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ABSORPTION AND INCREASE IN THE PRODUCTION OF PRENYLATED FLAVANONES IN *SOPHORA FLAVESCENS* CELL SUSPENSION CULTURES BY CORK PIECES

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Key Word Index—*Sophora flavescens*; Leguminosae; cell suspension cultures; prenylated flavanones; subcellular localization; protoplasts; cell wall; corks.

Abstract—The accumulation sites of prenylated flavanones in *Sophora flavescens* cell suspension cultures were investigated. Quantitative analysis of the contents of prenylated flavanones in the protoplasts revealed that these flavanones were localized mainly in the cell walls. To collect prenylated flavanones from the cultured cells, several absorbents such as liquid paraffin, Amberlite XAD-2, activated carbon and commercially available cork pieces were added to the culture medium. Amberlite XAD-2 collected about 50% of the total prenylated flavanones from the cells, whereas cork pieces collected more than 70%. By the addition of cork pieces, the production of prenylated flavanones could be increased by 2–5 times compared with that of the control. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

We have reported that *Sophora flavescens* callus cultures produce prenylated flavanones such as sophoraflavanone G and lehmannin [1, 2], which are localized only in the cork layers of the root systems of the intact plants [3]. To clarify the regulatory mechanism of their production, especially its relationship with morphological differentiation, we investigated the localization of prenylated flavanones in *S. flavescens* cultured cells. We also investigated the effect of absorbents on prenylated flavanone production.

RESULTS

Accumulation site of prenylated flavanones in suspension cultured cells

The content of prenylated flavanones in *S. flavescens* cells and in the protoplasts isolated from the cultured cells were determined (Table 1). The results revealed that prenylated flavanones were mainly accumulated (*ca* 95%) in the cell walls of *S. flavescens* cultured cells.

Effects of absorbents on the production and the accumulation of prenylated flavanones

To collect the prenylated flavanones accumulated in the cell walls of cultured cells, liquid paraffin [4], Amberlite XAD-2 [5], activated carbon [6] and commercially available cork pieces which had been solvent extracted were added to the medium, respectively, and cultured for 2 weeks (Fig. 1). Liquid paraffin and activated carbon did not absorb the flavanones, while inclusion of activated carbon inhibited cell growth. Amberlite XAD-2 absorbed about 50% of the flavanones, but the total yield was unchanged. Cork pieces absorbed about 70% of the total flavanones, and the total content of prenylated flavanones increased up to 2 mg per 20 ml medium (i.e. twice as high as in cultures without added corks). Furthermore, the cell growth was not inhibited by addition of cork, and the state of the cells such as the colour and the size were quite similar to that of the control cells. Interestingly, cork pieces covered with dialysis bag neither absorbed prenylated flavanones nor increased the yield. Accordingly, direct contact between cork pieces and cultured cells was necessary both for the absorption and the increase in production.

Effect of cork amount on the production of prenylated flavanones

As the total amount of cork pieces added was increased, both the yield and the absorption of prenylated flavanones increased (Fig. 2). On the addition of 100 mg cork, the yield reached 3.78 mg per 20 ml medium and *ca* 80% of it was recovered from the cork pieces. When 150 mg cork was added, the absorption percentage was unchanged but both cell growth and the yield of prenylated flavanones were slightly reduced.

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Table 1. Contents of prenylated flavanones in cells and protoplasts of *S. flavescens* cell suspension cultures

	Cell/protoplast number ($\times 10^6$)	Content of prenylated flavanones ($\mu g\ 10^{-6}$ cells)	Percentage of prenylated flavanones in the protoplasts (%)
Experiment 1			
Cells	42.128	3.45	100.0
Crude protoplasts	27.113	0.93	27.1
Pure protoplasts	7.157	0.17	4.9
Experiment 2			
Cells	38.425	2.61	100.0
Crude protoplasts	n.d.	n.d.	n.d.
Pure protoplasts	6.426	0.09	3.6

n.d.: not determined.

