ELSEVIER

Fitoterapia 71 (2000) 365–372

FITOTERAPIA

www.elsevier.com/locate/fitote

Isoflavonoid production by callus cultures of *Maackia amurensis*

S.A. Fedoreyev[a,*], T.V. Pokushalova[a], M.V. Veselova[a], L.I. Glebko[a], N.I. Kulesh[a], T.I. Muzarok[b], L.D. Seletskaya[b], V.P. Bulgakov[b], Yu.N. Zhuravlev[b]

[a] *Pacific Institute of Bioorganic Chemistry, Far East Branch of Russian Academy of Sciences, Vladivostok 690022, Russia*

[b] *The Institute of Biology and Soil Science of the Far East Branch of Russian Academy of Sciences, Vladivostok 690022, Russia*

Received 16 November 1999; accepted 19 December 1999

Abstract

Callus cultures were established from the different parts of *Maackia amurensis* plants and analyzed for isoflavonoids. The isoflavones daidzein, retuzin, genistein and formononetin and the pterocarpans maakiain and medicarpin were found to be produced by these cultures. The content of isoflavones and pterocarpans was essentially the same in cultures derived from leaf petioles, inflorescences and apical meristems of the plant. The maximal yield of isoflavones and pterocarpans in calluses was 20.8 mg/g cell dry wt., approximately four times higher than the content of the heartwood of *M. amurensis* plants. Unlike wild-growing plants, none of the cell cultures had the ability to accumulate stilbenes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Maackia amurensis*; Isoflavones; Pterocarpans; Callus culture

1. Introduction

Maackia amurensis Rupr. et Maxim. (Leguminosae) is the unique relict tree of tertiary flora [1]. A total of 11 isoflavonoid phytoalexins have been isolated from

* Corresponding author.

0367-326X/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0367-326X(00)00129-5

M. amurensis wood, collected in the Primorsky Region of Russia. These compounds have been identified as piceatannol and resveratrol (both stilbenes), formononetin, genistein, retuzin and afromozin (all isoflavones) [2], maackiasine (isoflavonostilbene) [3], scirpusin A, scirpusin B and maackin (all dimeric stilbenes) [4], and maackoline (stilbenolignan) [5]. The chemical composition of polyphenols of *M. amurensis* differs from that in the related species var. *buergeri* (Maxim.) C.K. Schneid., found in Japan and Korea [4–6]. Pterocarpans, which frequently occur in many Leguminosae species [7], were not previously found in samples of *M. amurensis* wood [5].

The polyphenolic complex from the heartwood of *M. amurensis* is an effective hepatoprotector which normalize structure and metabolic function of the liver following acute CCl_4-hepatitis [8,9]. Furthermore, the hepatoprotective agent 'Maxar' has been shown to enhance ability of prednisolone to inhibit proliferation of fibrotic tissue into hepatic lobuli, and collagen and glycosaminoglycane synthesis in liver homogenates in rats with chronic CCl_4-induced hepatitis [10]. Therefore, the establishment of cell cultures from this plant is thought to be an important goal for plant biotechnology.

In the present paper we report on the establishment of *M. amurensis* callus cultures and demonstrate that these cultures produce isoflavonoid compounds.

2. Experimental

2.1. Callus culture induction and development

One-year shoots of *M. amurensis* were isolated in March 1996 from mature plants growing in the southern Primorsky Region of the Russian Far East. Samples of the plants were authenticated at the Botany Department of the Institute of Biology and Soil Science. Shoots were kept in water at 20°C for 7–14 days and developing young shoots, leaves and inflorescences were used as explant sources. Explants were sterilized for 2–3 min in 0.2% commercial Diocide solution, washed extensively in sterilized water and placed on solid W-0 medium [11] supplemented with 0.5 mg/l 6-benzyladenin and 2.0 mg/l α-naphthaleneacetic acid ($W_{B/NAA}$ medium). Further explants were incubated in the dark at 25°C using 100-ml flasks containing 20 ml of $W_{B/NAA}$ medium. Calluses emerging on the explants were excised and cultivated on the same medium at 1-month intervals. Selection of fast-growing callus aggregates for three–four subcultures yielded the callus cultures that were subsequently grown under the described conditions for 6 months before analysis.

