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Tomoya Okunishi · Naohiro Takaku
Patcharawadee Wattanawikkit · Norikazu Sakakibara
Shiro Suzuki · Fukumi Sakai · Toshiaki Umezawa
Mikio Shimada

Lignan production in *Daphne odora* cell cultures

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Abstract Lignan production in callus and cell suspension cultures of *Daphne odora* is reported for the first time. The cell suspension culture produced pinoresinol, lariciresinol, secoisolariciresinol, matairesinol, and wikstromol. The production of matairesinol in the cell suspension culture was much higher than that in *Daphne odora* stem tissues.

Key words Lignan · *Daphne odora* · Thymelaeaceae · Suspension culture

Introduction

Recent stereochemical studies of lignan biosynthesis have indicated that the stereochemistry of the upstream lignans (i.e., furofuran and furan lignans) isolated from many plant species is complicated, suggesting that there is stereochemical diversity in upstream steps of lignan biosynthesis.[1–8] Thus, most furofuran and furan lignans are not optically pure; that is, they are composed of both (+)- and (−)-enantiomers. In addition, predominant enantiomers of these lignans vary with plant species. Among the downstream lignans in biosynthesis, however, all the dibenzylbutyrolactone lignans of which the enantiomeric compositions have been examined by chiral high-performance liquid chromatography (HPLC), were found to be optically pure (>99% e.e.).[4–9] In addition, most dibenzylbutyrolactone lignans are found to be levorotatory and have the same absolute configuration at C_8 and $C_{8'}$ with respect to the carbon skeleton.[4,5] In sharp contrast, this class of lignans isolated from Thymelaeaceae plants were dextrorotatory[4–8,10–16] except for one.[17]

These results indicate that there are two stereochemical diversities in lignan biosynthesis. One is the diversity in enantiomeric compositions of the upstream lignans, and the other is the difference in the enantiomers of optically pure dibenzylbutyrolactone lignans between Thymelaeaceae plants and other species.

In previous studies on the characterization of Thymelaeaceae lignans,[6–8] we employed stem tissues of *Wikstroemia sikokiana* and stem tissues with leaves of *Daphne genkwa* and *Daphne odora*. These plants grow rather slowly, however, which limits their use for biochemical studies, although pinoresinol/lariciresinol reductase activity could be detected in *D. genkwa*.[8] Herein we report on a suspension culture of *D. odora* producing lignans.

Experimental

Instruments and chromatography

^{1}H-nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT-NMR system (JEOL) with tetramethylsilane as an internal standard. Chemical shifts and coupling constants (J) were expressed in δ and hertz, respectively. Gas chromatography-mass spectrometry (GC-MS) and electron impact-mass spectrometry (EI-MS) were performed as previously described.[1,2,6–8,18] Silica gel column chromatography and silica gel thin-layer chromatography (TLC) employed Kieselgel 60 (Merck, 70–230 mesh) and Kieselgel 60 F_{254} (Merck, 20 × 20 cm, 0.5 and 0.25 mm), respectively. All the chemicals used were of reagent grade unless otherwise described.

T. Okunishi[1] · N. Takaku · P. Wattanawikkit[2] · N. Sakakibara · S. Suzuki · F. Sakai · T. Umezawa (✉) · M. Shimada
Wood Research Institute, Kyoto University, Uji, Kyoto 611-0011, Japan
Tel. +81-774-38-3625; Fax +81-774-38-3682
e-mail: tumezawa@kuwri.kyoto-u.ac.jp

Present addresses:
[1] National Food Research Institute, Ibaraki 305-8642, Japan
[2] Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, Bangkok 10903, Thailand

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Preparation of compounds

(±)-Pinoresinols,[18] (±)-lariciresinols,[19] (±)-secoisolariciresinols,[18] (±)-matairesinols,[20] (±)-wikstromols,[6] (±)-[9,9,9′,9′-$^2H_4$]pinoresinols,[7] and (±)-[9,9,9′,9′-$^2H_4$]lariciresinols[7] were prepared previously. (±)-[3,3′-OC^2H_3 × 2]Secoisolariciresinols and (±)-[3-OC^2H_3]matairesinols were prepared as for the corresponding unlabeled ones but with [OC^2H_3]vanillin[18,21] as a starting material (to be reported in detail elsewhere). (±)-[3,3′-OC^2H_3 × 2]Wikstromols were prepared as for the corresponding unlabeled ones[6] but with [OC^2H_3]vanillin[18,21] as a starting material.

