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# TANNIN PRODUCTION IN CALLUS CULTURES OF *QUERCUS ACUTISSIMA*

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**Key Word Index**—*Quercus acutissima*; Fagaceae; tannin; polyphenol; callus.

**Abstract**—From callus cultures of *Quercus acutissima* Carruthers, 13 phenolics, (+)-catechin, gallic acid, β-glucogallin, gallic acid 3-*O*-β-D-glucopyranoside, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranoside, 1-desgalloyleugeniin, eugeniin, pedunculagin, 1-*O*-galloylpedunculagin, casuarinin, stachyurin, castalagin and vescalagin were isolated. The removal of $NH_4NO_3$ from the culture media enhanced both the growth and tannin production of the calli. We found that calli cultured on MS medium with IAA-BA and 40 or 50 g $l^{-1}$ sucrose showed good growth and optimal tannin production.

## INTRODUCTION

*Quercus acutissima*, mainly distributed in subtropical regions of Asia, is well known as a rich source of high $M_r$ polyphenols (tannin). Several tannins (both hydrolysable and condensed types) from this plant have been isolated and their chemical structures elucidated [1–4]. In spite of numerous chemical studies on *Quercus* plants, only a few experiments on secondary metabolism with *in vitro* cultures of the genus (only *Quercus robur*) have been reported [5,6]. This report is the first study of tannin production in callus cultures of *Q. acutissima*.

## RESULTS AND DISCUSSION

The calli of *Q. acutissima* were derived from leaf segments of the plant cultured on Murashige-Skoog (MS) [7] solid medium supplemented with 2 mg $l^{-1}$ naphthaleneacetic acid (NAA) and 0.1 mg $l^{-1}$ benzyladenine (BA). The induced calli were subcultured on the same medium for over one year in the dark.

From calli cultured for four weeks, 13 phenolic compounds, (+)-catechin (**1**), gallic acid, β-glucogallin (**2**) [8], gallic acid 3-*O*-β-D-glucopyranoside [9], 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranoside (**3**) [10, 11], 1-desgalloyleugeniin [12], eugeniin [13], pedunculagin (**4**) [14], 1-*O*-galloylpedunculagin [14], casuarinin [15], stachyurin [15], castalagin (**5**) [15] and vescalagin [15] were isolated. Among these, **1** is a structural component of condensed tannins, while the others are hydrolysable tannins (gallotannin and ellagitannin) and related phenolics. This profile of polyphenol constituents produced in the calli was similar to that observed in extracts from the intact plants. From the bark of the intact plant of *Q. acutissima*, it was possible to isolate **1** (0.001%), **3** (0.003%), **4** (0.003%) and **5** (0.009%), together with 1-desgalloyleugeniin (0.013%), casuarinin (0.013%) and acutissimin A [2] (0.033%, a fresh wt). Compound **2** could not be detected in extracts from the bark.

For the determination of the growth and tannin production of *Q. acutissima* calli, three media; A (0.1 mg $l^{-1}$, 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg $l^{-1}$ BA); B (2 mg $l^{-1}$ NAA and 0.1 mg $l^{-1}$ BA) and C (3 mg $l^{-1}$ indole-3-acetic acid (IAA) and 0.1 mg $l^{-1}$ BA) were used. After inoculation into these three media, the biomass of the calli increased throughout the culture period (Fig. 1), with the most rapid weight gain observed from weeks 2 to 4. When cultured on medium C, the calli attained the highest (dry wt, 130 mg) at the end of the culture period (at week 8). Tannin (**1**–**5**) content in the calli on these media is shown in Fig. 2. On all media, the major constituent produced in the calli was a gallotannin **3** (2.10% as dry wt at week 6 on medium A, 3.41% as dry wt at week 4 on medium B and 3.73% as dry wt at week 4 on medium C). The contents of **1**, **2**, **4** and **5** remained at low levels (below 1% as dry wt) throughout the culture period on all media.

