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PRODUCTION OF MACROCYCLIC ELLAGITANNIN OLIGOMERS BY *OENOTHERA LACINIATA* CALLUS CULTURES

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Key Word Index—*Oenothera laciniata*; Onagraceae; plant cell cultures; macrocyclic ellagitannin oligomer; oenothein B; oenothein A.

Abstract—Callus cultures of *Oenothera laciniata* grown on LS agar medium supplemented with IAA and kinetin produced large amounts of the macrocyclic ellagitannin dimer, oenothein B, and a trimer, oenothein A, accompanied with related monomeric hydrolysable tannins. The content of the main compound oenothein B (65 mg/g dry wt) in calli cultured on modified LS medium containing 10 mM NH_4^+ and 5 mM NO_3^- was nearly two times higher than that in intact leaves. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

An ellagitannin dimer, oenothein B (**1**), and a trimer, oenothein A (**2**), were first isolated from *Oenothera erythrosepala*, and *O. biennis*, respectively, and were later found to be distributed in many onagraceous plant species [1, 2]. They are of considerable interest because of their unique macrocyclic structures and remarkable biological activities, such as host-mediated antitumour activity [3, 4] and suppression of mouse mammary tumour gene expression [5]. Oenothein A also shows a significant inhibitory effect on polygalacturonase, which is often related to plant diseases caused by infection with microbial pathogens [6]. Although some studies on ellagitannin production by plant cell cultures have been published [7–10], there has been no report of the establishment of cell cultures which are capable of producing oligomeric ellagitannins having macrocyclic structures. As a part of our studies on the production of biologically active compounds by plant cell cultures, we have now established callus cultures of *O. laciniata* Hill. These cultures produce large amounts of the macrocyclic ellagitannin dimer, oenothein B (**1**), together with a trimer, oenothein A (**2**), and related hydrolysable tannin monomers. This paper deals with the effects of phytohormones, sugar components and inorganic components of Linsmaier-Skoog's (LS) [11] medium, especially the NH_4^+ to NO_3^- ratio of the nitrogen sources, in order to optimize the production of the dimeric macrocircular ellagitannin by *O. laciniata* callus cultures.

RESULTS AND DISCUSSION

Callus tissues of *O. laciniata* were induced from axenic seedlings on LS agar medium containing 3% sucrose and 10^{-5} M indole-3-acetic acid (IAA) and 10^{-5} M kinetin. The callus cultures were maintained by subculturing on LS agar medium containing sucrose (3%) and the above hormones over 2 years at 25° in the dark.

The polyphenolic constituents produced by the cultured cells were characterized as follows. Fresh callus tissues were homogenized in aqueous acetone and the concentrated homogenate was extracted with *n*-hexane to remove lipids. Column chromatography of the aqueous portion on Dia-ion HP-20 and Toyopearl HW-40 yielded oenothein B (**1**) [1] and tellimagrandin I (**3**) [12] as major components of the callus tissues. A trimeric ellagitannin, oenothein A (**2**) [13], as well as four minor monomeric hydrolysable tannins, 1,6-di-*O*-galloyl-β-D-glucose, 1,2,3- and 1,2,6-tri-*O*-galloyl-β-D-glucose [14, 15] and gemin D [16], were also obtained. The composition of these phenolic metabolites in the callus tissues was found to be almost the same as that in intact plants [17].