(±)-[(OC^2H_3) × 2]Secoisolariciresinols: ^{1}H-NMR ($CDCl_3$): δ 1.86 (1H × 2, m), 2.64 (1H × 2, dd, J = 13.9, J = 6.6), 2.74 (1H × 2, dd, J = 13.9, J = 8.0), 3.55 (1H × 2, dd, J = 11.4, J = 4.6), 3.82 (1H × 2, dd, J = 11.4, J = 2.2), 6.57 (1H × 2, d, J = 2.0), 6.62 (1H × 2, dd, J = 8.0, J = 2.0), 6.80 (1H × 2, d, J = 8.0); MS m/z (%): 370 (1.1), 369 (6.0), 368 (21.6), 367 (1.1), 366 (0.5), 365 (0.2), 364 (0.3), 363 (0.1), 362 (0.1), 350 (18.0), 192 (9.8), 140 (100), 122 (8.5); high-resolution MS m/z (M^+): calculated for $C_{20}H_{20}{}^2H_6O_6$: 368.2106, found: 368.2115.

(±)-[3-OC^2H_3]Matairesinols: ^{1}H-NMR ($CDCl_3$): δ 2.40–2.66 (4H, m), 2.87 (1H, dd, J = 14.0, J = 7.0), 2.94 (1H, dd, J = 14.0, J = 5.2), 3.80 (3H, s), 3.88 (1H, dd, J = 9.0, J = 7.3), 4.14 (1H, dd, J = 9.0, J = 7.2), 6.40 (1H, d, J = 2.0), 6.50 (1H, dd, J = 8.0, J = 2.0), 6.57–6.61 (2H, m), 6.77–6.82 (2H, m); MS m/z (%): 363 (2.7), 362 (14.4), 361 (56.9), 360 (2.2), 359 (1.1), 358 (0.6), 224 (5.1), 164 (9.3), 140 (100), 138 (27.3), 137 (43.5), 122 (13.0); high-resolution MS m/z (M^+): calculated for $C_{20}H_{19}{}^2H_3O_6$: 361.1605, found: 361.1623.

(±)-[3,3′-OC^2H_3 × 2]Wikstromols: ^{1}H-NMR ($CDCl_3$): δ 2.46–2.97 (5H, m), 3.98–4.05 (2H, m), 6.59–6.84 (6H, m); MS m/z (%): 382 (0.8), 381 (2.1), 380 (15.3), 379 (0.8), 378 (0.2), 377 (0.2), 376 (0.1), 375 (0.1), 374 (0.1), 140 (100.0), 122 (5.2), 94 (2.6); high-resolution MS m/z (M^+): calculated for $C_{20}H_{16}{}^2H_6O_7$: 380.1742, found: 380.1729.

Plant material

Daphne odora Thunb. was obtained from a local nursery and maintained in the experimental forest of Wood Research Institute, Kyoto University. It was used as explant material for callus induction.

Tissue culture media

Murashige and Skoog (MS) medium (pH 5.6) was composed of MS inorganic salts (4.3 g/l, Flow Laboratories) supplemented with myoinositol (100 mg/l), sucrose (30 g/l), nicotinic acid (0.5 mg/l), pyridoxine hydrochloride (0.5 mg/l), thiamine hydrochloride (0.1 mg/l), and glycine (0.2 mg/l). Wolter and Skoog (WS) medium was prepared according to the literature[22] and supplemented with myoinositol (10 mg/l) and sucrose (20 g/l). Both media were further supplemented with auxin [1-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D)] (0.1, 1.0, or 5.0 μM) and cytokinin [6-benzyladenine (BA) or kinetin (K)] (0, 0.1 or 1.0 μM). The media were prepared in liquid form or solidified with agar (8 g/l).

Callus induction

Young leaves of *D. odora* were washed successively with tap water and 70% EtOH, then sterilized with 1% sodium hypochlorite containing 0.1% Tween 20 for 10 min followed by three washes in sterile distilled water. Any damaged tissue was removed. The explants thus obtained were put on sterilized agar media in Petri dishes. The Petri dishes were sealed with Parafilm M (American National Can) and maintained in a growth chamber at 25°C under a 16/8 h light/dark regime (ca. 1300 lux). Induced calli were subcultured onto fresh medium every 8 weeks.

