



Shikonin production by *p*-fluorophenylalanine resistant cells of *Lithospermum erythrorhizon*

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Abstract

Studies were conducted with a BK-39 callus culture of *Lithospermum erythrorhizon*, which produced seven shikonin derivatives (acetylshikonin, propionylshikonin, isobutyrylshikonin, β,β -dimethylacrylshikonin, isovalerylshikonin, β -hydroxyisovalerylshikonin and α -methyl-*n*-butyrylshikonin). A selection of cell aggregates of BK-39 culture on a medium containing *p*-fluorophenylalanine (PFP) yields a cell line possessing a higher resistance to the inhibitor than the initial culture. Selected BK-39F cultures produced almost the same profile of shikonin naphthoquinones as the initial culture. The shikonin derivative content of PFP-resistant culture was approximately two times higher than that of the control, reaching 12.6% of DW cell biomass. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Lithospermum erythrorhizon*; Callus culture; *p*-Fluorophenylalanine; Shikonin derivatives

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1. Introduction

Red naphthoquinone pigments (shikonin derivatives) of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) roots are known to possess antibacterial, anti-inflammatory and anti-tumor activity [1–3], and accelerate the proliferation of granulation tissue [4]. Therefore, numerous research projects have been carried out on the development of alternative raw sources of shikonin and its derivatives [5]. The production of shikonin by cell cultures, as well as by chemical synthesis, has been studied extensively over the last few decades [5,6].

The key step, linking precursors from the phenylpropanoid and isoprenoid (mevalonate) pathways in the biosynthetic chain leading to shikonin, is the formation of *m*-geranyl-*p*-hydroxybenzoic acid from *p*-hydroxybenzoic acid and geranylpyrophosphate (Fig. 1) [7–11]. Previously, we have shown that addition of mevalonates to the culture medium did not alter shikonin production by cultured *L. erythrorhizon* cells, whereas the addition of *p*-hydroxybenzoic acid strongly increases shikonin formation [12]. Yazaki et al. [13] reported that the content of *p*-hydroxybenzoic acid was much higher in shikonin-producing cells than in shikonin-free cells and that exogenous addition of *p*-hydroxybenzoate increased

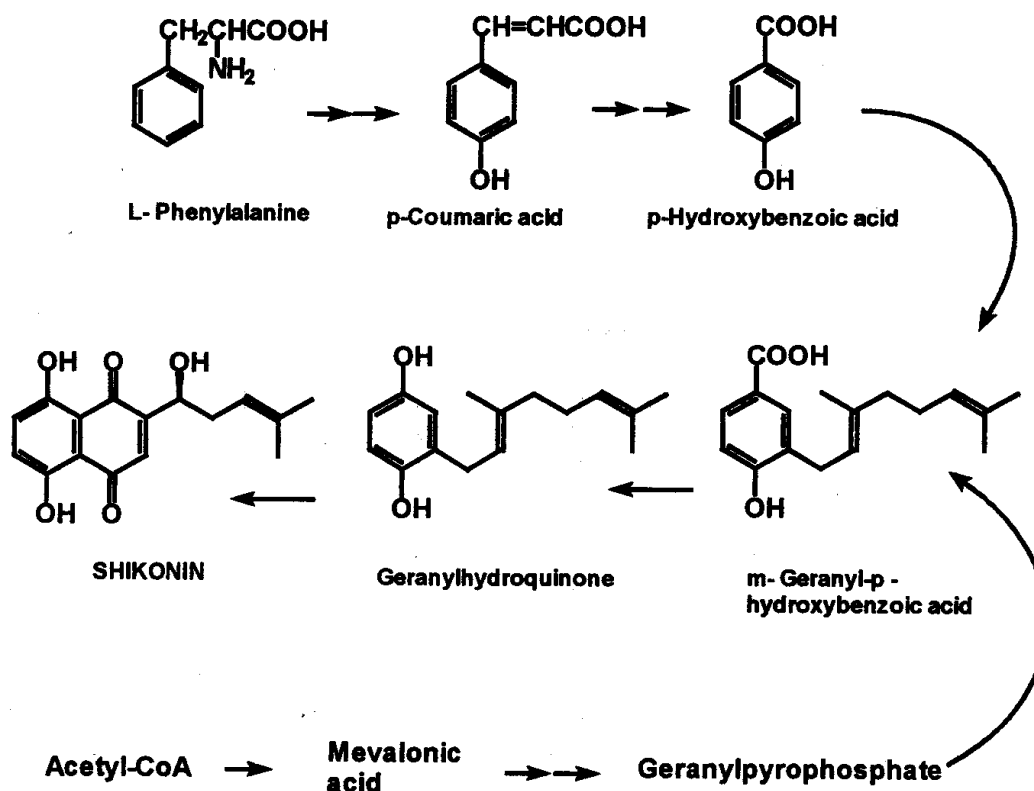


Fig. 1. Biosynthetic pathway of shikonin formation.

shikonin production. Recently, to stimulate shikonin biosynthesis in *L. erythrorhizon* cells, Sommer et al. performed genetic engineering of a metabolic pathway leading to *p*-hydroxybenzoic acid [14].

