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PRODUCTION OF TANNIC ACID BY *RHUS JAVANICA* CELL CULTURES

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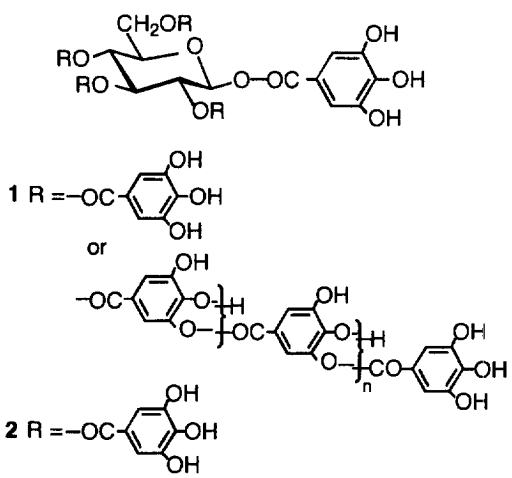
Key Word Index—*Rhus javanica*; Anacardiaceae; cell culture; gallotannin; tannic acid; pentagalloylglucose.

Abstract—Cell cultures of *Rhus javanica* producing large amounts of tannic acid, which is a mixture of tetra- to decagalloylglucoses, were established. The calli cultured on Linsmaier–Skoog gellan gum medium containing 10^{-5} M 2,4-dichlorophenoxyacetic acid and 10^{-5} M benzyladenine showed high gallotannin production as well as a high growth rate. The main component in a mixture of polygalloylglucoses detected in cultured cells was heptagalloylglucose, while octagalloylglucose was the representative compound in intact plants. The parent compound was confirmed by isolating pentagalloylglucose produced upon methanolysis of the depside linkage of polygalloylglucoses in an ethyl acetate fraction of the callus extract. © 1997 Elsevier Science Ltd

INTRODUCTION

Chinese nutgalls, containing a large amount of tannic acid, a mixture of polygalloylglucoses (**1**), is an official crude drug used as an astringent and gargle in Japan [1]. The galls are formed on the leaves of *Rhus javanica* in response to an insect, *Melaphis chinensis*, and, thus, the supply of tannic acid is dependent on the spontaneous formation of insect galls. In the present study, we have attempted to establish callus and suspension cultures of *R. javanica* producing gallotannin to offer an alternative to galls as a stable source of tannic acid.



RESULTS AND DISCUSSION

Callus tissue was induced from the petioles of *R. javanica* in various combinations of phytohormones on Linsmaier–Skoog's (LS) [2] agar medium. As an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) showed most effective callus formation. Out of 14 combinations of plant hormones tested in the present study, the medium containing 10^{-5} M 2,4-D and 10^{-5} M benzyladenine (BA) was found to be most suitable for the callus induction. The calli subcultured on 1% agar medium containing this hormone combination, grew very slow to form fairly hard tissue, while a low concentration (0.1%) of gellan gum in place of agar gave soft calli with a high growth rate. Thus, callus tissues subcultured on the 0.1% gellan gum medium were used in the following experiments.

Normal-phase HPLC analysis of the aqueous acetone extract of the cultured cells showed a typical chromatogram of tannic acid (**1**) suggesting that calli were capable of producing gallotannins. Tannic acid is a complex mixture of polygalloylglucoses of various M_rs having several galloyl residues attached through depside linkages to pentagalloylglucose as the core structure [3, 4]. In order to characterize the phenolic metabolites in the callus tissue, the crude tannin fraction was methanolysed under mild conditions to yield

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Table 1. Effects of hormone composition on total tannin production (mg per flask) at first and third passages in callus cultures of *Rhus javanica*

BA concn (M)	2,4-D concentration (M) 0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
0				4.7(3.0)	
10^{-7}				4.7(7.1)	
10^{-6}				6.4(9.0)	
			4.6(5.2)*	8.1(9.5)*	
10^{-5}	1.3(2.9)	1.6(3.2)	4.2(8.7)	2.6(7.4)	0.3(1.5)
				8.3(14.3)*	
10^{-4}				0.2(7.8)	

Cell-growth (g fr. wt per flask) shown in parentheses.
* Third passage.

the parent compound, penta-*O*-galloyl-β-D-glucose (**2**), which was identified by comparison with an authentic sample.

