liter 2,4-D and 1.0 mg/liter kinetin. The calluses
with imperfectly differentiated rootlets were induced within 4–6 weeks and cultivation was performed after the succeeding 6–8 weeks at 30°C in the dark.

Analytical procedure. Cultured tissue harvested and plant tissues were dried at temperature below 60°C or freeze-dried. Dried materials were subjected to extraction with ethyl ether, acetone or chloroform in a Soxhlet’s extractor for 10–24 hr. The extracts were concentrated and used for the analyses.

The extracts of derris plant and cultured tissues were analyzed by thin layer chromatography (TLC) of Kieselgel GF254 (Merck) using chloroform-acetone-acetic acid (196:3:1)\textsuperscript{3} as the solvent system. The spots were detected by iodine vapor.

Gas liquid chromatography (GLC) was performed with a Hitachi model 063 gas liquid chromatograph equipped with flame ionization detector. A glass column (3 mm × 1 m) packed with 2% Silicone OV-17 on Chromosorb WAW DCMS (60–80 mesh) was employed. Helium gas was used as carrier at 0.5 atm.

Identification of rotenoids was carried out with TLC followed by gas liquid chromatograph mass spectrometer (GC-MS). The chloroform extracts obtained from preparative TLC were separated by GLC on the condition described above, and analyzed with combined mass spectrometer Hitachi RMU-6. Rotenoids were identified by the spectra recorded at 40 eV of ionizing potential.

Colorimetric determination of rotenoids was carried out according to the method described by Goodhue,\textsuperscript{4} which was the modification of Gross and Smith’s method.\textsuperscript{5} The content of rotenoids was calculated as rotenone from the calibration curve drawn with the maximum optical density (O.D.) of coloration. A Shimadzu Spectronic 20 A spectrophotometer was used for the measurement of O.D. at 540 nm.

RESULTS

Callus culture
Callus tissue of derris was fragile and suspended easily in the liquid medium. Rapid growth was observed in subculture, especially in cell suspension culture. The minimum doubling time was 1.5 day in shaking culture.

Content of rotenoids in callus tissue
On colorimetric determination, 2.9 µg/gDW of rotenoids was detected in the 4 month subcultured tissue but not in the cultured broth, while only 0.6 µg/gDW of rotenoids was detected in 14 month subcultured callus tissue. The ability of rotenoid biosynthesis decreased gradually during further subculture, and was finally lost completely. Rotenoids were not detected even if the callus tissue was cultured on the same medium replacing 2,4-D (2.0 mg/liter) with naphthaleneacetic acid (2.0 mg/liter), or it was transferred into the liquid medium, in which the plant hormone composition was modified as to promote re-differentiation (kinetin 1.0 mg/liter and no auxin).

Induction of calluses with imperfectly differentiated rootlets
The imperfectly differentiated organ was induced together with callus tissues within 4–6 week incubation of derris leaf or stem on the induction medium in which the plant hormone composition was altered from that of the callus induction medium. The organ is morphologically similar to a root as shown in Fig. 1, and we named this organ “root-like organ.”

IAA concentration in the medium had a remarkable effect on induction and its growth of root-like organ as shown in Table I.

\begin{table}[h]
\centering
\caption{Root-like organ induction and its growth in various IAA concentrations}
\begin{tabular}{|c|c|c|c|c|}
\hline
IAA concn. (µg/ml) & 10 & 20 & 50 & 100 \\
\hline
Root-like organ induction & ± & ++ & + & ± \\
Growth & ± & + & ++ & ± \\
\hline
\end{tabular}
\end{table}

±, poor; +, good; ++, excellent.
Purification and identification of rotenoids

Chloroform extract of dried root-like organ was chromatographed on silica gel TLC plate as shown in Fig. 2. Figure 2 suggests the presence of rotenoids in root-like organ. We obtained the extract of fraction of rotenoids by preparative TLC and applied to GC-MS. In gas chromatogram shown in Fig. 3, we could find out the peaks which coincided with authentic rotenoids (deguelin and rotenone) in retention time. The mass spectra of these peaks and authentic rotenoids are shown in Fig. 4. From these spectra, the peaks were identified as deguelin and rotenone, respectively.

These rotenoids were also isolated and identified from the root of original plant.

Content of the rotenoids in root-like organ

Table II shows the content of rotenoids in root-like organ and original plant, determined by colorimetric method. In original plant, rotenoids were found in large amounts in thick roots (8 mmφ), and small amounts in fine roots (0.5~1.0 mmφ). In root-like organ which was induced within 4~6 weeks and cultured for 6~8 weeks, 160 μg/gDW of rotenoids were detected. However, rotenoids were hardly found out in callus tissue accompanied with root-like organ.

Root-like organ was able to be subcultured. More rapid growth was, however, observed in callus tissue accompanied with root-like organ than root-like organ itself.

DISCUSSION

Only trace amounts of rotenoids were detected in undifferentiated callus tissue induced from leaves of D. elliptica. This result

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<th>Rotenoid content</th>
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<td>Root (8 mmφ)</td>
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<td>Root (0.5~1.0 mmφ)</td>
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<td>Root-like cultured organ</td>
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agrees with the well-known phenomena that callus tissues often contain none or little amounts of secondary metabolites compared with intact plants. Moreover, when we transfer the callus tissues frequently, we might select the cell line that grows rapidly but is not suitable for producing secondary metabolites.

On the other hand, it has been observed generally that redifferentiated organ from callus tissues could synthesize the secondary metabolites as well as intact plants. However, a long cultivation time is needed for small callus tissue to redifferentiate and produce the secondary metabolites. In this respect, the root-like organ grows more rapidly than root organ cultured in similar condition, and still maintains the ability of rotenoid biosynthesis. The imperfectly differentiated organ is suitable for producing rotenoids by tissue culture.

But the results of this investigation pointed out the difficulties in carrying out a large scale suspension culture of these organs, because the calluses accompanied with root-like organ grows more rapidly than root-like organ itself, and these calluses hardly synthesize the rotenoids. Therefore we should transfer the root-like organs selectively and cultivate them with the static culture method for the production of rotenoids. Using these techniques we can exclude the calluses that are incapable of producing rotenoids.

So, it can be said that these results demonstrate the possibility of producing secondary metabolites by incompletely differentiated tissue culture.

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**REFERENCES**