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EFFECT OF NUTRITIONAL FACTORS ON SHIKONIN DERIVATIVE FORMATION IN *LITHOSPERMUM* CALLUS CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; plant tissue culture; regulation of biosynthesis; nutritional factors; 1,4-naphthoquinone; shikonin derivatives.

Abstract—Some nutritional factors affecting the biosynthesis of shikonin derivatives in callus cultures of *Lithospermum erythrorhizon* were examined. High sucrose concentrations increased the content of shikonin derivatives, but neither glucose nor fructose was effective for shikonin derivative formation. High concentrations of nitrogen sources inhibited or retarded shikonin derivative formation and streptomycin sulphate stimulated their biosynthesis. Addition of ascorbic acid increased the content of shikonin derivatives. Among some precursors tested only L-phenylalanine had a positive effect. At high concentrations, Ca^{2+} and Fe^{2+} inhibited the biosynthesis of shikonin derivatives.

INTRODUCTION

Part of our efforts to determine which factors regulate shikonin derivative production by callus cultures of *Lithospermum erythrorhizon* included experiments on the effects of auxin and light, which we reported previously [1, 2]. In this paper, we describe the effects of some nutritional factors on biosynthesis of shikonin derivatives.

RESULTS AND DISCUSSION

Carbohydrates

Ikeda *et al.* [3] reported that the ubiquinone content in tobacco suspension cultures tended to increase with low sugar concentration (1%). In contrast, Zenk *et al.* [4] reported that in *Morinda* suspension cultures the optimal concentration of sucrose for anthraquinone production was rather high (7%), but optimal growth was independent of optimal anthraquinone production. To determine the optimal concentration of sucrose needed for growth and for shikonin derivative formation, we used 1–10% sucrose in combination with the other ingredients of Linsmaier–Skoog's basal medium [5] containing 10^{-6} M IAA and 10^{-5} M kinetin. As shown in Fig. 1, both growth and formation of shikonin derivatives were highest with 5% sucrose concentration. At this concentration, the content of shikonin derivatives was 1.52 mg per g fr. wt of callus (20 mg per flask containing 40 ml agar medium). With a concentration of 1% sucrose, the content of shikonin derivatives was only about 0.01 mg per g fr. wt of callus, i.e. less than 1% of that for 5% sucrose concentration; however, growth was maintained at about 50% of that for 5% sucrose concentration.

Matsumoto *et al.* [6] reported that sucrose, glucose and fructose were effective for anthocyanin synthesis in *Populus* suspension cultures. It was also reported by Ikeda *et al.* [3] that little difference was observed between sucrose and glucose as carbon sources in ubiquinine formation in tobacco suspension cultures. Zenk *et al.* [4] reported, however, that sucrose was much superior to all other carbohydrates for anthraquinone production in *Morinda* suspension cultures.

The influence of different carbon sources at 3% concentration, normally used as the sucrose concentration in Linsmaier–Skoog's medium, on *Lithospermum* callus cultures is shown in Table 1. Both glucose and fructose supported growth to a limited extent; however, they were much inferior to sucrose for the formation of shikonin derivatives even when added in a 1:1 ratio of glucose to fructose, the ratio found in sucrose. These results showed that only sucrose is an effective carbon source for shikonin derivative production in callus cultures.

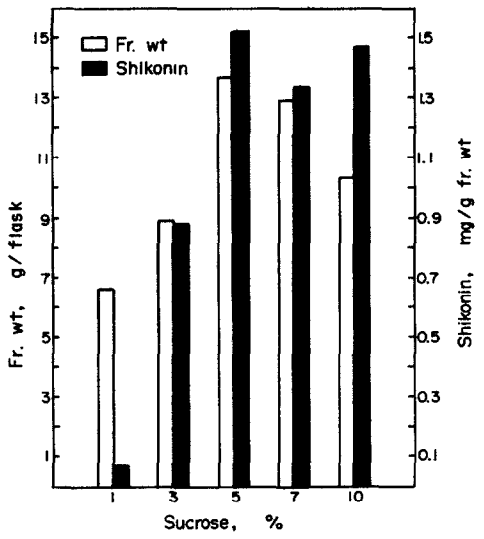

Fig. 1. Effects of sucrose concentration on growth and shikonin derivative formation.

Table 1. Effects of various carbon sources (3%) on growth and shikonin derivative formation

Carbon source	Fr. wt of callus (g/flask)	Content of shikonin derivatives (mg/g fr. wt)
Sucrose	9.8 ± 0.5*	1.04 ± 0.09*
Glucose	7.2 ± 0.8	0.02 ± 0.01
Fructose	5.6 ± 1.0	0.01 ± 0.002
Glucose + Fructose	3.9 ± 0.5	0.01 ± 0.002

* Mean ± s.e.

Nitrogen sources

The concentration of N sources in the basal medium (normally 67 mM as N) was changed without altering the ratio of NH_4^+ and NO_3^- (1:1.1). As shown in Table 2, the optimal N concentration was 104 mM for both growth and shikonin derivative formation. Above 134 mM N concentration, shikonin derivative formation was inhibited more strongly than was growth. Furthermore, the addition of organic-bound N sources such as peptone, casein hydrolysate and yeast extract to the basal medium at 0.3% concentration caused a significant decrease in content of shikonin derivatives (Fig. 2).