The time-course of oenothein B production and cell growth are shown in Fig. 1. The callus grew constantly

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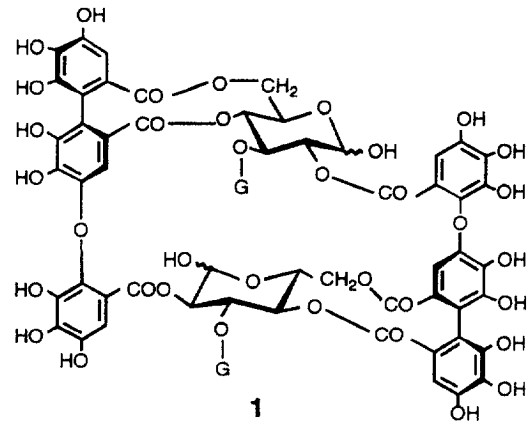

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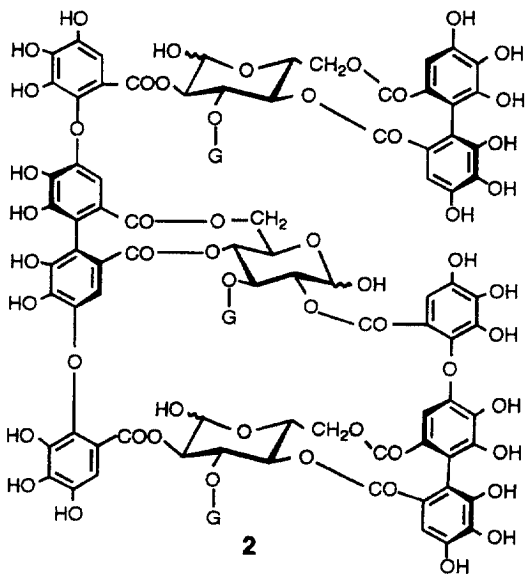

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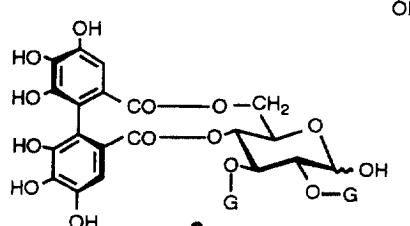

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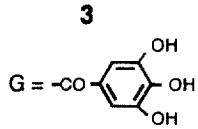


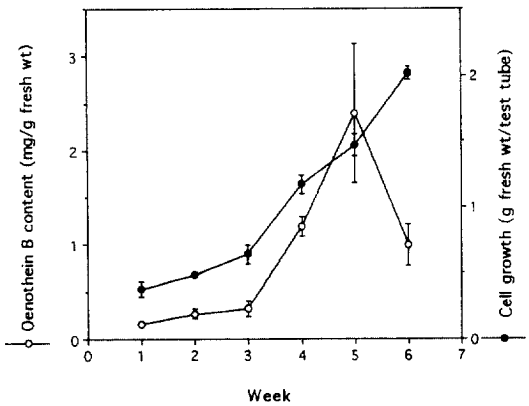
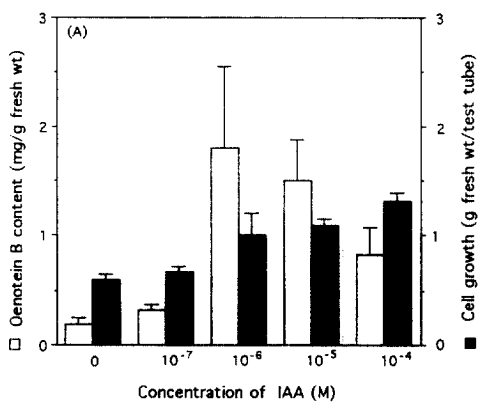


Fig. 1. Time-course of oenothein B content and growth of *O. laciniata* callus cultured on LS agar medium containing 10^{-5} M IAA and 10^{-5} M kinetin and 3% (w/v) sucrose. Error bars indicate standard deviation of the mean of three replicates.



