

Prenylated Flavanone Production in Callus Cultures of *Sophora flavescens* var. *angustifolia*

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Callus cultures of *Sophora flavescens* var. *angustifolia* established on Murashige-Skoog medium containing 1 μ M 2,4-dichlorophenoxyacetic acid and 1 μ M kinetin produced the prenylated flavanones (2*S*)-5,7,2',4'-tetrahydroxy-8-lavandulylflavanone (sophoraflavanone G) and (2*S*)-7,2',4'-trihydroxy-8-lavandulylflavanone (lehmanning). In addition, maackiain and its 3-O- β -glucoside (trifolirhizin) were also produced in the callus. Upon transfer to White's medium or M9 medium, the content of prenylated flavanones, in particular lehmanning, was increased, whereas that of pterocarpan was decreased. Time-course experiments indicated that the production of pterocarpan was closely related with cell growth. On the other hand, an inverse relationship existed between cell growth and the production of prenylated flavanones.

Introduction

Dry roots of *Sophora flavescens* Solander ex Aiton var. *angustifolia* Kitagawa (= *Sophora flavescens* Solander ex Aiton) (Leguminosae), which have been used as a bitter medicine in China and Japan, contain pterocarpan and prenylated flavonoids possessing a lavandulyl group on their A-ring [1, 2] as well as quinolizidine alkaloids [3]. Many attractive pharmacological activities of these flavonoids were reported, for example, an antitumor activity [4, 5], antimicrobial and anti-dermatophytic activities [6], an antifungal activity [7], and an antiarrhythmic activity [8]. Moreover, some prenylated flavonoids in *S. flavescens* roots were found to have an inhibitory effect on adenosine 3',5'-cyclic monophosphate phosphodiesterase activity [9].

Callus cultures of *S. flavescens* were first established by Furuya and Ikuta, and they found that the callus produced (*R*)-maackiain and pterocarpin [10]. Recently, Saito *et al.* demonstrated that green callus and multiple shoots of *S. flavescens* produced matrine and other quinolizidine alkaloids [11]. However, the production of prenylated flavonoids in cell cultures of *S. flavescens* has never been reported. In the present study, we estab-

lished callus cultures of *S. flavescens* to produce prenylated flavanones as well as pterocarpan. We also investigated the effect of various media on the production of flavanones.

Materials and Methods

Callus cultures

For induction of callus, segments of the surface-sterilized young stems of *Sophora flavescens* Solander ex Aiton var. *angustifolia* Kitagawa grown in the Botanical Garden of Gifu Pharmaceutical University were placed on Murashige-Skoog (MS) [12] medium containing 1 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μ M kinetin, solidified by 0.3% gellan gum (Wako Pure Chem. Ltd., Japan), and cultured at 25 °C in the dark. Callus was subcultured on the same medium at 25 °C in the dark, at intervals of 1 month for over 1.5 years. To test the effect of various media on flavonoid production, 0.3 g of fresh cells were inoculated on 10 ml of different media containing 3% sucrose, 1 μ M 2,4-D and 1 μ M kinetin, solidified by 0.3% gellan gum (3 replicates), and subcultured for 1 month under the same culture conditions as described above. The media used in the present study were as follows; B5 medium (Gamborg *et al.*, [13]), LS medium (Linsmaier and Skoog, [14]), M9 medium (Fujita *et al.*, [15]), NN medium (Nitsch and Nitsch, [16]), and White's medium (White, [17]). In kinetic studies on cell

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growth and the flavonoid production in callus cultures, the dry weight of cells and the flavonoid contents in culture samples (3 replicates) were measured every 5 days during the culture period of 60 days.

Isolation and identification of flavonoids

Lyophilized cells (150 g) were Soxhlet-extracted with acetone for 36 h. The acetone extract was evaporated to dryness, and the residue dissolved in distilled water was extracted with CHCl_3 and EtOAc, successively. The CHCl_3 extract (3.2 g) was chromatographed on a column (6 cm I.D. \times 50 cm) of silica gel (silica gel BW-300, Fuji Devision Chem., Japan) using hexane and acetone as solvents.