Effects of larger amounts of synthetic absorbents on the production of prenylated flavanones

When larger amounts of Amberlite XAD-2 (0.8–1.6 g per 20 ml medium) were added to the medium, they absorbed about 80–90% of the prenylated flavanones but did not affect their production (Fig. 3). Diaion HP-20, another synthetic absorbent, also absorbed 80–90% of the flavanones. In the latter case, with the increase of the added polymer amounts, the yield also increased. On addition of 1.6 g Diaion HP-20 to the medium, the total yield of prenylated flavanones reached 1.1 mg per 20 ml medium (Fig. 3).

Effects of cork components on the production of prenylated flavanones

Cork tissues mainly consist of cellulose, polyphenols and long-chain fatty acids [7]. The effects of individual cork components on prenylated flavanone production were therefore investigated. Cellulose had no effect either on cell growth or prenylated flavanone production when tested at 2, 20 and 200 mg per 20 ml of medium (data not shown). 0.1–1 mM of suberic acid, obtained from the cork by acidic hydrolysis, increased the yield of prenylated flavanones about two-fold compared with the control, which was almost the same a level of response to that induced by 50 mg cork pieces addition (Fig. 4). However, in the case of suberic acid, cell growth was inhibited about 50% compared with the control, and the colour of cells turned from pale-yellow to dark brown. By contrast, the colour of cells cultured in the cork pieces-supplemented medium did not change during a 2-week culture period and these cells were able to be successfully subcultured into fresh medium. We also investigated the effects of $CHCl_3$, MeOH and H_2O extracts of corks (2 mg extract per 20 ml medium) on the production of prenylated

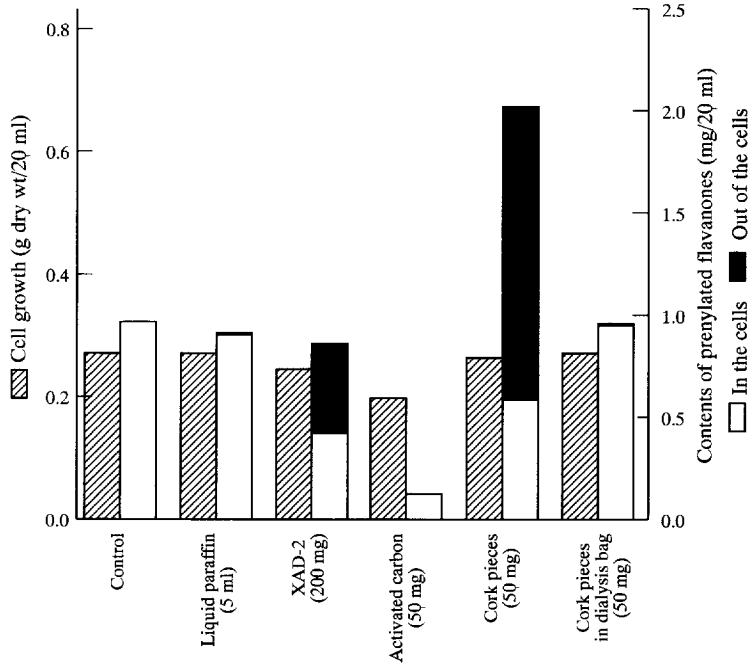

Fig. 1. Effects of absorbents on the production of prenylated flavanones in *S. flavescens* cell suspension cultures.