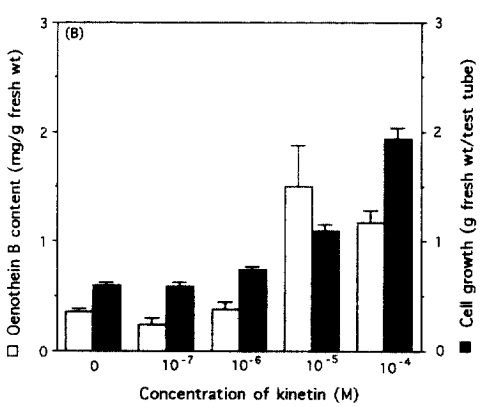


Fig. 2(A). Effects of IAA concentration on oenothein B content and cell growth of callus cultures of *O. laciniata* grown in the presence of 10^{-5} M kinetin. (B). Effects of kinetin concentration on oenothein B content and cell growth of callus cultures of *O. laciniata* grown in the presence of 10^{-5} M IAA. Calli were cultured on LS agar medium with 3% (w/v) sucrose in the dark at 25° for 28 days before harvest. Error bars indicate standard deviation of the mean of three replicates.

to reach 3 times the inoculum weight in 6 weeks. The oenothein B content in the callus tissues increased rapidly after a lag phase of 3 weeks, reached its maximum (2.4 mg/g fr. wt) 5 weeks after inoculation, and then markedly decreased in the 6th week, although the calli continued to grow.

The effects of IAA and kinetin on tannin production and cell growth are shown in Fig. 2 (A, B). Cell growth increased proportionally to increasing concentrations of both IAA and kinetin. The best cell growth was observed upon addition of 10^{-5} M IAA and 10^{-4} M

kinetin. High concentration of phytohormones, however, caused suppression of the tannin productivity. For the highest tannin content in the callus (at first passage), a combination of 10^{-6} M IAA and 10^{-5} M kinetin appeared to be optimal.

Figure 3 shows the effects of sucrose concentrations in the range of 0–5% (w/v) on cell growth and the tannin content of *Oenothera* calli. The best cell growth was observed on 3% of sucrose. On 5% sucrose an almost 2 fold increase of oenothein B was observed, whereas cell growth was significantly suppressed in comparison with that on the standard sucrose concentration of LS medium (3% w/v sucrose). We also examined the effects of other carbohydrates (glucose, fructose and maltose), but sucrose was found to be the most suitable for oenothein B production. This is a similar result to the earlier findings reported for polyphenol production in other tissue and cell cultures [18, 19]. In all subsequent experiments, 3% sucrose was used as the carbon source.

The influence of inorganic elements and vitamins in LS medium was examined to optimize cell growth and oenothein B production (Fig. 4). Although cell growth was not strongly affected by the inorganic element concentration of LS medium, the oenothein B content increased in the presence of lower concentration of inorganic elements than in the normal LS medium. The diluted LS medium (1/4) gave more than a 2-fold increase of oenothein B content in calli compared to standard LS medium.

Nitrogen source is known to often have important effects on secondary metabolism in plant cell cultures [20]. Gallotannin and ellagitannin production in *Sapium* [8] and *Quercus* cell cultures [10], respectively, were reported to be affected by omission of NH_4NO_3 from the growth media. Figure 5 shows the effects of NH_4^+/NO_3^- ratio in LS basal medium on cell growth

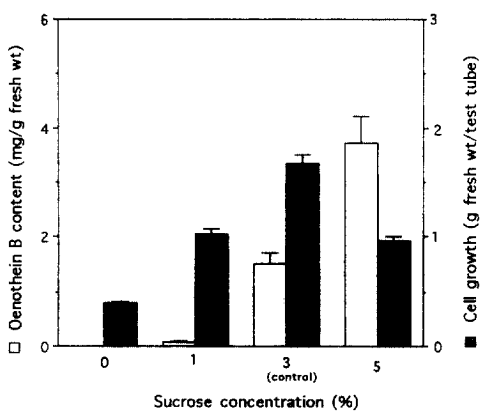


Fig. 3. Effects of sucrose concentration on oenothein B content and cell growth of callus cultures of *O. laciniata*. Calli were cultured on LS agar medium containing 0 to 5% of sucrose, as well as 10^{-5} M IAA and 10^{-5} M kinetin. Cultures were grown at 25° in the dark for 28 days before harvest. Error bars show standard deviation of the mean for three replicates.