(*R*)-Maackiain: The 25% acetone (in hexane) fraction was evaporated to dryness, and the residue dissolved in MeOH was chromatographed on a column (2 cm I.D. \times 30 cm) of Sephadex LH-20 (MeOH as a solvent). The fraction containing (*R*)-maackiain was evaporated and recrystallized from benzene to afford it as colorless needles (231 mg).

Sophoraflavanone G (renamed from norkurarinone by Shirataki *et al.*, [18]) and lehmannin: The 33% acetone (in hexane) fraction was rechromatographed on a column (2 cm I.D. \times 50 cm) of silica gel (hexane:acetone = 2:1 as a solvent) and Sephadex LH-20 (2 cm I.D. \times 30 cm, MeOH as a solvent), successively. Sophoraflavanone G was obtained as colorless needles from benzene (138 mg). The fraction containing lehmannin was evaporated and separated by preparative TLC (Kieselgel 60 F₂₅₄, 20 cm \times 20 cm, Merck) using CHCl_3 :MeOH = 15:1. Lehmannin was obtained as an amorphous powder from hexane/EtOAc (14 mg).

Trifolirhizin: The EtOAc extract (4.3 g) was chromatographed on a column (6 cm I.D. \times 50 cm) of silica gel using CHCl_3 and MeOH as sol-

vents. The 10% MeOH (in CHCl_3) fraction was evaporated and chromatographed on Sephadex LH-20 column (2 cm I.D. \times 40 cm, MeOH). Trifolirhizin was recrystallized from acetone/MeOH (707 mg).

Maackiain, sophoraflavanone G and trifolirhizin were identified by direct comparison with the authentic samples (UV, m.p., EI-MS, ^1H - and ^{13}C -NMR, CD) and lehmannin was identified by the comparison of its physicochemical properties reported by Bakirov *et al.* [19].

Quantitative analysis of flavonoids

Lyophilized cells (100 mg) Soxhlet-extracted with MeOH (10 ml) for 3 h were assayed by reverse phase HPLC to measure the quantities of flavonoids on the basis of the area of an absorption peak at 294 nm by Chromatopak C-R6A (Shimadzu, Japan) using *p*-tert-butylphenol as an internal standard. The conditions of HPLC were as follows; column: Capcellpak C₁₈ AG-120A (5 μm , 4.6 mm I.D. \times 250 mm, Shiseido, Japan), solvent: MeCN/ H_2O gradient of 35% MeCN to 50% MeCN in 10 min, to 60% MeCN in another 10 min, then to 90% MeCN in 1 min; flow rate: 1 ml/min.

Results

Flavonoids produced in callus cultures

TLC analysis of the MeOH extract of pale yellow callus of *S. flavescens* indicated that the callus produced some phenolic compounds. As shown in Fig. 1 the callus contained two pterocarpan (maackiain and trifolirhizin) and two prenylated flavanones (sophoraflavanone G and lehmannin). Maackiain and trifolirhizin occurred both in callus and in dry roots of *S. flavescens*. On the other hand, 5-methylated flavanones such as kurarinone

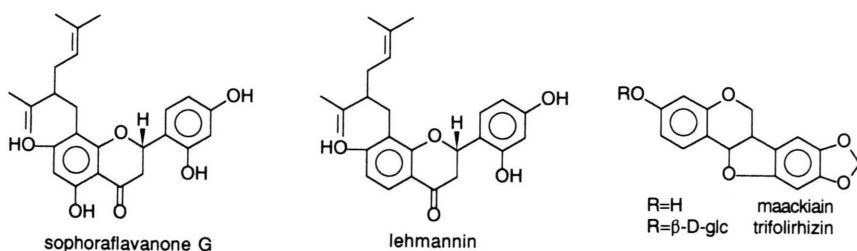


Fig. 1. Flavonoids isolated from callus of *Sophora flavescens*.

(7,2',4'-trihydroxy-8-lavandulyl-5-methoxy-flavanone) were major constituents in the intact plant whereas such a flavanone was not detected in the callus, which indicated that the methylation step was suppressed in the callus. Furthermore, it is noteworthy that lehmanningin, which has never been isolated from the parent plant, was produced in the callus.