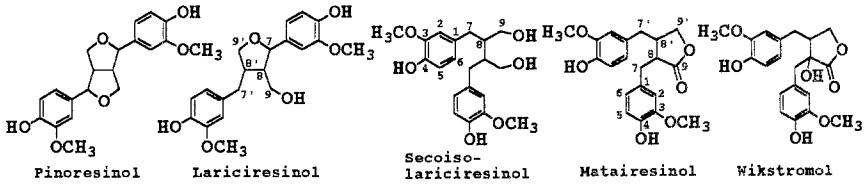
Suspension cultures

The callus (ca. 1.5 g fresh weight), maintained for 2 years on WS medium supplemented with 2,4-D (1.0 μM) and BA (1.0 μM), was suspended in WS liquid medium (30 ml) with 2,4-D (1.0 μM) and BA (1.0 μM) in a 100-ml Erlenmeyer flask and shaken on a rotary shaker at 120 rpm at 25°C under a 16/8 h light/dark regime (ca. 1300 lux). Following incubation for 21 days, the cell suspension was filtered through an autoclaved tea strainer. About 2.0 g of the cells trapped on the strainer and 10 ml of the filtrate were transferred to fresh medium (20 ml) in a 100-ml Erlenmeyer flask; they were incubated under the same conditions.

Growth measurements of suspension cultures and lignan analyses

After subculturing, triplicate flasks were harvested every 4 days. The culture medium in each flask was filtered by suction filtration through a filter paper (No. 5A; Kiriyama Co.), and the cells trapped on the paper were subjected to measurement of fresh weight and dry weight after freeze-drying. The filtrate was also freeze-dried, and the weight of the residue was measured. The dried cells were ground into powder and extracted three times with MeOH at 60°C. The combined MeOH extracts were evaporated to dryness, and an aliquot (5%) of the MeOH extracts was mixed with five deuterium-labeled lignans as internal standards: (±)-[9,9,9′,9′-$^2H_4$]pinoresinols; (±)-[9,9,9′,9′-$^2H_4$]lariciresinols; (±)-[OC^2H_3 × 2]secoisolariciresinols; (±)-[3-OC^2H_3]matairesinols; (±)-[3,3′-OC^2H_3 × 2]wikstromols. The mixture was then suspended in a solution (500 μl) of β-glucosidase [Sigma G-0395, 4 mg (13.6 units)/ml] in 0.1 M NaOAc buffer (pH 5.0). Following incubation at 33°C for 24 h, the hydrolysate was extracted with 500 μl of EtOAc. The extract was evaporated to dryness and subjected to GC-MS analysis; the content of lignans was determined by comparing the intensities of the molecular ion peaks with those of deuterium-labeled internal standards in mass chromatograms as previously reported.[2,7,8]

Fig. 1. Structures of lignans in *Daphne odora* cell suspension culture



Results and discussion

Callus induction from leaf explants was assessed on MS and WS media supplemented with various concentrations of plant hormones. No callus formation occurred on MS medium, whereas WS medium supplemented with the following concentrations of auxin and cytokinin supported callus induction and growth: BA 1.0μM and 2,4-D 1.0μM (WS1010); BA 1.0μM and 2,4-D 5.0μM (WS1050); BA 0.1μM and 2,4-D 1.0μM (WS0110); BA 0.1μM and 2,4-D 5.0μM (WS0150); BA 0μM and 2,4-D 5.0μM (WS0050). When 2,4-D/K, NAA/BA, and NAA/K were employed, active callus growth was not observed.

Since we reported the isolation of four lignans – matairesinol, secoisolariciresinol, lariciresinol, pinoresinol – from *D. odora* and *D. genkwa*,[8] production of the lignans by callus grown on WS1010, WS1050, WS0110, and WS0150 media was examined. The MeOH extracts of the calli were treated with β-glucosidase and subjected to GC-MS, which indicated that the extracts contained the four lignans, probably as glycosides (data not shown).