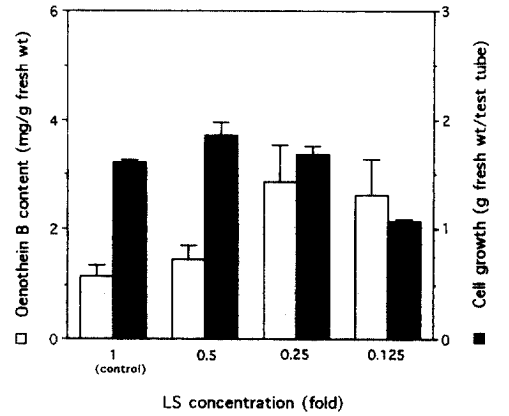


Fig. 4. Effects of inorganic elements in LS medium on oenothein B content and cell growth of callus cultures of *O. laciniata*. Calli were cultured on diluted LS medium (1- to 8-fold) which was supplemented with 3% (w/v) sucrose, 10^{-5} M IAA and 10^{-5} M kinetin. Cultures were grown at 25° in the dark for 28 days before harvest. Error bars are standard deviation of the mean of three replicates.

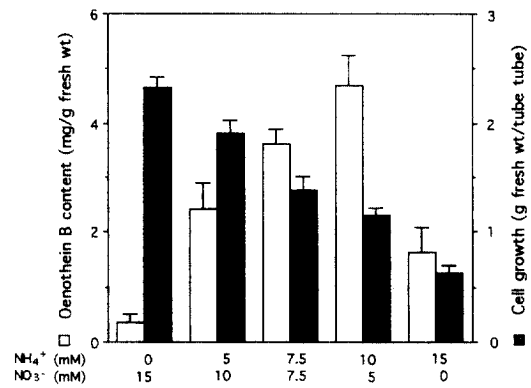


Fig. 5. Effects of NH_4^+/NO_3^- ratio on oenothein B content and cell growth of callus cultures of *O. laciniata*. Different ratios of NH_4^+/NO_3^- (total 15 mM) were added to nitrogen source-free LS medium containing 3% (w/v) sucrose, 10^{-5} M IAA and 10^{-5} M kinetin. Calli were cultured 25° in the dark for 28 days. NH_4Cl and KNO_3 were used as nitrogen sources. Error bars show standard deviation of the mean of three replicates.

and oenothein B content, where total nitrogen was adjusted to 15 mM which corresponds to a nitrogen concentration of 1/4 LS medium. The absence of NH_4^+ gave the best cell growth of *Oenothera* calli, whereas oenothein B production was strongly suppressed in the absence of NH_4^+. The increment of NH_4^+ ratio gave a remarkable enhancement of oenothein B production up to a NH_4^+/NO_3^- ratio of 2 to 1, whereas cell growth was accordingly depressed. Both cell growth and oenothein B production were suppressed in the absence of the nitrate ion. Based on the above data, the combination of 10 mM NH_4^+ and 5 mM NO_3^- was selected as the nitrogen source for maximal oenothein B production (65 mg/g dry wt). In

this medium, tannin production was 4-fold better than that obtained on standard LS medium with 20 mM NH_4^+ and 40 mM NO_3^-. Cell growth in the modified LS medium was almost the same as that in the standard LS medium. It is noteworthy that the oenothein B content in the callus was 1.8 times higher than that of intact leaves.

The present study is the first example of the establishment of a callus producing a macrocyclic ellagitannin oligomer in large amounts (65 mg/g dry wt). The *Oenothera* cultures might represent a stable source of the bioactive tannins, and also offer a suitable system for biosynthetic studies on a tannin with a unique macrocyclic structure.