Hatayama and Komatsu [1] isolated 22.8 g of prenylated flavonoids (0.114%) from 20 kg of dry roots of *S. flavescens*, and Honda and Tabata [6] isolated 663 mg of maackiain from 3.6 kg of dry roots (0.018%) of *S. flavescens*, while callus cultured on MS medium for 30 days contained 0.15–0.20% of prenylated flavanones and 0.50–0.96% of pterocarpan.

Effects of various media on cell growth and flavonoid production

With the aim to increase the content of prenylated flavanones, effects of various media on cell growth and flavonoid production were examined (Fig. 2). LS and MS media were the best among the media examined for their effect on cell growth, followed by B5 and NN media. Interestingly, the content of prenylated flavanones, especially lehmanningin, was markedly increased both on White's medium and on M9 medium, although cell growth was reduced. The content of pterocarpan was, on the other hand, decreased both on White's medium and on M9 medium. On NN medium, the con-

tent of pterocarpan was about two times higher than on MS medium in spite of cell growth and the content of prenylated flavanones being equal.

Kinetic studies on cell growth and flavonoid production

Time-courses of cell growth and flavonoid production on MS medium and on White's medium are shown in Fig. 3 and Fig. 4, respectively. On MS medium (Fig. 3), the pterocarpan content was increased almost in parallel with cell growth, and reached the maximal value at the early stationary growth stage (30 days after inoculation), and finally decreased. The content of sophoraflavanone G increased similarly that of the pterocarpan, but its maximal value was only one third of that of the total pterocarpan. The content of lehmanningin was almost unchanged throughout the culture period. On White's medium (Fig. 4), the callus grew more slowly, and produced only three fourth of the dry weight produced on MS medium. Pterocarpan were increased during the logarithmic and the early linear growth stages, and decreased before the stationary growth stage. The prenylated flavanones increased continuously to reach their maximum at the middle of the stationary growth stage (45–50 days after inoculation). These results suggested that the pterocarpan production is closely associated with cell growth, whereas the prenylated flavanone production is inversely correlated to cell growth.

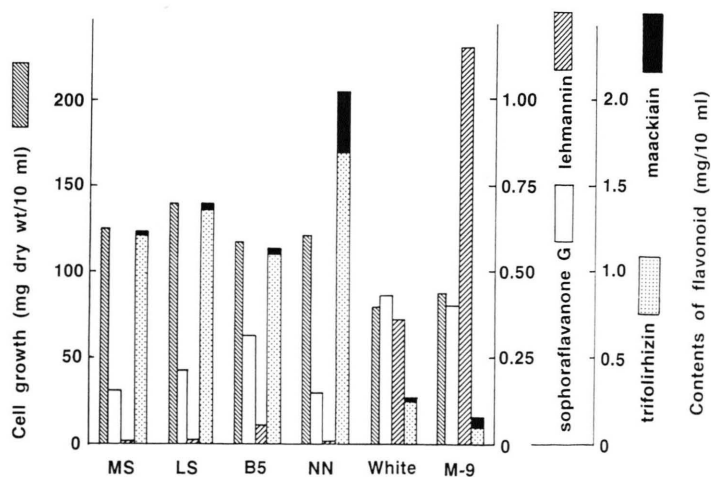


Fig. 2. Effects of various media on cell growth and flavonoid production in callus cultures of *Sophora flavescens*. 0.3 g of cells inoculated on each medium were cultured for 30 days in the dark at 25 °C.