The calli maintained on WS1010 medium plate appeared soft and friable and were transferred to the equivalent liquid medium. After incubation for 21 days, the cells and filtrate were harvested, freeze-dried, and extracted with hot MeOH. GC-MS analysis indicated that lignans were not detected in the medium filtrate residue after β-glucosidase treatment. On the other hand, the MeOH extracts of cells after β-glucosidase treatment were found to contain five lignans – matairesinol, lariciresinol, pinoresinol, secoisolariciresinol, wikstromol (Fig. 1) – which, except for pinoresinol, were identified by direct comparison of their mass spectral data (Fig. 2) and retention times (t_R) on GC (matairesinol 14.8 min, lariciresinol 13.7 min, secoisolariciresinol 10.5 min, wikstromol 12.6 min) with those of chemically synthesized authentic samples (t_R: matairesinol 14.7 min, lariciresinol 13.7 min, secoisolariciresinol 10.5 min, wikstromol 12.7 min). Pinoresinol was identified mass chromatographically, which gave the correct t_R and relative intensities of the molecular and important daughter ions (Fig. 3) when compared with those of a chemically synthesized authentic sample.

Next, the suspension cultures were transferred to fresh WS liquid medium, and cell growth and lignan production were monitored. The cells in the medium were pale yellow-green, and most of them were dispersed with small numbers of aggregated cells. Growth of the cells measured by fresh and dry weights showed a sigmoid curve with an exponential phase of 4–30 days followed by a stationary phase, as

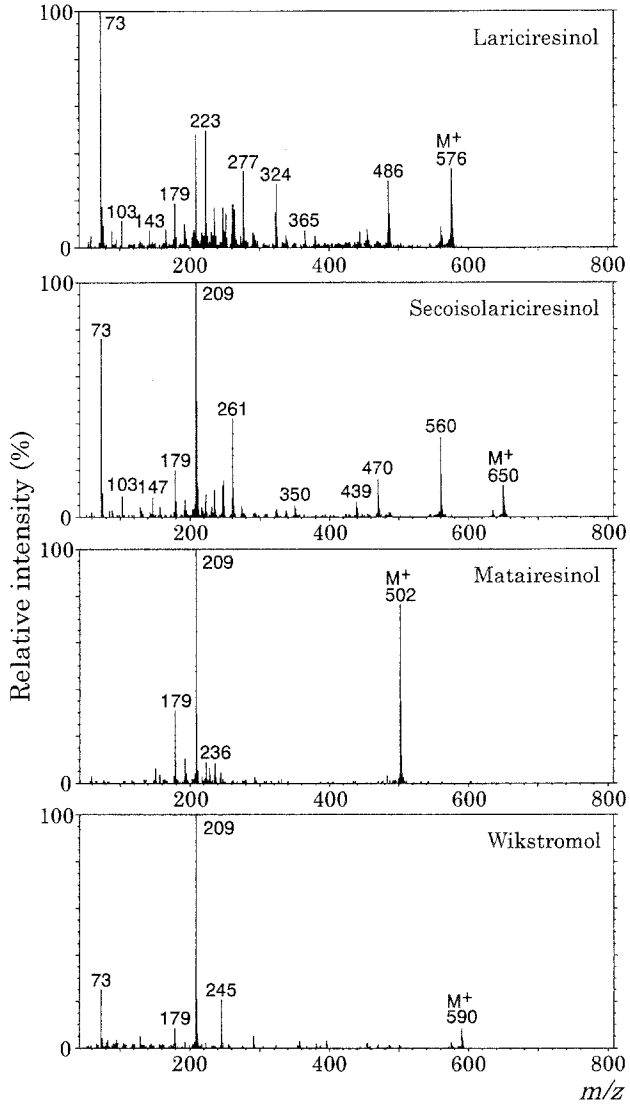


Fig. 2. Mass spectra of lignans [trimethylsilyl (TMS) ethers] in *D. odora* cell suspension culture

shown in Fig. 4A. This accorded with the decrease in the dry residue of the medium (Fig. 4A).