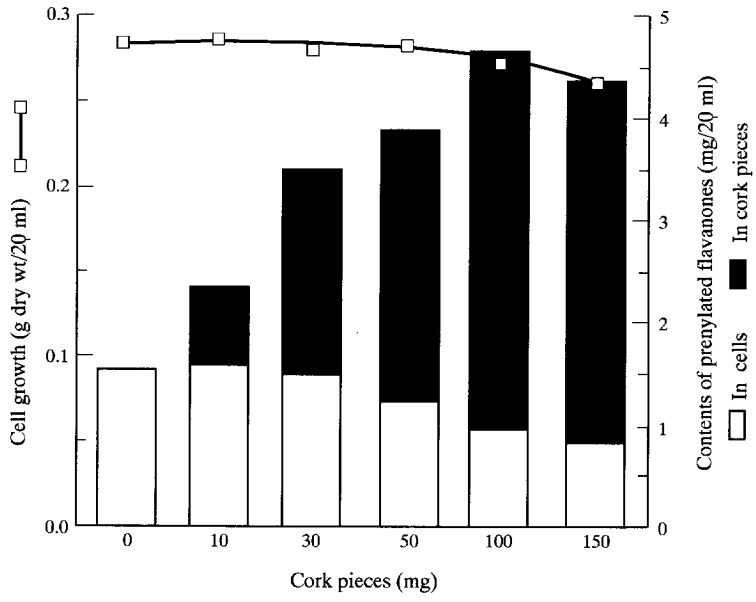

Fig. 2. Effect of different amounts of cork on the production of prenylated flavanones in *S. flavescens* cell suspension cultures.

flavanones but these had no effects on either cell growth or production of prenylated flavanones (data not shown).

Degradation of prenylated flavanones by cell cultures

The stability of prenylated flavanones in the culture medium was also examined (Table 2). Used medium decomposed only 7.6% of exogenous sophoraflavanone G whereas a 16.6% loss was noted in freshly prepared medium, indicating that cell debris present in the used medium may act as an absorbent that prevents flavanone degradation. In the case of cultured cells (Table 3), 14% (without cork) and 10% (with cork) of exogenous sophoraflavanone G was decomposed, but the absolute amounts of degraded sophoraflavanone G (0.12 mg and 0.08 mg, respectively) did not correspond to the additional amounts of sophoraflavanone G induced by inclusion of cork pieces (2.4 mg).

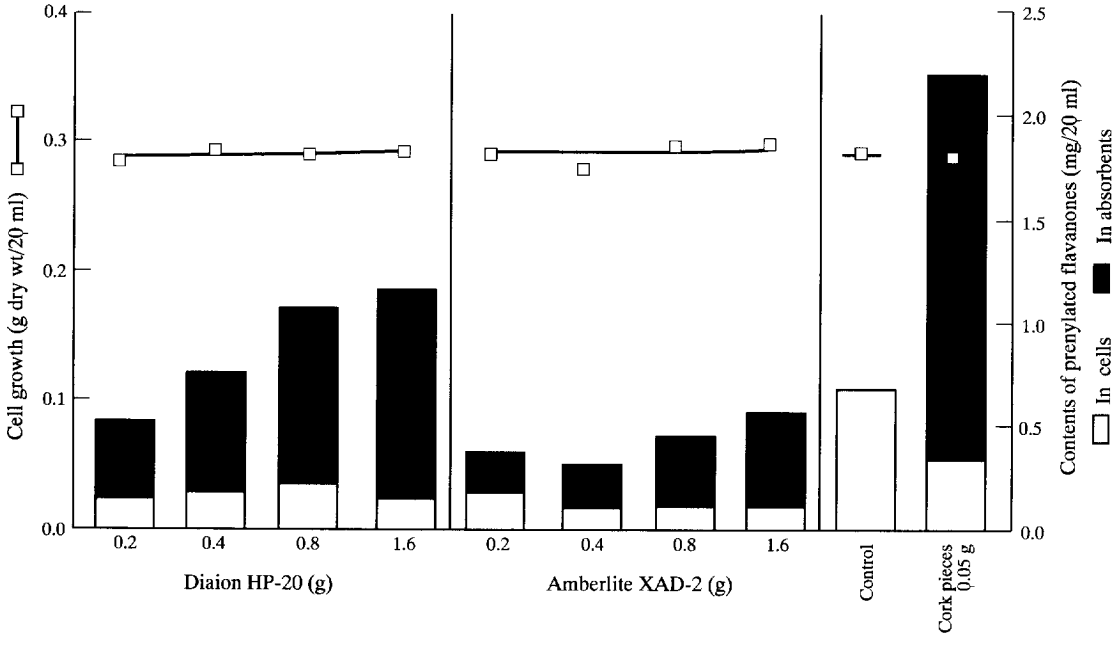

Fig. 3. Effect of the amounts of synthetic polymers on the production of prenylated flavanones in *S. flavescens* cell suspension cultures.