2.2. Extraction

Calluses dried under hot air flow and powdered (5 g) were extracted with 50 ml of 95% EtOH for 2 h. Extracts were filtered through filter paper and the filtrate was evaporated under reduced pressure at 40°C to yield a residue, which was

Table 1
Content of polyphenols (mg/g dry wt.) in heartwood and leaf petiole-derived calluses of *M. amurensis*

Constituents[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Heart-wood[b]	6.32 ± 0.52	6.10 ± 0.18	Trace	1.15 ± 0.06	1.92 ± 0.23	1.15 ± 0.19	0.35 ± 0.06	0.28 ± 0.05
Calluses[c]	–	–	0.52 ± 0.18	0.91 ± 0.34	2.53 ± 0.42	4.23 ± 1.01	5.78 ± 1.94	2.68 ± 0.45

[a] See Section 2.3 for definition of compounds **1**–**8**.
[b] Values are mean ± S.E., based on four separate samples of plants collected in November 1998.
[c] Values are mean ± S.E., based on four separate samples of leaf petiole-derived callus culture cultivated on $W_{B/NAA}$ medium.

partitioned between EtOAc (5 ml) and H_2O (2 ml). The EtOAc layer (1 ml) was evaporated and the resulting solid residue was dissolved in 1 ml EtOH containing 0.5 mg acetate 2-naphthol. After filtration (0.45 μm Millipore) the solution was used for HPLC analysis [12].

2.3. Chemicals and standards

Reagents for the preparation of nutrient media were obtained from Sigma ('Tissue culture' grade). The standards of polyphenols: piceatannol (1), resveratrol (2), daidzein (3), retuzin (4), genistein (5), formononetin (6), maackiain (7) and medicarpin (8) were previously isolated from the heartwood [3,4], as well as from callus cultures of *M. amurensis* in the course of the present series of investigations and identified by ^1H- and ^{13}C-NMR. Acetate 2-naphthol (9) was used as the internal standard.

2.4. High-performance liquid chromatography

The polyphenolic composition was analyzed qualitatively and quantitatively by HPLC with dual pump 114 M (Beckman), solvent programmer 420 (Altex) and wavelength monitor detector 2151 (LKB). Column: 250-4.6 Si 100: Polyol RP-18, 5 μm (Serva). For gradient elution two solvents were used: (A) H_2O-AcOH 100:2; and (B) MeCN. Elution profile: 0–20 min, 24% B in A; 20–60 min, 30% B in A. The flow rate was 1 ml/min and UV detection was at 282 nm. The injection mixture consisted of 10 μl of ethanol solution, containing 0.5 mg/ml standard and 0.5 g/ml extract.