In plant tissue cultures, nitrogen source is often an important factor for growth and for production of polyphenols [16–19]. The effects of $NH_4NO_3$ on the growth and tannin production of *Q. acutissima* calli were therefore investigated. Three media (A′, B′ and C′) whose constituents were identical to those of media A, B and C, respectively, except for the omission of $NH_4NO_3$, were prepared. The growth and tannin (**1**–**5**) content of the

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calli cultured on media A', B' and C' were much greater than those observed on media A, B and C (containing $NH_4NO_3$) (Fig. 3). Particularly the levels of **1**–**3** were markedly increased in the media without $NH_4NO_3$. The highest content of tannins (total content of **1**–**5**) was obtained on medium C' (**1**: 0.61%, **2**: 2.29%, **3**: 11.05%, **4**: 0.22% and **5**: 0.18% as dry wt). Although the data are not shown in the figures, the calli cultured on the media without $NH_4NO_3$ also yielded a large amount (*ca* 2.5 times compared with those on the media with $NH_4NO_3$) of polygalloylglucoses whose profile was similar to that of *Q. infectoria* [20]. Therefore, the removal of $NH_4NO_3$ from the culture medium seemed to promote the biosynthesis of gallotannins in the callus cultures of *Q. acutissima*.

Sucrose concentrations in the culture media are known to affect secondary metabolism of cultured plant cells [21–23]. The growth and tannin (**1**–**5**) production of *Q. acutissima* calli cultured on medium C containing different concentrations of sucrose (20–100 $g\,l^{-1}$) were determined (Fig. 4). On the media containing 40 or 50 $g\,l^{-1}$ sucrose, the calli showed good growth and high yields of **1**–**5**. The growth level (dry wt of the callus) on these two media was almost two times greater than that on the medium with 30 $g\,l^{-1}$ sucrose (original medium C). Among the different sucrose concentration tested, the maximum content of **1**–**5** was observed on the medium with 50 $g\,l^{-1}$ sucrose; **1**: 0.24%, **2**: 0.55%, **3**: 6.59%, **4**: 1.01% and **5**: 0.41% as dry wt. On the medium containing 20 $g\,l^{-1}$ sucrose, callus growth was relatively poor.

Both the high level of production and the close similarity between the metabolite profiles of cultured and whole plant tissues, make *Q. acutissima* calli a suitable system for the study of hydrolysable tannin biosynthesis.

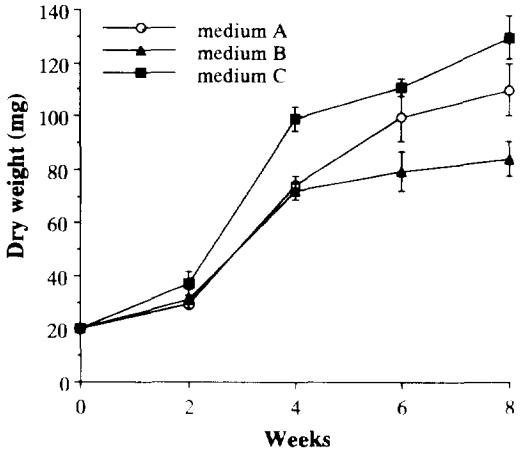


Fig. 1. Growth of callus cultures of *Quercus acutissima* (bars represent standard errors).

## EXPERIMENTAL

$^1H$ NMR was measured at 270 MHz, locked to the major deuterium resonance of the solvent ($(CD_3)_2CO$). TLC was conducted on silica gel and spots were detected under UV lights and visualized by spraying with 2% $FeCl_3$ and 10% $H_2SO_4$. MS solid medium used for the experiments contained 30 $g\,l^{-1}$ sucrose. All media were adjusted to pH 5.7 before autoclaving at 121° for 15 min. Cultures were placed in the dark at 25°. Pieces of calli were cultured in sterile Petri dishes (9 cm in the diameter, three pieces of the calli per dish) containing 25 ml medium. Data shown in the figures are the means of 3 samples (calli).

