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PRODUCTION OF PODOPHYLLOTOXIN IN *JUNIPERUS CHINENSIS* CALLUS CULTURES TREATED WITH OLIGOSACCHARIDES AND A BIOGENETIC PRECURSOR

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Juniperus chinensis*; Cupressaceae; Callus cultures; podophyllotoxin; oligosaccharide; biogenetic precursor.

Abstract—Calli were induced from the leaves of young trees of *Juniperus chinensis* on Schenk and Hildebrandt medium supplemented with napthalenacetic acid and kinetin and subcultured on the same medium. Podophyllotoxin, a strong anti-tumor agent, was isolated from the extractives of calli and found that calli produced podophyllotoxin. The podophyllotoxin in the calli derived from the leaves constituted 0.005% of dry weight. In contrast, the content of podophyllotoxin in intact plant was 0.0025% of dry weight. The cultures of the calli produced twice as much podophyllotoxin as did those of the intact plant.

The production of podophyllotoxin was increased fifteen-fold by addition of chito-oligosaccharides, an elicitor, to the calli. And furthermore, the production increased eleven-fold by addition of phenylalanine, a biogenetic precursor of podophyllotoxin.

INTRODUCTION

Byakushin (*Juniperus chinensis*) which has green, scaly and persistent foliage is a tall tree grown in Japan, Korea and China [1]. We have examined the extractives of *J. chinensis* leaves [2] and found that podophyllotoxin is present as a potential source of this compound, although it is present in small amounts. Podophyllotoxin is also found in Podophyllum species [3], *Diphylleia cymosa* and *D. grayi* [4], respectively, several studies have indicated that podophyllotoxin has strong anti-leukemic and tumor-inhibitory effects [5, 6, 7]. Therefore, for podophyllotoxin to be utilized as a forest resource product for *J. chinensis*, its production needs to be increased. Production of podophyllotoxin by tissue culture of rhizomes of *Podophyllum peltatum* has been reported [8, 9]. However, there have been no reports about production of podophyllotoxin by callus cultures of *J. chinensis*. Therefore, we examined its production in *J. chinensis* callus cultures and the effect of oligosaccharides and a biogenetic precursor on its formation.

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RESULTS AND DISCUSSION

Induction and growth of calli from J. chinensis

Five media, Murashige and Skoog (MS) [10], M8 (one-third the normal concentration of MS salts) [11], Gamborg's B5 (B5) [12], Schenk and Hildebrandt (SH) [13] and Woody plant (WP) [14] were used for the induction of calli from the leaves of *J. chinensis*. Naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D), as auxins and kinetin, as a kind of cytokinin, were used as growth regulators. No callus formation occurred on media lacking growth regulators. Callus was induced from sterile leaves of *J. chinensis* on SH medium supplemented with NAA ($1.0\,mgl^{-1}$) and kinetin ($0.2\,mgl^{-1}$) or 2,4-D ($1.0\,mgl^{-1}$) and kinetin ($0.2\,mgl^{-1}$) solidified with agar (1%) in the dark. However, callus was not induced from the leaves of *J. chinensis* on MS, M8, B5, or WP medium supplemented with NAA and kinetin or 2,4-D and kinetin solidified with agar (1%). To find the optimum combination and concentration of growth regulators for induction of the calli, eight combinations of growth regulators [(A)–(H), see Experimental section] were added to the SH medium. The best callus induction was obtained on SH medium

with NAA (3.0 mgl^{-1}) and kinetin (0.2 mgl^{-1}): the calli initiated from the leaves within 20 days of culture. Induced calli generally appeared white to light yellowish brown. The contamination level during callus initiation was about 40% with garden-grown plant tissues.

SH medium supplemented with NAA and kinetin supported callus growth without regeneration. To find the optimum concentration of NAA and kinetin for the proliferation of the calli derived from the leaves, four combinations [(E)–(H), see Experimental section] of each growth regulator were added to the SH medium. The combination of optimum concentrations of NAA and kinetin for proliferation of the calli was 3.0 mgl^{-1} of NAA and 0.2 mgl^{-1} of kinetin. Callus growth was increased about 11-fold over that of the starting ones for 40 days. Subculturing on this SH medium led to a reduction in the proliferation of calli after five subcultures and, ultimately, complete loss of proliferation after ten subcultures.

In addition, SH medium supplemented with 2,4-D and kinetin also supported callus growth without regeneration. However, the rate of proliferation of the callus on SH medium supplemented with 2,4-D and kinetin was about two-thirds that on SH medium supplemented with NAA and kinetin. SH medium supplemented with NAA and kinetin was also superior to SH medium supplemented with 2,4-D and kinetin for callus maintenance.