Effects of 2,4-D and BA concentration on cell-growth and polyphenol production are shown in Table 1. Various concentrations of 2,4-D and BA were added to the medium, on which callus tissues were cultured for 1 month. The quantity of each galloylglucose in the cultured cells was estimated from the peak area detected by normal phase HPLC and calculated as pentagalloylglucose. In the presence of 10^{-5} M BA, calli grew very well on the medium containing either 10^{-5} M or 10^{-6} M of 2,4-D, whereas tannin contents were not strongly affected by 2,4-D at low concentrations. Both cell-growth and tannin production were suppressed by a high concentration of 2,4-D (10^{-4} M). On the other hand, cell-growth was hardly affected by BA concentration in contrast to 2,4-D, although tannin production was apparently suppressed at high concentrations (10^{-4} M). As far as the first passage, the best combination for total productivity of tannin per litre medium was 10^{-5} M 2,4-D and 10^{-6} M BA. In order to optimise tannin productivity for long culture periods, tannin contents and cell-growth were further evaluated after a third passage, culturing the calli on media containing both plant hormones (Table 1). Sufficient cell-growth was shown, after the third passage of culture, an inverse relationship between tannin contents and cell-growth being observed. Thus, the medium containing 10^{-5} M 2,4-D and 10^{-5} M BA was optimal for continuous tannin production of *Rhus* calli on the solid medium.

The time-course of total galloylglucose production in the calli cultured under the above conditions is shown in Fig. 1(A). Under these conditions, calli showed a stable high growth-rate over the long subculture period, and they grew to *ca* four-times of inoculum weight in four weeks. The amount of polygalloylglucoses produced by the calli decreased transiently at the early logarithmic phase, but then increased during the log phase. A marked increase in tannin content was observed, when cell-growth reached the stationary phase (*ca* 5 weeks after inoculation). The pattern of galloylglucose components

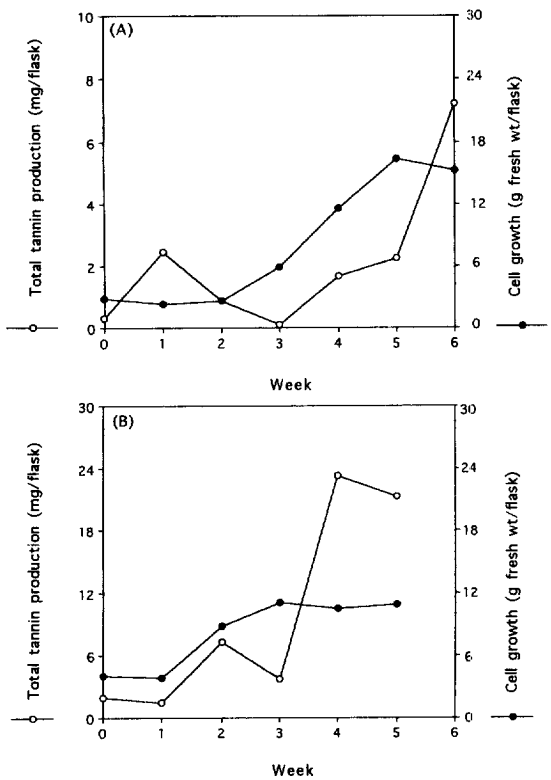

Fig. 1. (A) Time-course of total tannin production and cell-growth in callus cultures of *Rhus javanica* in LS medium containing 10^{-5} M 2,4-D and 10^{-5} M BA. (B) Time-course of total tannin production and cell-growth in suspension cultures of *Rhus javanica* in LS medium containing 10^{-6} M 2,4-D and 10^{-6} M BA.

(average ratio: 5-GG 19%, 6-GG 24%, 7-GG 28%, 8-GG 18%, 9-GG 10% and 10-GG 1%) detected by HPLC did not change significantly through the culture period. The main component of the tannic acid produced in the *Rhus* cells was heptagalloylglucose, although octagalloylglucose is the most abundant component in intact plants. The cell suspension cultures were also induced by transferring callus tissues into liquid medium, to which 10^{-6} M of both 2,4-D and BA were supplemented. Although the concentrations of 2,4-D and BA were different from those

used for callus cultures, they gave the highest tannin production in suspension cultures. The time-course of tannin production and cell-growth of suspension cultures is shown in Fig. 1(B). In the liquid medium, cells grew much faster than on solid medium, and the log-time of tannin production was also shorter than that on the solid medium. The production pattern of tannin was found to be similar to callus culture: the tannin content in cells also increased rapidly after reaching the stationary phase.