*Plant material and induction of the callus.* Leaf segments of *Quercus acutissima* Carruthers collected in April 1991 at Saga City (Japan) were surface sterilized and placed aseptically on MS solid medium (solidified with 2.5 $g\,l^{-1}$ gerlite) supplemented with 2 $mg\,l^{-1}$ NAA and 0.1 $mg\,l^{-1}$ BA (medium B). After 2 months culture, the calli derived on the segments were transferred to the same medium and subcultured at 6 weeks intervals for over 1 year. Voucher specimens are deposited at Faculty of Agriculture (Saga University, Japan).

*Extraction and isolation of tannins.* (a) *From callus.* Lyophilized calli (36.4 g) were mashed and extracted at room temp. with 80% aq. $Me_2CO$ (200 ml × 3). The extract, after concn under red. pres., was subjected to Sephadex LH-20 (3.0 × 18.5 cm) CC and eluted by $H_2O$ increasing amount of MeOH to afford 4 frs (frs 1–4). Fr. 1 was applied on MCI GEL CHP 20P ($H_2O$–MeOH) CC to give **4** (161 mg), **5** (130 mg) and vescalagin (80 mg). Fr. 2 was separated by Fuji gel ODS G3 ($H_2O$–MeOH) CC

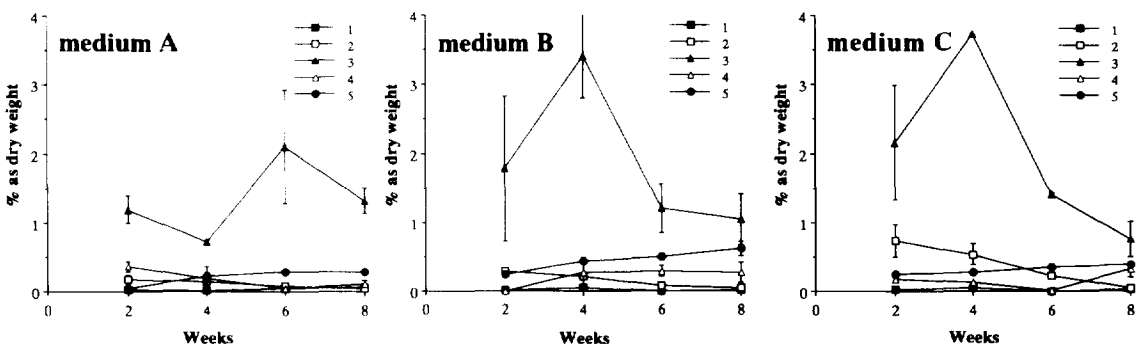


Fig. 2. Tannin contents in callus cultures of *Quercus acutissima* (bars represent standard errors).

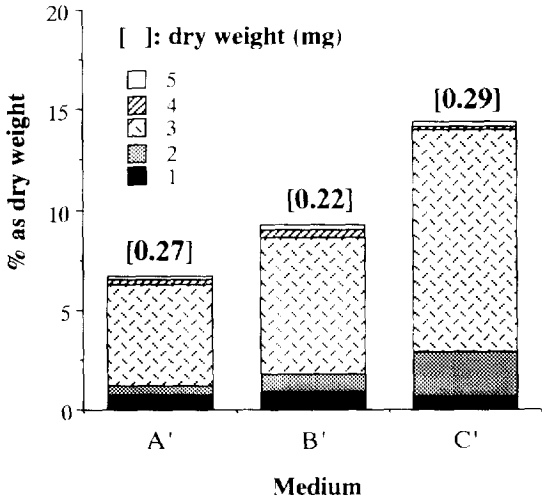


Fig. 3. Growth (dry weight (mg) given in square brackets) and tannin contents of *Quercus acutissima* calli cultured for 4 weeks in the dark