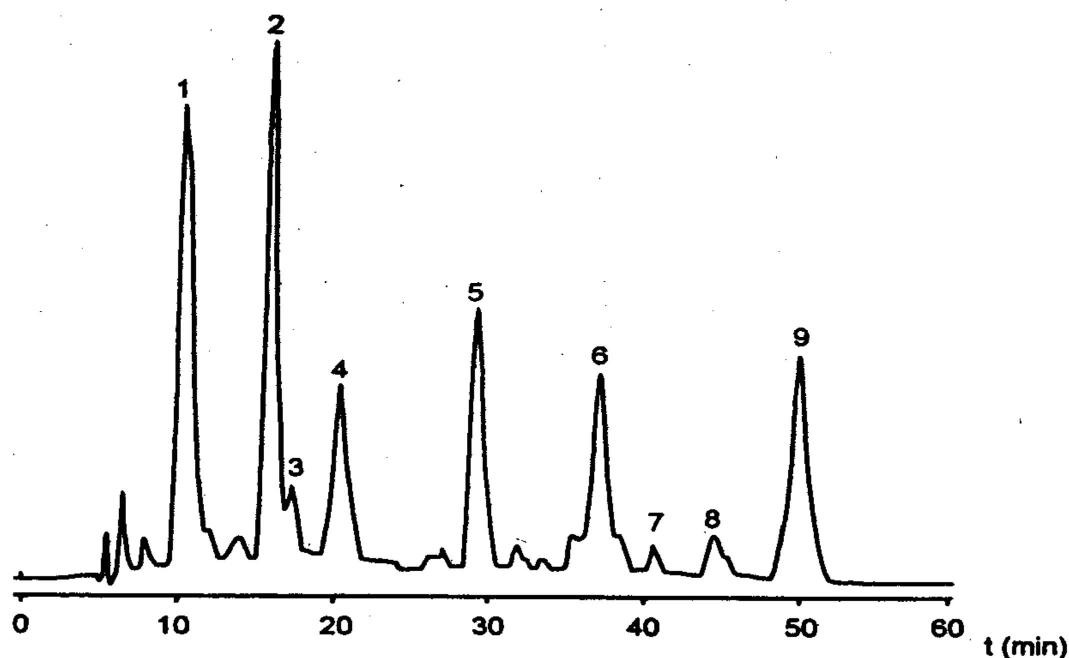

Fig. 1. HPLC profile of polyphenol fraction from *M. amurensis* heartwood. See Section 2.3 for definition of compounds 1–9.

3. Results and discussion

Leaf petiole-derived calluses were grown on $W_{B/NAA}$ medium, then dried and stored until analyses. Polyphenols were extracted from these samples as well as from heartwood of *M. amurensis* and separated by HPLC [12]. Results of the analysis are shown in Figs. 1 and 2. Stilbenes **1** and **2** were identified as the main components of polyphenolic complex of heartwood (Fig. 1, Table 1). On the contrary, stilbenes were not found in the leaf petiole-derived callus culture. This culture, however, accumulated significant amounts of isoflavones and pterocarpans (Fig. 2), approximately two and 13 times higher than those of the wild-growing plants, respectively (Table 1). The total content of polyphenols was 17.27 and 16.65 mg/g dry wt. for *M. amurensis* heartwood and calluses, respectively.

Callus cultures of *M. amurensis* derived from leaf petioles, inflorescences, leaves and apical meristems were grown for 1 month on $W_{B/NAA}$ medium and quantitatively analyzed by HPLC. All of these cultures contained isoflavonoids **3–8**, although in different quantities. The total amounts of isoflavonoids in leaf petiole-, inflorescence-, leaf- and apical meristem-derived cultures were 13.6, 11.5 (Table 2), 30.7 and 10.4 mg/g dry wt. (data not shown), respectively. No stilbenes were found in any culture.

R i/p index, which we use to clarify peculiarities in isoflavonoid biosynthesis, indicates the ratio between isoflavone and pterocarpan production. In our cultures, inflorescence-, leaf- and apical meristem-derived cultures have an index < 1 (0.57–0.82), and leaf petiole-derived culture > 1 (1.1–2.4). This difference in