We proposed that increased shikonin production by *Lithospermum* cells could potentially be achieved by stimulation of the metabolite flux via a phenylpropanoid pathway. It is well established that selection for resistance to the phenylalanine analogue *p*-fluorophenylalanine (PFP) can result in the hyperaccumulation of aromatic compounds in cultured plant cells [15]. Hence, it was of interest to determine the effect that a selection of *Lithospermum* cells in the presence of PFP would have on pigment production. Doubt has been expressed about whether an increase in the production of shikonin may be achieved by the over-expression of a single metabolic pathway [14]. However, the result described in this report was as predicted: PFP-resistant cells accumulated higher levels of the shikonin naphthoquinones compared with non-treated control cells.

2. Experimental

2.1. Stock culture

The callus culture BK-39 was established from the roots of wild-growing *L. erythrorhizon* plants collected in the Primorye Region of Russia [16]. The culture was deposited at the Russian Collection of Plant Cell Cultures as a source of shikonin pigments [17]. Calluses were cultivated on solid W-0 medium [18], supplemented with kinetin (2.0 mg/l), indole-3-acetic acid (0.2 mg/l) and CuSO₄ (0.25 mg/l) in the dark at 25°C with 30-day subculture intervals.

2.2. Selection

p-Fluoro-DL-phenylalanine (Sigma) was dissolved in hot water (10 mg/ml), sterilized by Millipore filtration and added into autoclaved media. Cell aggregates (1–2 mm) of BK-39 culture were transferred to the media containing PFP. Well-growing aggregates were selected on media containing gradually increased concentrations (10, 30 and 100 mg/l) of the inhibitor, according to the described selection procedure [15].

2.3. Extraction and isolation

Dried powdered samples of the cell cultures were completely extracted with hexane. After evaporation of the solvent, the residue was dissolved in EtOH and naphthoquinones were precipitated with Cu(OAc)₂ to form Cu-pigment complex [16,19]. After evaporation of EtOH the precipitate was exhaustively extracted by hexane and subsequently by acetone. Dried Cu-pigment complex was decomposed by conc. HCl and shikonin pigments were extracted with diethyl ether and dried.

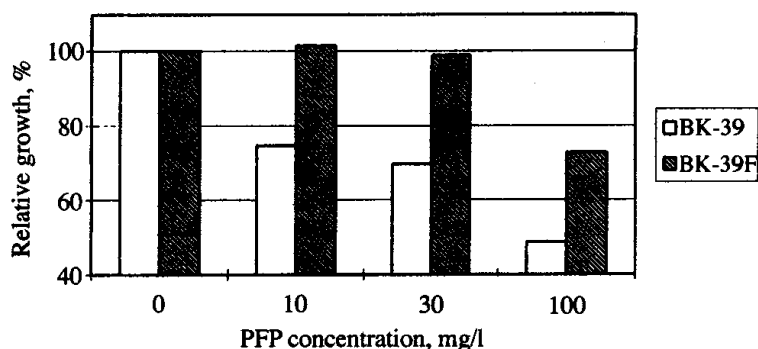


Fig. 2. Growth of BK-39 and BK-39F cultures in the presence of *p*-fluorophenylalanine (PFP).

2.4. Shikonin derivatives content

The total content of shikonin derivatives was determined in 30-day cultures photometrically at 520 nm [16].

2.5. $^1\text{H-NMR}$ analysis of shikonin derivatives

The composition of shikonin derivatives was determined by $^1\text{H-NMR}$ analysis [16]. The proportions of the individual esters were calculated according to the intensities of the signals of the methyl group of the acyl substituents (0.94–2.16 ppm).