In order to examine whether the productivity of tannin in cultured cells is dependent on the tannin content of the individual intact plant used for callus induction, several callus strains were induced from leaves of three different *R. javanica* plants growing at different places in which tannin content varied significantly. Total tannin content (mg g^{-1} dry wt) of the leaves of plant A was 4-times higher than that of plant B, and *ca* 20-times higher compared with plant C. However, the calli derived from plants A, B or C showed no correlation with the tannin productivity of the intact plants. The total tannin content of callus (44 mg g^{-1} dry wt) was comparable to that of plant A (57 mg g^{-1} dry wt).

There have been some reports concerning production of gallotannins by cell cultures of other species, e.g. *Quercus robur* [5] and *Cornus officinalis* [6]. The main component of the gallotannins of *Cornus* cultures was tetragalloylglucose, which has lower astringency than the gallotannins of higher M_r [7]. However, the callus and suspension cell cultures of *R. javanica* established in the present study produce polygalloylglucoses with a similar composition to those in the intact plant. This *Rhus* culture system would thus be useful for producing a stable supply of tannins, as well as for basic studies on gallotannin biosynthesis and on biotransformation utilizing its galloylation potency.

EXPERIMENTAL

Plant material and culture methods. For induction of callus tissues, segments of surface-sterilized (8% antiformin containing 0.5% Tween 80, 30 min) leaves of *R. javanica* L., grown in the botanical garden of the Faculty of Pharmaceutical Sciences in Okayama University, were placed on LS agar medium containing an auxin and a cytokinin in various combinations and cultured at 25° in the dark. Callus tissues induced from petioles, were then subcultured on a medium containing 0.1% gellan gum, 10^{-5} M 2,4-D and 10^{-5} M BA, at intervals of 1 month for over 3 years. To establish cell suspension cultures, the callus was transferred into LS liquid medium containing 10^{-6} M 2,4-D and 10^{-6} M BA, and cells subcultured every 3 weeks on a rotary shaker (100 rpm).

Influence of plant hormones on cell-growth and tannin content. Callus cells precultured on LS medium with 10^{-5} M of 2,4-D and BA were inoculated (inoculum size 3 g) on LS gellan gum medium (30 ml) containing 2,4-D and BA at various concns in 100 ml Erlenmeyer flasks with 3 replicates. They were cultured under the same conditions as described above and harvested after 4 weeks.

Comparison of productivity of tannin between intact plants and callus tissues. Three callus strains were induced from petioles obtained from three different plants growing in the campus of Okayama University in May, and subcultured under identical condition over 1 year. Callus tissues were inoculated (inoculum size 1 g) on LS medium (15 ml) containing 10^{-5} M 2,4-D and 10^{-5} M BA, in 50 ml Erlenmeyer flasks with 3 replicates. They were cultured under the same condition as described above.

Time-course experiment. Callus cultures were initiated by placing callus cells (inoculum size 3 g) on LS gellan gum medium and the cells were harvested every 7 days. For cell suspension cultures, 4.5 g cells were inoculated in LS liquid medium (30 ml medium in 100 ml Erlenmeyer flasks) and harvested every 7 days by filtration in order to analyse tannin content. Values are the means of 3 replicates.

Isolation and identification of pentagalloylglucose. Dride callus tissues (5 g) were homogenized in 70% Me_2CO (70 ml × 3). The extract was concd *in vacuo* and partitioned between *n*-hexane (7 ml × 3) and EtOAc (7 ml × 10), successively. The EtOAc extract (78.5 mg), which was rich in gallotannins, was dissolved in 8.8 ml of MeOH–acetate buffer (pH 5.6) (10:1) and left at 37° for 24 hr. The reaction mixt. was evapd to dryness and the residue partitioned between Et_2O and H_2O to extract methyl gallate into the organic phase. The aq. layer was concd and subjected to CC on Tyoperal HW-40 (Tosho, Japan; coarse grade) (11 × 450 mm) with 50% EtOH and 70% Me_2CO as eluants. The crude methanolysate eluted by 50% EtOH was dissolved in dil. HCl and then passed through Sep-pak C18 with H_2O and MeOH to give **2** (8.8 mg), which was identified by comparison with an authentic sample by ^{1}H NMR (500 MHz).

Quantitative analysis of tannins. Fr. cells (1 g) or dried leaves (0.1 g) were homogenized in 70% Me_2CO (5 ml) and centrifuged to remove cell debris. A portion (1 ml) of each supernatant was evapd to dryness and the residue dissolved in MeOH and analysed by HPLC using the following condition: YMC PACK SIL A-003 (4.6 × 250 mm) with the solvent system, *n*-hexane–MeOH–THF–HCO_2OH (55:33:11:1) containing oxalic acid (450 mg l^{-1}), at a flow rate of 1.5 ml min^{-1} at room temp., detecting at 280 nm.

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