Production of podophyllotoxin in J. chinensis callus cultures

Podophyllotoxin was isolated from the fresh calli subcultured on the SH medium (see Experimental section) and identified by comparison of its ^{13}CNMR spectral data with that of an authentic sample and with that of literature values [15]. It was also identified by the mixed-melting-point test of the isolated podophyllotoxin with authentic podophyllotoxins purchased from Sigma Chemical Company and isolated from *J. chinensis* [2]. However, podophyllotoxin glucoside was not found in the extractives of calli by comparison with an authentic sample by TLC analysis.

For podophyllotoxin analyses, callus subcultured thrice on the SH medium were freeze-dried and extracted with methanol and water (1:1 v/v). The extractives were partitioned between dichloromethane and water (1:1 v/v) and gave dichloromethane solubles. The dichloromethane solubles were analyzed by HPLC by reference to the method of Berlin *et al* [16]. and Cairnes *et al* [17]. Furthermore, the podophyllotoxin was identified by comparing its retention time with that of authentic podophyllotoxin isolated from *J. chinensis* [2] and by adding authentic podophyllotoxin. The podophyllotoxin in the calli derived from the leaves constituted 0.005% of dry weight, as opposed to 0.0025% for the intact leaves. Therefore, the calli of the leaves of *J. chinensis* produced twice as much podophyllotoxin as did the intact plant. In addition, no podophyllotoxin was found in the agar medium.

This is the first report about production of podophyllotoxin by callus cultures of *J. chinensis*. Kadkade [8, 9] studied the production of podophyllotoxin by tissue cultures of rhizomes of *Podophyllum peltatum* and calculated the yield from the calli to be 0.64% of dry weight. As the content of podophyllotoxin was increased by callus cultures of *J. chinensis*, it was considered that the production of podophyllotoxin by callus cultures of *J. chinensis* could be enhanced by several methods, for example, treatment with an elicitor.

Effect of oligosaccharides on production of podophyllotoxin by callus cultures of J. chinensis

Two kinds of oligosaccharides, chito-oligosaccharides (COS) [18, 19] and laminaran enzyme-hydrolyzates (LEH) [20] were used to increase the podophyllotoxin yield. An aqueous solution of each elicitor was added to the calli derived from the leaves and incubation carried out on SH medium for 15 and 30 days, respectively. The amounts of the elicitors added to the calli are given in the Experimental section. After incubation, the podophyllotoxin content of the extractives was determined by HPLC analysis. The results are shown in Figs 1 and 2. As shown in Fig. 1, the yield of podophyllotoxin increased significantly when COS were added to the calli. The production of podophyllotoxin was enhanced by 15-fold over that of the control (no addition of COS) when 1 mg of COS was added to fresh calli (1 g) and the mixture incubated on SH medium for 30 days at 25°C in the dark. However, the increase in production was less pronounced after only 15 day's incubation. This suggests that production of podophyllotoxin in the calli following treatment with COS is stimulated after 15 day's incubation.

Figure 2 shows the effect of LEH on production of podophyllotoxin in the calli. The increase in production was less marked on treatment with LEH than COS, with only about 3.5-fold increase over the control.

There have been no reports on the enhancement of podophyllotoxin yield in callus cultures of *J. chinensis* by treatment of oligosaccharide. Yoshida *et al* [19]. found that the production of taxol by callus cultures of *Taxus cuspidata var. nana* Rehder was increased by about 4-fold when COS was added. In the case of callus cultures of *J. chinensis*, the enhancement of production of podophyllotoxin by treatment of COS was about 4 times that of production of taxol.

Kobayashi *et al* [20]. reported oligosaccharides prepared by enzyme hydrolysis of algal laminaran with tunicase had elicitor-activity and stimulated the production of antifungal compounds in alfalfa. However, in the callus cultures of *J. chinensis*, the enhancement effect of LEH on podophyllotoxin production was