EXPERIMENTAL

Plant material and culture methods

Surface-sterilized seeds of *O. laciniata*, collected in the Botanical Garden of the Faculty of Pharmaceutical Sciences, Okayama University on June 1993, were placed on sugar-free LS agar medium under continuous light (3000 lx, 12 h/day) for 1 month. Callus tissue was induced from axenic seedlings on LS agar medium containing 14 combinations of auxin (2,4-D, IAA, IBA, NAA) and cytokinin (BA, kinetin), and cultured at 25° in the dark. Calli cultured on the medium supplemented with a combination of 10^{-5} M IAA and 10^{-5} M kinetin were selected as the most favorable ones for both tannin production and cell growth, and were subcultured on the same medium at 1 month intervals for over 2 years in the dark.

Time-course experiment

Cultures were initiated by placing callus tissues (inoculum size 0.6 g) on LS agar medium containing 10^{-5} M IAA and 10^{-5} M kinetin. Cells were cultured in the same way as above and harvested every 7 days.

Optimization of culture medium

LS medium was used as the basal medium for inorganic elements and vitamins and was supplemented with 1% agar, 3% sucrose, 10^{-5} M IAA and kinetin. The callus tissues were inoculated (inoculum size 0.6 g) on 10 ml agar medium in a test tube with 3 replicates. They were cultured at 25° in the dark, and then harvested after culturing for 28 days. Various concentrations of IAA in the presence of 10^{-5} M kinetin, as well as various concentrations of kinetin in the presence of 10^{-5} M IAA in the LS medium were used for the experiment reported in Fig. 2(A,B). The effects of sucrose levels (0, 1, 3 or 5%) were tested using standard LS medium containing 10^{-5} M IAA and 10^{-5} M kinetin. In the experiment reported in Fig. 4, diluted LS basal medium was used by phytohormones and sucrose, were fixed as mentioned above. In the experiment reported in Fig. 5, NH_4Cl and KNO_3 (total 15 mM nitrogen) were added to nitrogen-free LS medium.

Isolation of tannins

Fresh callus tissues (250 g) were homogenized in 70% Me_2CO (70 ml × 3). The homogenate was concd *in vacuo* and extracted with *n*-hexane. The aq. layer was concd and subjected to CC over Dia-ion HP-20 (30 × 200 mm) eluted in stepwise mode with $H_2O \rightarrow 20 \rightarrow 40 \rightarrow 60 \rightarrow 100\%$ MeOH → 70% Me_2CO (each 300 ml). The 40% MeOH eluate (797 mg) was rechromatographed over Toyopearl HW-40 (fine, 22 × 480 mm) to yield 1,6-di-*O*-galloyl-β-D-glucose (6.6 mg), 1,2,3-tri-*O*-galloyl-β-D-glucose (5.3 mg), 1,2,6-tri-*O*-galloyl-β-D-glucose (8.2 mg), gemin D (13.0 mg) and tellimagrandin I (126.6 mg) from the eluate with 70% EtOH, and oenothein B (261.5 mg) and oenothein A (29.2 mg) from the eluate with 70% Me_2CO. These metabolites were identified, respectively, by direct comparisons of their ^1H NMR (500 MHz) spectra and HPLC (normal and reversed-phase) properties with those of authentic samples.

Quantitative analyses of tannins

Fresh cells (1.0 g) were homogenized in 70% Me_2CO (5 ml) and centrifuged. An aliquot (1 ml) of the supernatant was evapd to dryness, and the residue disolved in MeOH was analyzed by normal phase HPLC using the following condition; YMC PACK SIL A-003 (4.6 × 250 mm) with *n*-hexane–MeOH-THF-HCOOH (60:45:15:1) containing oxalic acid (450 mg/l) at a flow rate of 1.5 ml/min at room temp. with detection at 280 nm. The quantity of oenothein B in the callus tissues was determined from the peak area referenced with an authentic specimen.

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