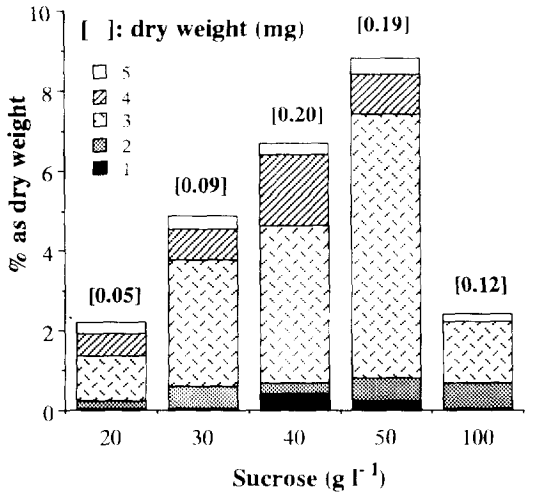


Fig. 4. Growth (dry weight (mg) given in square brackets) and tannin contents of *Quercus acutissima* calli cultured on medium C with various concentrations of sucrose.

to afford **1** (5 mg), gallic acid (30 mg), gallic acid 3-*O*-β-D-glucopyranoside (15 mg), 1-*O*-galloylpedunculagin (36 mg) and eugeniin (43 mg). Frs 3 and 4 were, respectively, purified by MCI GEL CHP 20P ($H_2O$–MeOH) and Fuji gel ODS G3 ($H_2O$ MeOH) CC to give **2** (28 mg), 1-desgalloyleugeniin (42 mg), casuarinin (83 mg) and stachyurin (68 mg) (from fr. 3) and **3** (230 mg) (from fr. 4). (b) *From bark of the intact plant.* Fresh bark (6.1 kg), collected in September 1982, was extracted and separated by the above method to afford 40 tannins and related phenolics. The experimental details and results will be reported elsewhere.

*Growth and tannin production of the callus.* Fresh calli (*ca* 0.2 g), subcultured on medium B, were transferred to three MS solid media A (with 0.1 mg $l^{-1}$ 2,4-D and 0.1 mg $l^{-1}$ BA), B and C (with 3 mg $l^{-1}$ IAA and 0.1 mg $l^{-1}$ BA) and cultured for 8 weeks. These cultures were harvested every 2 weeks, and the growth (fresh and dry wt) and tannin (**1**–**5**) production (by HPLC) were determined.

*Quantitative determination of tannins by HPLC.* Lyophilized calli (*ca* 20–30 mg) were mashed and extracted with MeOH (2 ml) for 16 hr at room temp. Each extract, after filtration through a millipore filter (0.45 μm), was subjected to HPLC analysis; column: TSK gel ODS 80Ts (4.6 mm × 250 mm), mobile phase; 1 mM tetrabutylammonium (adjusted to pH 2.9 with HOAc–MeCN (9:1 → 1:4, in 32 min), flow rate: 0.6 ml $min^{-1}$, column temp.: 40°, detect.: 280 nm (UV), $R_t$ (min) : **2** (6.5), **5** (12.2), **4** (14.6), **1** (17.4), **3** (23.9) and polygalloylglucoses (major peaks, 24.9 and 25.5).

*Effects of the removal of $NH_4NO_3$.* Pieces of fresh calli (*ca* 0.2 g), cultured on media A, B and C for 4 weeks, were inoculated, respectively, onto media A', B' and C' (without $NH_4NO_3$) and cultured for **4** weeks. The growth (fresh and dry wt) and tannin (**1**–**5**) production of the calli were determined.

*Effects of sucrose concentration.* Pieces of fresh calli (*ca* 0.2 g), cultured on medium C for 4 weeks, were inoculated separately onto medium C containing various concns of sucrose (20, 30, 40, 50 and 100 g $l^{-1}$) and cultured for 4 weeks. The growth (fresh and dry wt) and tannin (**1**–**5**) production of the calli were determined.

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