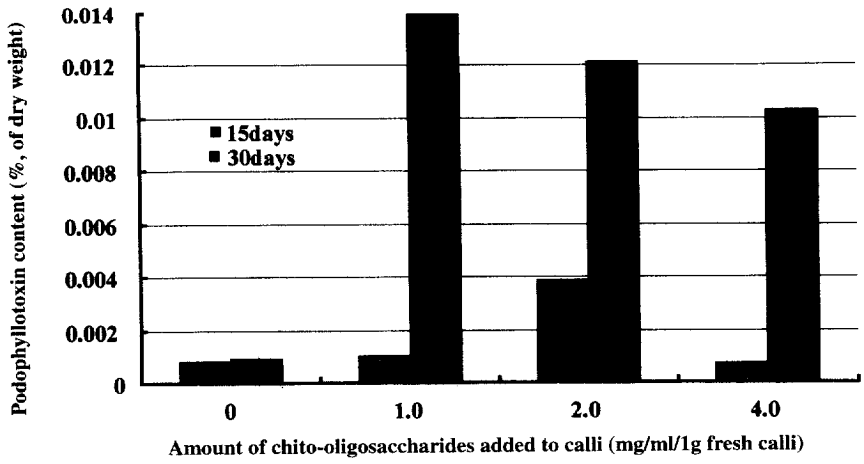

Fig. 1. Effect of chito-oligosaccharides on podophyllotoxin yield of calli from *J. chinensis* L.

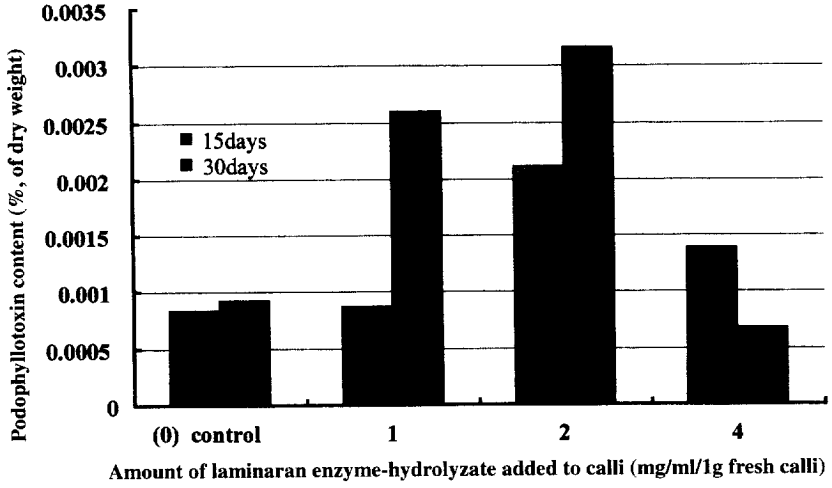

Fig. 2. Effect of laminaran enzyme-hydrolyzates on podophyllotoxin yield of calli from *J. chinensis* L.

less than that of the antifungal compounds in alfalfa. These results suggest that COS and LEH may act as an elicitor to each callus, though the degree to which they elicit podophyllotoxin production may vary.

Effect of a biogenetic precursor on production of podophyllotoxin by callus cultures of J. chinensis

Phenylalanine, a biogenetic precursor of podophyllotoxin [21], was used to stimulate the production of podophyllotoxin by callus cultures of *J. chinensis*. An aqueous solution of phenylalanine (see Experimental section) was added and the calli incubated on the SH medium for 15 and 30 days, respectively. The amount of podophyllotoxin in the extractives was determined by HPLC analysis. As shown in Fig. 3, after 15 days incubation, the podophyllotoxin yield was 11 times that of the control when 0.5 mg of the precursor was added to the fresh calli (1 g), whereas it was lower than that of the control when 2.0 mg of the precursor was added. After 30 days incubation with the precursor, the amount of podophyllotoxin had merely increased to between 1.5 to 2.2 times that of control.

The increase in podophyllotoxin yield in callus cultures of *J. chinensis* induced by treatment of the precursor is considered to involve incorporation of the precursor into a biogenetic route for production of podophyllotoxin in the calli. However, as shown in Fig. 3, as the incubation time became longer, the amount of podophyllotoxin increased relative to the added amount of the precursor. The reason is unclear. However, if the incubation time is longer, it may be considered that the amount of production of podophyllotoxin in callus itself may be increased by addition of the precursor.

EXPERIMENTAL

Mps were determined on a Yanagimoto melting point apparatus and were uncorr. The ^{13}CNMR spectra were recorded at 100 MHz with TMS as an internal