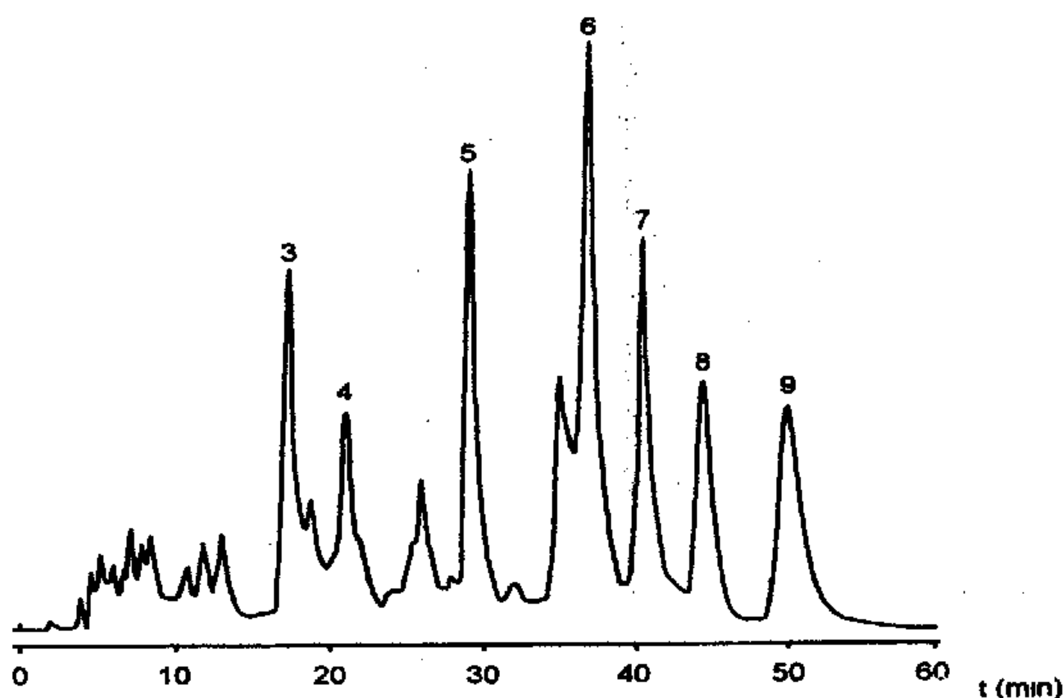

Fig. 2. HPLC profile of polyphenol fraction from *M. amurensis* leaf petiole-derived callus culture. See Section 2.3 for definition of compounds **1–9**.

Table 2
Production parameters of compounds 3–8 (mg/g dry wt.) in *M. amurensis* cultures growing in the presence of different growth regulators[a]

Medium[b]	Fresh cell biomass (g)	3	4	5	6	7	8	Total	R i/p	Isoflavonoid accumulation (mg/flask)
Inflorescence-derived calluses										
$W_{B/NAA}$	1.30 ± 0.12	0.5	0.7	1.4	2.8	5.8	2.4	13.6	0.7	1.41
$W_{B/2,4D}$	1.39 ± 0.20	0.9	0.8	1.5	4.3	5.2	6.9	19.6	0.6	2.18
$W_{Kin/NAA}$	1.32 ± 0.16	0.2	0.5	0.6	0.5	1.4	-	3.2	1.3	0.34
Leaf petiole-derived calluses										
$W_{B/NAA}$	1.90 ± 0.12	0.6	-	3.0	2.9	3.0	2.0	11.5	1.3	1.75
$W_{B/2,4\text{-}D}$	1.43 ± 0.09	2.5	0.8	3.7	5.3	3.3	5.2	20.8	1.4	2.37
$W_{Kin/NAA}$	1.71 ± 0.16	-	-	0.5	0.2	0.5	-	1.2	1.4	0.16

[a]See Section 2.3 for definition of compounds 3–8. B, 6-benzyladenin; 2,4-D, 2,4-dichlorophenoxyacetic acid; Kin, kinetin; NAA, α-naphthaleneacetic acid. Inoculum mass of calli was approximately 100 mg. R i/p, isoflavone/pterocarpan production ratio.

[b]Growth regulators (mg/l) in media: $W_{B/NAA}$ (0.5 B + 2.0 NAA); $W_{B/2,4D}$ (0.5 B + 1.0 2,4-D); $W_{Kin/NAA}$ (0.2 Kin + 1.0 NAA).