Figure 4B shows the production of the lignans versus time. Production of matairesinol was highest among the lignans and reached a maximum (ca. 2 mg/g dry weight cells) at day 8, after which levels started to decrease. The contents of the other lignans also had a similar tendency (Fig. 4B). The contents of the lignans lariciresinol, pinoresinol, secoisolariciresinol, and wikstromol were high-

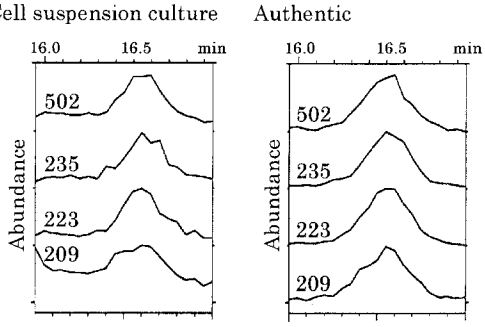


Fig. 3. Mass chromatograms of molecular and important fragment ions of the TMS ethers of pinoresinols. *Cell suspension culture*: pinoresinol in *D. odora* cell suspension culture. *Authentic*: chemically synthesized racemic (±)-pinoresinols

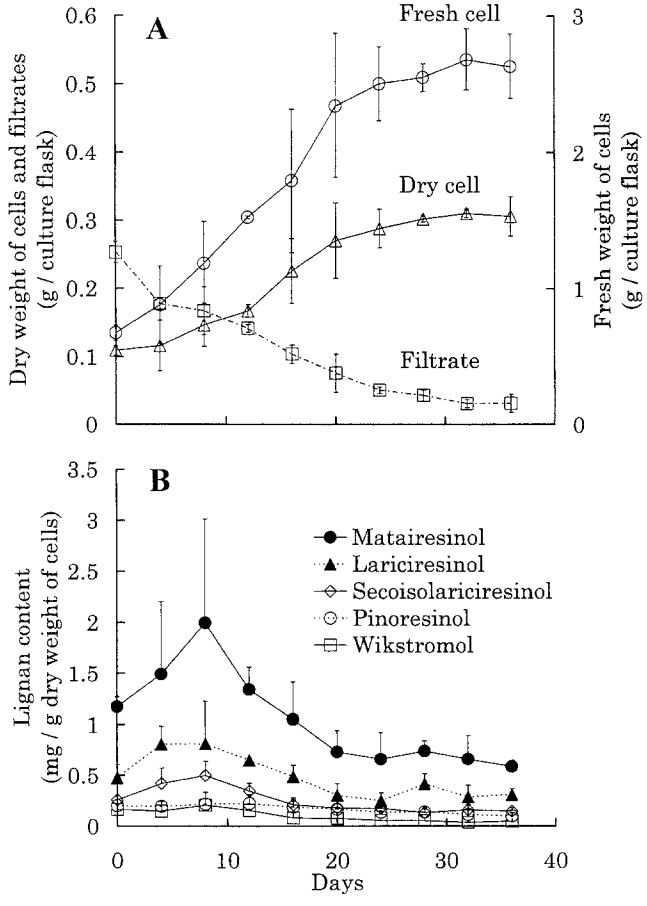


Fig. 4. Growth pattern (**A**) and lignan production (**B**) in *D. odora* cell suspension culture. Values are expressed as the means and standard deviations at the 95% confidence level

est (ca. 0.8, 0.2, 0.5, and 0.2 mg/g dry weight cells, respectively) at day 8, after which the levels decreased.

Although the biochemical background underlying the observation that the lignan contents were highest at the early stage of the cell growth curve is unknown, emphasis is placed on the high production of lignans in the *D. odora* suspension cells. The content of matairesinol (2 mg/g dry weight cells and 0.25 mg/g fresh weight cells) in the suspension cells was much higher than the yield of the lignan isolated from the stem and leaf tissues (0.015 mg/g fresh weight).[8] The high production of the lignans together with good growth of the cells suggests strongly that the cell suspension culture is useful for biochemical studies of lignan biosynthesis.

In conclusion, the present study reports for the first time that the cell suspension culture of *D. odora* produces lignans.

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T.O. and N.T. Contributed equally to this work.