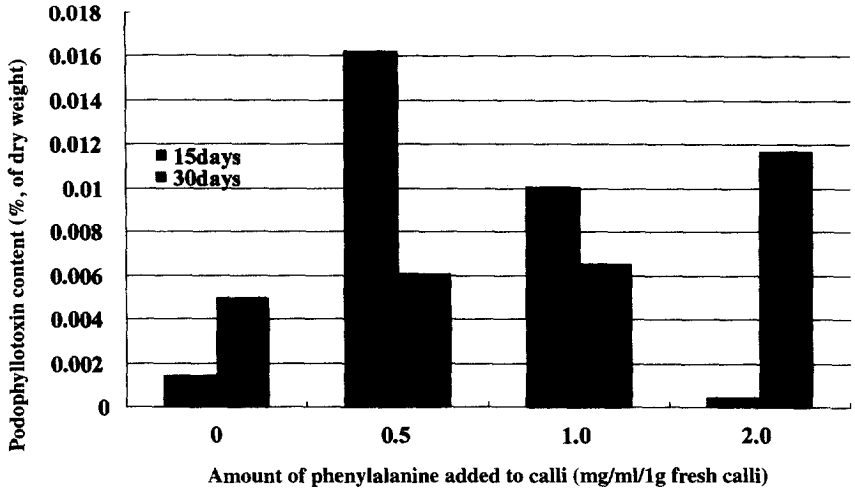

Fig. 3. Effect of phenylalanine on podophyllotoxin yield of calli from *J. chinensis* L.

standard. MS (70 eV) were taken with a direct inlet. Authentic podophyllotoxin was purchased from Sigma Chemical Company and authentic podophyllotoxin was also isolated from the leaves of *J. chinensis* [2]. Phenylalanine was purchased from Tokyo Kasei Co. Ltd. Authentic podophyllotoxin glucoside was isolated from podophyllum resin(from *Podophyllum peltatum*) purchased from Sigma Chemical Company.

Tissue culture media

Murashige and Skoog (SM) [10], M8 (one-third MS salts concentration) [11], Gamborg's B5 (B5) [12], Schenk and Hildebrandt (SH) [13] and Woody plant (WP) [14] media were prepared as described. In each case, the sucrose content was $30\,g^{-1}$. The pH of MS, M8, B5 and SH media was adjusted to 5.8, in the case of WP medium to 5.9. The growth regulators added to each medium for induction of callus from the leaves of *J. chinensis* were 2,4-D ($1.0\,mgl^{-1}$) and kinetin ($0.2\,mgl^{-1}$) and NAA ($1.0\,mgl^{-1}$) and kinetin ($0.2\,mgl^{-1}$), respectively. Furthermore, in the case of SH medium, eight combinations [(A)–(H)] of two kinds of growth regulators were also added to the medium to determine the optimum concentration of growth regulators for induction of calli from the leaves. The combinations of the growth regulators were as follows: (A): 2,4-D ($1.0\,mg^{-1}$) and kinetin ($0.2\,mg^{-1}$); (B): 2,4-D ($3.0\,mg^{-1}$) and kinetin ($0.2\,mg^{-1}$); (C): 2,4-D ($1.0\,mg^{-1}$) and kinetin ($0.5\,mg^{-1}$); (D): 2,4-D ($3.0\,mg^{-1}$) and kinetin ($0.5\,mg^{-1}$); (E): NAA ($1.0\,mg^{-1}$) and kinetin ($0.2\,mg^{-1}$); (F): NAA ($3.0\,mg^{-1}$) and kinetin ($0.2\,mg^{-1}$); (G): NAA ($1.0\,mg^{-1}$) and kinetin ($0.5\,mg^{-1}$); (H): NAA ($3.0\,mg^{-1}$) and kinetin ($0.5\,mg^{-1}$). Media were solidified with 1.0% w/v agar. To find the optimum concentration of growth regulators for proliferation of callus, the combinations [(E)–(H)] were used.

Preparation of explants

Fresh leaves of young trees of garden-grown *J. chinensis* were used as explant source material. The leaves were collected in the suburbs of Matsuyama and Uwajima Cities, Ehime Prefecture.

Callus initiation and culture

Explants were placed on the surface of agar-solidified medium (30 ml) in 100 ml Erlenmeyer flasks, or on 20 ml of solidified medium in 9 cm Petri dishes. Petri dishes were sealed with Nescofilm. Cultures were maintained at 25°C in the dark. Eight combinations of two kinds of growth regulators [(A)–(H), see above] were added to the SH medium for measurement of the optimum concentration of the growth regulators for the induction of the calli. The calli (about 2–3 g fresh weight) induced were transferred to 200 ml Erlenmeyer flasks, each having 70 ml of SH medium supplemented with growth regulators and agar (1%). Four combinations [(E)–(H), see above] of two kinds of growth regulators were added to the medium to determine the optimum concentration of growth regulators for proliferation of the calli. The cultures were maintained at 25°C in the dark and were routinely subcultured onto fresh agar solidified SH medium supplemented with NAA ($3.0\,mg^{-1}$) and kinetin ($0.2\,mg^{-1}$) every 40 days.

Growth measurement

Fr. wts were measured directly and dry wts after freeze drying of tissue. Growth rate was calculated from the increase in the weight of calli to incubation time (day).