Table 3
The effect of L-phenylalanine and benzoic acid on isoflavonoid content (mg/g dry wt.) in leaf petiole-derived calluses of *M. amurensis* growing on $W_{B/NAA}$ medium[a]

Precursors	3	4	5	6	7	8	Total	R *i/p*
None (control)	0.5	0.4	2.1	3.5	2.2	2.1	10.8	1.5
L-Phenylalanine	1.4	0.4	2.8	6.2	5.3	4.5	20.6	1.1
Benzoic acid	0.9	0.6	2.4	3.0	1.5	1.4	9.8	2.4

[a]See Table 2 for definitions.

isoflavonoid accumulation within the cultures remained constant during subculturing.

The influence of some growth regulators on biomass accumulation and polyphenol production by *M. amurensis* callus cultures was tested. Kinetin and α-naphthaleneacetic acid, which have been reported to be appropriate regulators, supporting high level of stilbenes accumulation in *Vitis vinifera* cell suspension culture [13], were found to be ineffective for the stimulation of stilbene production in our cultures. Moreover, these regulators inhibited isoflavonoid production (Table 2). The replacement of α-naphthaleneacetic acid by 2,4-dichlorophenoxyacetic acid in the nutrient media resulted in an increase of isoflavonoid production, as well as increased total yield of the target substances per flask (Table 2).

To determine whether the absence of stilbenes in *M. amurensis* calluses was caused by depletion of precursors, L-phenylalanine was added to the growth media. Just as in other experiments, stilbenes were still not detectable in the callus cultures. A comparison of the amounts of isoflavones and pterocarpans in calluses growing either on phenylalanine-free or phenylalanine-containing media indicates that phenylalanine addition increases yield of isoflavonoids (Table 3). In contrast, benzoic acid did not increase production of these substances. These results are consistent with the supposed importance of L-phenylalanine as an early intermediate in the synthesis of isoflavonoids [14].

Thus, the callus cultures of *M. amurensis* produce a substantial quantity of polyphenols (Tables 1–3). However, the set of polyphenols found in these cultures significantly differed from that in the wild-growing plants. Although the calluses produce more isoflavones and pterocarpans than wild-growing plants, they did not produce stilbenes. Despite the fact that much research is carried out on the isolation and determination of polyphenols from plants, little is known about regulation of isoflavonoid and stilbene production in plants or in culture. Efforts are being made to determine whether the production characteristics of the *M. amurensis* cells could be affected by changes of cultivation conditions, changes of metabolite fluxes or by the transfer of specific genes.

References

[1] Maksimov OB, Kulesh NI, Gorovoy PG. Rastit Resursy 1992;28:157.

[2] Maksimov OB, Krivoschekova OE, Stepanenko LS, Boguslavskaya LV. Khim Prirodn Soed 1985;6:775.
[3] Krivoschekova OE, Stepanenko LS, Maksimov OB. Khim Prirodn Soed 1986;1:39.
[4] Kulesh NI, Isakov VV, Maksimov OB. Khim Prirodn Soed 1992;5:468.
[5] Kulesh NI, Denisenko VA, Maksimov OB. Phytochemistry 1995;40:1001.
[6] Takai M, Yamacuchi H, Saitoh T, Shibata S. Chem Pharm Bull 1972;20:2488.
[7] Kobayashi A, Akiyama K, Kawazu K. Phytochemistry 1993;32:77.
[8] Vengerovsky AI, Sedih IM, Vlasova TV, Saratikov AS. Rastit Resursy 1993;3:95.
[9] Vengerovsky AI, Sedih IM, Saratikov AS. Eksp Klin Farmakol 1993;56:47.
[10] Vengerovsky AI, Kovalenko MYu, Arbusov AG et al. Rastit Resursy 1998;3:91.
[11] Bulgakov VP, Zhuravlev YuN, Radchenko SV et al. Fitoterapia 1996;6:238.
[12] Pokushalova TV, Glebko LI, Maksimov OB. Khim Prirodn Soed 1988;6:801.
[13] Teguo PW, Decendit A, Krisa S, Deffieux G, Vercauteren J, Merillon LM. J Nat Prod 1996;59:1189.
[14] Goodwin TW, Mercer EI. Introduction to plant biochemistry. Oxford, New York, Toronto: Pergamon Press, 1983.