Table 2. Degradation of sophoraflavanone G (SFG) by *S. flavescens* culture medium

Medium	Conditions: Cork	Conditions: SFG	Total SFG (mg)	Decrease of exogenous SFG (%)
2-week-old	−	+	0.290	7.64
culture medium	+	+	0.331	−5.41
Fresh medium	−	+	0.262	16.56
	+	+	0.305	2.87

Sophoraflavanone G (0.321 mg/0.1 ml EtOH) was added to 5 ml of 2-week-old culture medium of *S. flavescens* cells in test tubes, and incubated for 1 week at 25° on a reciprocal shaker at a speed of 150 strokes min^{-1}.

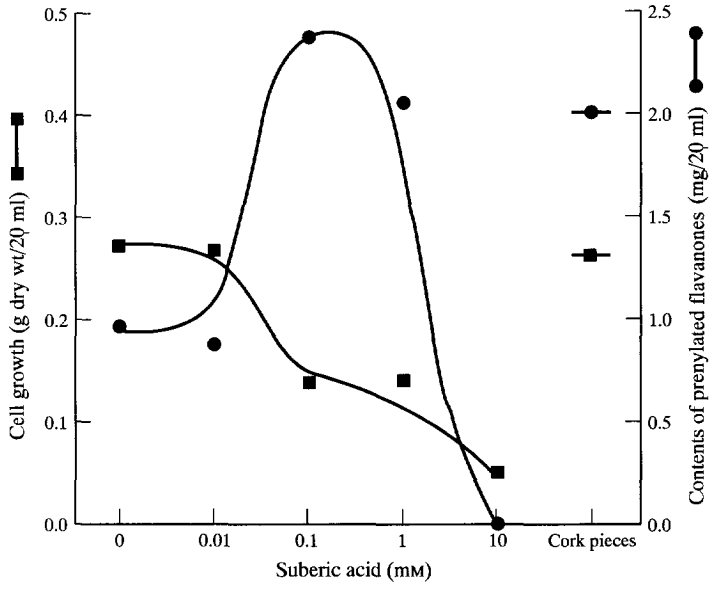

Fig. 4. Effect of suberic acid on the production of prenylated flavanones in *S. flavescens* cell suspension cultures.

Table 3. Degradation of exogenously added sophoraflavanone G (SFG) by cultured cells of *S. flavescens*

Condition: Cork	Condition: SFG	Total SFG (mg)	Increase of SFG (mg)	Decrease of exogenous SFG (%)	Increase of SFG by addition of corks (mg)
−	−	1.768	—	—	—
−	+	2.480	0.712	14.01	—
+	−	4.212	—	—	2.444
+	+	4.957	0.745	10.02	2.477

Sophoraflavanone G (0.828 mg per 0.2 ml EtOH) was added to 1-week cultured *S. flavescens* cells in 100 ml Erlenmeyer flasks (20 ml medium), and incubated for 1 week at 25° on a rotary shaker at a speed of 100 rpm.

DISCUSSION

In the present study, we have demonstrated that prenylated flavanones are accumulated in the cell walls of *S. flavescens* suspension cultured cells, and they are easily collected by use of appropriate absorbents such as Amberlite XAD-2 or cork pieces. Several workers have tried to collect secondary metabolites from cultured cells by use of absorbents. Deno *et al.* efficiently collected shikonin derivatives from *Lithospermum erythrorhizon* cell suspension cultures by use of liquid paraffin, but the production of the pigment was not changed [4]. In contrast to shikonin, the benzoquinone echinofuran B was isolated when activated carbon was added to the same *Lithospermum* cell cultures [6]. Furthermore, Berlin and coworkers reported the formation of mono- and di- terpenoids by the addition of Migloyl to *Thuja occidentalis* cell suspension cultures [8, 9]. In the latter two cases, enhanced production of the constituents of interest was observed, presumably because of prevention of degradation, or volatilization, by the addition of absorbents. In *Galium vernum* cells [5], anthraquinones that were usually accumulated in the cells were adsorbed extracellularly by the addition