2.6. High-performance liquid chromatography

The shikonin derivatives were analyzed qualitatively and quantitatively by HPLC with dual pump 114 M (Beckman), solvent programmer 420 (Altex) and wavelength monitor detector 2151 (LKB). Column: 150-3 mm: SGX C-18, 5 μm (Tessek Ltd., Praha). For elution, two solvents were used: 60% $\text{H}_2\text{O}/\text{HCOOH}$ (96:4), and 40% MeCN/HCOOH (96:4). The flow rate was 1 ml/min and UV detection was at 282 nm.

3. Results and discussion

The selection of PFP-resistant lines was conducted by the step-wise exposure of the parent line to increased levels of the analogue, as described in Section 2. Sequential selection yielded callus lines capable of growing normally on the medium containing concentrations of PFP which significantly inhibited growth of the parent line (Fig. 2). The BK-39F culture possessing the highest growth rate on PFP-containing media was chosen for further investigation. This culture grew normally in the presence of 10–30 mg/l of PFP (Fig. 2) and continued to grow at

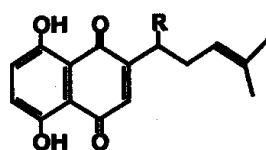
Table 1
Biomass accumulation and shikonin derivative production by initial BK-39 and PFP-resistant (BK-39F) callus cultures of *Lithospermum erythrorhizon*^a

Cell line	Fresh biomass (g/l)	Dry biomass (g/l)	Shikonin derivative content (% DW)	Shikonin derivative production (g/l)
BK-39	282 ± 36	18.9 ± 1.1	7.2 ± 0.6	1.36 ± 0.30
BK-39F	295 ± 32	21.0 ± 1.5	12.6 ± 0.8	2.64 ± 0.45

^a Values are mean ± S.E. based on five separate replicate samples harvested at 3-month intervals.

concentrations of PFP as high as 300 mg/l (1640 μM). The dose of PFP which decreases the rate of cell growth by half (LD₅₀), was found to be 940 μM PFP for the BK-39F line. This value is in the same order as those reported for *Anchusa officinalis* and *Nicotiana tabacum* PFP-resistant cells (1820 and 800 μM PFP, respectively) [15] and exceeds the value reported for *Catharantus roseus* cells (175 μM PFP) [15].

The examination of naphthoquinone levels in the sensitive and resistant lines was started after 12 subcultures of the calluses had been grown on the media



R	Compound	Content, %	
		BK-39	BK-39F
1 H	Deoxyshikonin	tr.	tr.
2 OCOCH ₃	Acetylshikonin	27.3	24.0
3 OCOCH ₂ CH ₃	Propionylshikonin	3.4	4.0
4 OCOCH(CH ₃) ₂	Isobutyrylshikonin	38.3	36.0
5 OCOCH=C(CH ₃) ₂	β,β-Dimethylacrylshikonin	1.8	Nf.
6 OCOCH ₂ CH(CH ₃) ₂	Isovalerylshikonin	12.5	18.0
7 OCOCH ₂ C(CH ₃) ₂ OH	β-Hydroxyisovalerylshikonin	9.1	8.0
8 OCOCH(CH ₃)CH ₂ CH ₃	α-Methylbutyrylshikonin	7.3	7.5

Fig. 3. Composition of shikonin derivatives produced by BK-39 and BK-39F cultures. tr, trace amount of pigment; Nf, pigment not found.

Analysis revealed that three clusters were found among samples, in which the first cluster consisted of five samples of *L. erythrorhizon* roots of the different origin (Chinese, Japanese, Korean and Russian). The second cluster consisted of Korean roots (sample I) and our cultures (BK-39 and BK-39F), while the third cluster comprised Japanese root sample II and Japanese cell culture (Fig. 4). Much variation was found for samples of the natural roots. On the other hand, the naphthoquinone composition of BK-39F culture does not differ significantly from that of the initial BK-39 culture. Naphthoquinones were accumulated in both cultures in similar proportions as in a sample of Korean cultured roots.

There are abundant data which reveal that plant cells in *in vitro* culture conditions lose their ability to produce secondary metabolites. However, examining the production parameters of BK-39F culture, we have found that this culture possesses a high and stable biosynthetic ability during long-term cultivation (Table 1, unpublished observations). Moreover, the induction of shikonin ether synthesis in this culture paralleled the rise in biomass accumulation (data not shown). It allowed us to use BK-39F culture for the one-stage cultivation process. This is economically important because other *L. erythrorhizon* cultures described so far were adapted for two-stage cultivation processes [5]. The biomass of the BK-39F culture proved to be a good source for the production of a new drug composition, so-called 'Shikonin oil', which has been used for the treatment of important skin diseases [21].

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