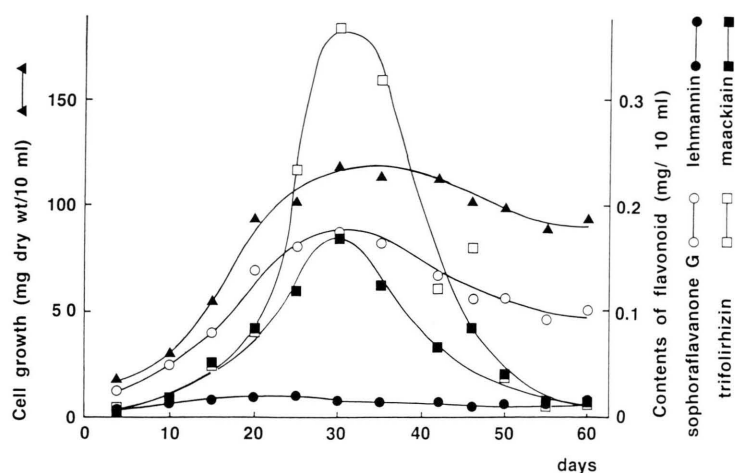


Fig. 3. Time-course of cell growth and flavonoid production in callus cultures of *Sophora flavescens* cultured on Murashige-Skoog medium. ▲: Cell growth (mg dry wt/10 ml), ■: maackiain (mg/10 ml), □: trifolirhizin, ●: lehmanning and ○: sophoraflavanone G.

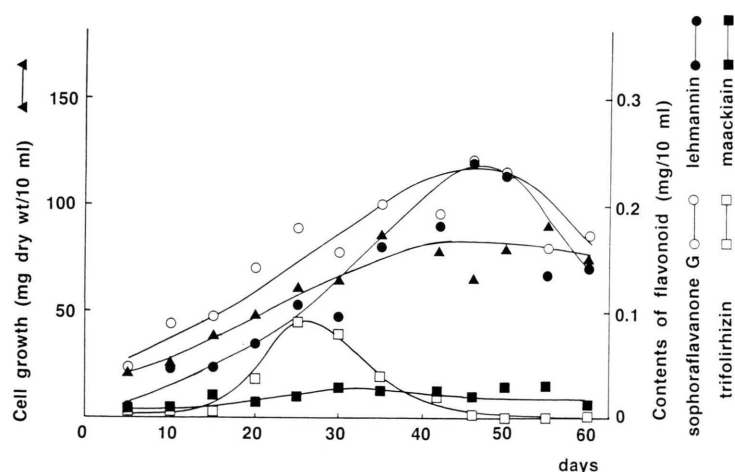


Fig. 4. Time-course of cell growth and flavonoid production in callus cultures of *Sophora flavescens* cultured on White's medium. ▲: Cell growth (mg dry wt/10 ml), ■: maackiain (mg/10 ml), □: trifolirhizin, ●: lehmanning and ○: sophoraflavanone G.

Discussion

Although the formation of prenylated flavonoids in plant tissue cultures has been reported by some workers [20–22], none of them has described factors controlling their production. In the present study, we have established a system suitable for studies on the biosynthesis of prenylated flavanones and pterocarpan in leguminous plants.

The enhancement of prenylated flavanone production in *S. flavescens* callus cultures on White's medium or on M9 medium indicated that nutritional factors play an important role in the regulation of prenylated flavanone and pterocarpan production. Fujita *et al.* [23] showed that ammonium ions inhibited the production of shikonin de-

rivatives, while Cu^{2+} enhanced their production in cell suspension cultures of *Lithospermum erythrorhizon*, and they established a new medium suitable for the production of shikonin derivatives, namely M9 medium [15]. It is interesting that the production of both naphthoquinones and prenylated flavanones, which belong to different classes of secondary metabolites, was stimulated by culturing cells on White's or M9 medium. Similar control mechanisms may exist in *Sophora* and *Lithospermum* cells. Studies on the effect of individual nutrients on the production of prenylated flavanones in *Sophora* cells are now in progress.

In the roots of *S. flavescens*, all prenylated flavonoids previously isolated possess hydroxyl or

methoxyl groups at C-5, whereas isoflavones and pterocarpan lack the oxygen-function at this position (5-deoxy derivatives). The occurrence of lehmanningin, a 5-deoxy prenylated flavanone, in callus tissues suggests that some differences of flavonoid synthesis exist between callus tissues and the intact plant of *S. flavescens*. 5-Deoxyflavanone prenyltransferase which must be suppressed in the parent plant may be expressed in callus tissues, or the flavanone/isoflavone isomerization may be inhibited

in callus tissues cultured on White's medium, so that unutilized 5-deoxyflavanone may flow into the prenylated flavanone biosynthetic pathway. Moreover, it may be speculated that the flavanone/isoflavone isomerization step will play a regulatory role in flavonoid production in *S. flavescens* cells as was shown to be the case of pterocarpan synthesis in soybean plants and cell cultures [24].

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