of Amberlite XAD-2 or Wofatite ES (30–60 g l^{-1} of medium) with an increase in overall productivity. These authors speculated on the possibility of a release from feedback inhibition of anthraquinone biosynthesis cause by removal of the products from the cells.

In *Sophora* cells, cork pieces not only absorbed the prenylated flavanones but also increased their yield. We have considered three possibilities for this phenomenon. First, it is possible that the production of prenylated flavanones is normally regulated by feedback inhibition and that their removal from the cells results in the increasing production. Second, the absorption by cork pieces of prenylated flavanones that are usually degraded by enzymes secreted into the cultured medium could result in an increased yield of the product. Third, cork may act as an elicitor, so that the production is stimulated. However, none of these hypotheses is tenable. Larger amounts of Amberlite XAD-2 removed prenylated flavanones efficiently from the cultured cells but their production did not increase (Fig. 2), indicating that any release of a feedback regulatory mechanism is negligible. The prevention of the degradation also appears to be of minor importance considering the results of the fate of exogenously added sophoraflavanone G (Tables 2 and 3). The suggestion that cork pieces might act as elicitors also appears unlikely because the growth and appearance of cells cultured with cork pieces were similar to those of the cells cultured without them. Suberic acid did stimulate production, but it also inhibited cell growth and caused the cells to turn dark brown (Fig. 3). These responses are characteristic of the cells treated with other elicitors such as mannan purified from yeast extract, or pectin [10]. Thus, suberic acid acted as an elicitor or a stress inducer, and the mechanism of the yield stimulation by suberic acid must be different from that caused by cork pieces. It thus appears that cork pieces may contain unknown factors that do not affect the cell growth but are capable of stimulating prenylated flavanone production.

Cork tissues in plants have been regarded mainly as protective barriers against their environments [7]. They also act as biological barriers insofar as they control the diffusion of molecules such as waters and minerals [7]. In some cases, such as wounding, the deposition of phenolic compounds which act as chemical defense systems within the cork tissues occurs [11], but their production is triggered by wounding, an exogenous stimulus. It is quite unexpected to find that cork tissues themselves stimulate the production of phenolics.

EXPERIMENTAL

General. Cork sheet (2 mm thickness); Dainaga Cork Co. Ltd, Osaka, Japan. Amberlite XAD-2; Organo Co., Japan. Diaion HP-20; Mitsubishi Kasei Co., Japan. Cellulase Onozuka RS; Yakult Honsha Co. Japan. Pectolyase Y-23; Seishin Co., Japan. Activated carbon; Wako Pure Chemical Industries Ltd, Japan.

Plant material and culture method. The origin and subculturing of callus cultures of *Sophora flavescens* were described previously [1]. For establishment of cell suspension cultures, callus cells subcultured for 4 years (3 g fr. wt) were transferred to 20 ml MS liquid medium [12] containing 1 μM 2,4-D and 1 μM kinetin (100 ml flasks) and agitated on a rotary shaker at a speed of 100 rpm at 23° in the dark and subcultured every 2 weeks for more than 1 year. For each experiment, 1 g cells were inoculated in 20 ml culture medium containing additives and cultured for 2 weeks. All additives were added to the medium before autoclaving. In all experiments, triplicated samples were used and operations were repeated at least twice.

Quantitative analysis of flavonoids. Extraction and quantitative analysis of flavonoids produced by cultured cells were carried out according to the method of ref. [2].