Extraction and isolation

The fresh calli (479.2 g, dry wt 62.3 g) subcultured thrice on the SH medium were freeze-dried for 5 days,

ground with a pestle and extracted twice with methanol and water (1:1 v/v) (600 ml) for 2 days at room temperature. The resulting extractives were then concentrated under red. pres. and partitioned between dichloromethane and water (1:1 v/v). The dichloromethane layer was dried with dry Na_2SO_4 and evaporated (dichloromethane solubles, 4.17 g). The dichloromethane solubles were then chromatographed on a silica gel column repeatedly followed by recrystallization from EtOH and chloroform to give podophyllotoxin as colorless crystals (2.1 mg), mp 181–183°C. The isolated podophyllotoxin was confirmed to be identical with authentic podophyllotoxin (mixed m.pt and comparison of ^{13}CNMR spectrum). EIMS *m/z* (rel. int.): 414[M^+](100), 399(8), 396(16), 352(3), 339(3), 309(2), 279(2), 249(2), 201(10), 189(11), 181(15), 169(23), 168(50), 153(28), 125(9), 115(13), 111(10), 97(12), 69(22), 57(35). ^{13}CNMR ($CDCl_3$): δ 40.8(C8), 44.1(C7), 45.3(C8), 56.3(C10), 56.3(C12), 60.8(C11), 71.3(C9), 72.8(C8), 101.5(C10), 106.3(C2), 108.5(C2), 108.5(C6), 109.8(C5), 131.2(C6), 133.1(C1), 135.4(C1), 137.3(C4), 147.7(C3), 147.8(C4), 152.6(C3), 152.6(C5), 174.4(C9).

Thin layer chromatography

TLC was carried out using 0.2 mm (analytical) layer of silica gel (Merck Kieselgel G F_{254}). Lignan bands were located by UV light and by spraying with HOAc-conc HNO_3 (10:3), then heating with a hot air dryer [3]. Sugar bands were detected by spraying with aniline hydrogen phthalate reagent [4] and heating at 100°C for 10 min.

Determination of podophyllotoxin in the calli from J. chinensis

Extraction of podophyllotoxin from the calli derived from *J. chinensis* leaves used the methods of Berlin *et al* [16]. and Cairnes *et al* [17]. The calli subcultured thrice on the SH medium were freeze-dried for 4 days, ground with a pestle and extracted twice with methanol and water (1:1 v/v) for 2 days at room temperature. The resulting extractives were evaporated in vacuo to give crude residues. The crude residues were subsequently partitioned between dichloromethane and water (1:1 v/v) to yield, after evaporation of the solvent and drying in vacuo, dichloromethane solubles. The amount of podophyllotoxin in each sample was determined by HPLC performed on a reversed-phase column (Hitachi Gel # 3056) in a Hitachi 655 liquid chromatograph equipped with a L-4000 UV (ultraviolet)detector (wave length: 290 nm) by isocratic elution with a water–acetonitrile (2:1 v/v) mobile phase. The flow rate was 0.8 ml/min and all chromatograms were plotted at the absorption maximum of podophyllotoxin, 290 nm. The podophyllotoxin in the dichloromethane extract was identified by comparing its retention time with that of authentic podophyllotoxin isolated from *J. chinensis* [2] and by adding authentic podophyllotoxin. A calibration curve was obtained using authentic podophyllotoxin. For comparison with the content of podophyllotoxin in the cultured calli, intact leaves were extracted similarly and were subjected to HPLC analysis.

Treatment of calli from J. chinensis with oligosaccharides and a biogenetic precursor

Chito-oligosaccharides [18] and laminaran enzyme-hydrolyzates [19], and phenylalanine were used as oligosaccharides and a biogenetic precursor, respectively. The chito-oligosaccharides used were prepared from chitosan by the method of Kikkawa *et al* [18]. Laminaran enzyme-hydrolyzates were prepared from algal laminaran with tunicase by the method of Kobayashi *et al* [19]. One ml of aqueous solution of the chito-oligosaccharides (0, 1.0 g, 2.0 g and 4.0 mg) and laminaran enzyme-hydrolyzates (0, 1.0 g, 2.0 and 4.0 mg) was added to the fresh calli (1 g), respectively. In the case of phenylalanine, 1 ml of aqueous solution of phenylalanine (0, 0.5 g, 1.0 g and 2.0 mg) was added to the calli (1 g). After addition of the compounds, the calli were incubated for 15 and 30 days. The amount of podophyllotoxin was determined as described above. Each experiment was repeated independently thrice and the results shown, are the average of three measurements, the deviation of each experimental value being within 12%.

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