References

1. Umezawa T, Shimada M (1996) Formation of the lignan (+)-secoisolariciresinol by cell-free extracts of *Arctium lappa*. Biosci Biotechnol Biochem 60:736–737
2. Suzuki S, Umezawa T, Shimada M (1998) Stereochemical difference in secoisolariciresinol formation between cell-free extracts from petioles and from ripening seeds of *Arctium lappa* L. Biosci Biotechnol Biochem 62:1468–1470
3. Umezawa T (1996) Biological activity and biosynthesis of lignans (in Japanese). Mokuzai Gakkaishi 42:911–920
4. Umezawa T, Okunishi T, Shimada M (1997) Stereochemical diversity in lignan biosynthesis. Wood Res 84:62–75
5. Umezawa T (2001) Biosynthesis of lignans and related phenylpropanoid compounds. Chem Regul Plants 36:57–67
6. Umezawa T, Shimada M (1996) Enantiomeric composition of (−)-pinoresinol, (+)-matairesinol and (+)-wikstromol isolated from *Wikstroemia sikokiana*. Mokuzai Gakkaishi 42:180–185
7. Okunishi T, Umezawa T, Shimada M (2000) Enantiomeric compositions and biosynthesis of *Wikstroemia sikokiana* lignans. J Wood Sci 46:234–242
8. Okunishi T, Umezawa T, Shimada M (2001) Isolation and enzymatic formation of lignans of *Daphne genkwa* and *Daphne odora*. J Wood Sci 47:383–388
9. Kawai S, Sugishita K, Ohashi H (1999) Identification of *Thuja occidentalis* lignans and its biosynthetic relationship. Phytochemistry 51:243–247
10. Tandon S, Rastogi RP (1976) Wikstromol, a new lignan from *Wikstroemia viridiflora*. Phytochemistry 15:1789–1791
11. Kogiso S, Wada K, Munakata K (1977) Nematicidal substances from *Daphne odora*. Report of the 26th International Congress of Pure and Applied Chemistry, Tokyo, p 291
12. Kato A, Hashimoto Y, Kidokoro M (1979) (+)-Nortrachelogenin, a new pharmacologically active lignan from *Wikstroemia indica*. J Nat Prod 42:159–162
13. Torrance SJ, Hoffmann JJ, Cole JR (1979) Wikstromol, antitumor lignan from *Wikstroemia foetida* var. *oahuensis* Gray and *Wikstroemia uva-ursi* Gray (Thymelaeaceae). J Pharm Sci 68:664–665
14. Lee K-H, Tagahara K, Suzuki H, Wu R-Y, Haruna M, Hall IH, Huang H-C, Ito K, Iida T, Lai J-S (1981) Antitumor agents. 49. Tricin, kaempferol-3-*O*-β-D-glucopyranoside and (+)-nortrachelogenin, antileukemic principles from *Wikstroemia indica*. J Nat Prod 44:530–535
15. Suzuki H, Lee K-H, Haruna M, Iida T, Ito K, Huang H-C (1982) (+)-Arctigenin, a lignan from *Wikstroemia indica*. Phytochemistry 21:1824–1825
16. Guo J-X, Handa SS, Pezzuto JM, Kinghorn AD, Farnsworth NR (1984) Plant anticancer agents. XXXIII. Constituents of *Passerina vulgaris*. Planta Med 50:264–265

17. Tatematsu H, Kurokawa M, Niwa M, Hirata Y (1984) Piscicidal constituents of *Stellera chamaejasme* L. II. Chem Pharm Bull 32:1612–1613
18. Umezawa T, Kuroda H, Isohata T, Higuchi T, Shimada M (1994) Enantioselective lignan synthesis by cell-free extracts of *Forsythia koreana*. Biosci Biotechnol Biochem 58:230–234
19. Umezawa T, Shimada M (1994) Syntheses of (±)-lariciresinols. Mokuzai Gakkaishi 40:231–235
20. Umezawa T, Isohata T, Kuroda H, Higuchi T, Shimada M (1992) Chiral HPLC and LC-MS analysis of several lignans. In: Kuwahara M, Shimada M (eds) Biotechnology in pulp and paper industry. Uni, Tokyo, pp 507–512
21. Umezawa T, Davin LB, Lewis NG (1991) Formation of lignans (−)-secoisolariciresinol and (−)-matairesinol with *Forsythia intermedia* cell-free extracts. J Biol Chem 266:10210–10217
22. Wolter KE, Skoog F (1966) Nutritional requirements of *Fraxinus* callus cultures. Am J Bot 53:263–269