Protoplast isolation. Seven to ten day-cultured cells (10 g) were suspended in 1 mM phosphate buffer (pH 6.0) supplemented with 10 mM $CaCl_2$, 0.5 M glucose (buffer A, 50 ml) containing 3% Cellulase Onozuka RS and 0.3% Pectolyase Y-23, and agitated on a rotary shaker at a speed of 60 rpm at 28° for 18 hr. To remove cell debris, the medium was passed through the nylon mesh (62 μm) and then centrifuged (100 *g*, 10 min). The pellets were washed ×3 with buffer A to give the crude protoplasts. The crude protoplasts, resuspended in buffer A (20 ml), were loaded on the surface of 21% sucrose soln (30 ml) and centrifuged for 10 min at 700 *g*. The purified protoplasts were collected from the surface between buffer A and the sucrose soln. The obtained soln was diluted by 5 vol of buffer A, and the pure protoplasts precipitated by centrifugation (100 *g*, 10 min). The purity of protoplasts was checked by microscopic observation. To count the cell numbers, fresh cells (100 mg) were suspended in 8% CrO_3 (8 ml) and heated at 70° for 3 hr to yield single cells. Numbers of cells and protoplasts were counted in a Fuchs-Rosenthal haemocyclometer.

Absorption of prenylated flavanones. Absorbents used in this study were as follows; liquid paraffin (5 ml), Amberlite XAD-2 (washed with EtOH, 200 mg), activated carbon (50 mg) and cork pieces (50 mg, 5 pieces) treated as follows: cork sheet (63 g) was cut into 5 mm squares, extracted successively with $CHCl_3$ (×3) and MeOH (×5) using a Soxhlet extraction apparatus (6 h each), then washed with H_2O (121°, 15 min, 15 times) and dried in an oven (50°). The amounts of each extract and of washed corks were 4.4 g, 2.9 g, 4.2 g and 51.3 g, respectively. Sepn of liquid paraffin from the cultured medium and extraction of prenylated flavanones from the liquid paraffin was carried out according to the procedure of ref. [4]. Amberlite XAD-2 floated in the medium during the culture period and was collected carefully by pipetting and extracted with MeOH. Cork pieces were removed with tweezers and extracted with MeOH. Activated carbon was difficult to sep from the cells, so in this study, it was not sepd. For extraction of prenylated flavanones from activated carbon-cell mixtures, they

were extracted successively with MeOH, Me_2CO and benzene, and all of extracts were combined and evapd.

In the case of larger quantities of synthetic polymer additions, Amberlite XAD-2 (washed with EtOH) or Diaion HP-20 (washed with Me_2CO) were added to the medium. After the sepn of the polymers, they were successively extracted with MeOH (×2), Me_2CO (×1) and $CHCl_3$ (×1). The extracts were combined and evapd.

Degradation of prenylated flavanones by cultured medium. A soln of sophoraflavanone G (0.3 mg) in EtOH (0.1 ml) was added aseptically to 5 ml freshly prepared LS medium, or 2-week-cultured medium, in test tubes, respectively, and incubated at 25° for 1 week on a reciprocal shaker at a speed of 150 strokes min^{-1}. To examine the effect of cork pieces on the degradation of sophoraflavanone G, autoclaved cork pieces (3 pieces) were added to each medium. After the incubation, each medium was extracted (×3) with *n*-BuOH and the flavanone content of the BuOH extract was measured.

Degradation of exogenously added prenylated flavanones by cultured cells. A soln of sophoraflavanone G (0.8 mg) in EtOH (0.2 ml) was added aseptically to *S. flavescens* cells cultured for 1 week, and cultured for an additional week under the same cultured condition as mentioned above. Autoclaved corks (5 pieces) were added to the medium at the same time as the sophoraflavanone G addition.

Effects of cork components on prenylated flavanones production. $CHCl_3$ and MeOH extracts of cork pieces were dissolved in EtOH (0.2 ml) and added to the medium, respectively. Lyophilized powders of H_2O extracts and cellulose were directly added to the medium, respectively. Suberic acid dissolved in 0.1 M NaOH was added to the medium and pH was adjusted to 6.0–6.2 prior to autoclaving.

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