Because urea is decomposed to ammonia and carbon dioxide in plant cells, it was used as a possible N source for cultured plant cells. The addition of urea at the end of the second week of culture retarded the initiation of shikonin derivative formation without affecting the growth curve (Fig. 3). The concentration of urea added was 360 mg/l. of medium and corresponded to 66% of the total amount of N in the basal medium. These results suggest that at high concentrations N sources inhibit shikonin formation in *Lithospermum* callus cultures.

Table 2. Effects of nitrogen* on growth and shikonin derivative formation

Nitrogen concentrated (mM)	Fr. wt of callus (g/flask)	Content of shikonin derivatives (mg/g fr. wt)
67	2.9 ± 0.4†	1.31 ± 0.26†
104	4.1 ± 0.5	1.49 ± 0.08
134	3.5 ± 0.3	1.12 ± 0.06
268	1.6 ± 0.4	0.07 ± 0.005

* Added as NH_4NO_3 and KNO_3 † Mean ± s.e.

Table 3. Effects of streptomycin sulphate on growth and shikonin derivative formation

Conc of streptomycin sulphate (ppm)	Fr. wt of callus (g/flask)	Content of shikonin derivatives (mg/g fr. wt)
0	7.5 ± 0.7*	0.24 ± 0.04*
1	4.9 ± 0.2	0.57 ± 0.08
10	4.6 ± 0.9	0.82 ± 0.06
50	4.0 ± 0.4	1.21 ± 0.43
100	2.9 ± 0.3	1.20 ± 0.15

* Mean ± s.e.

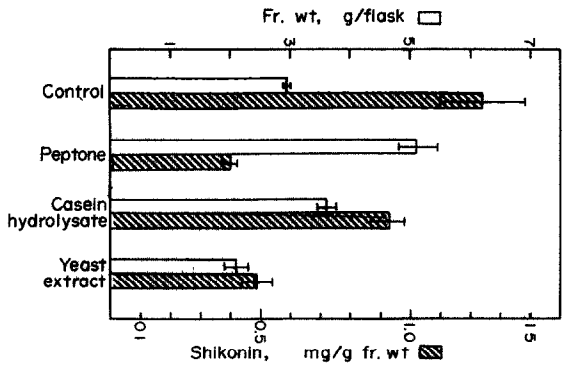

Fig. 2. Effects of various organic-bound nitrogen sources (0.3% concentration) on growth and shikonin derivative formation.

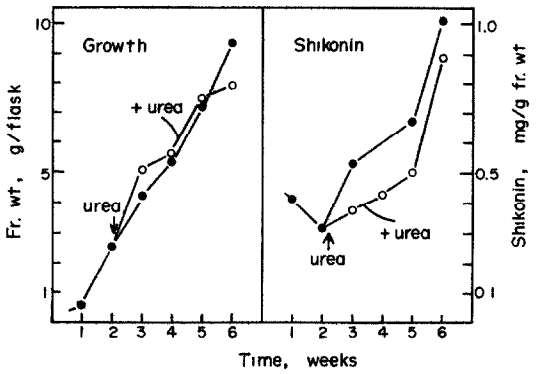

Fig. 3. Effects of urea on growth and shikonin derivative formation. Urea was administrered to the medium at the end of the second week of culture.

The time-course of shikonin derivative formation and synthesis of water-soluble protein in cultured cells is shown in Fig. 4. The initiation of shikonin derivative formation was observed when the content of water-soluble protein decreased. Furthermore, streptomycin sulphate, known to inhibit protein synthesis, promoted shikonin derivative formation in the cultured cells, as shown in Table 3.

These results suggest that biosynthesis of shikonin derivatives proceeds when protein synthesis in cultured

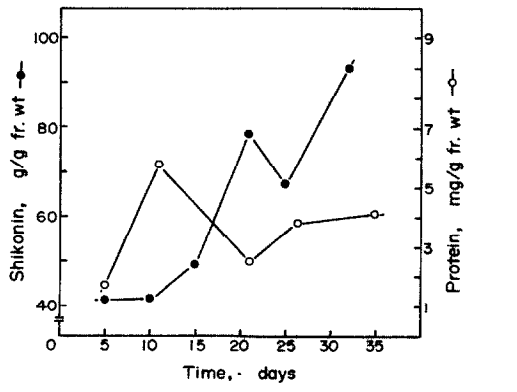

Fig. 4. Time-course of synthesis of water-soluble protein and of shikonin derivative formation.

Table 4. Effects of ascorbic acid on growth and shikonin derivative formation

Conc of ascorbic acid (M)	Fr. wt of callus (g/flask)	Content of shikonin derivatives (mg/g fr. wt)
0	9.1*	0.63*
10^{-6}	8.6	0.44
10^{-5}	8.7	0.76
10^{-4}	10.6	1.08
10^{-3}	10.8	1.05

* Mean of 4 replicates.

cells has ceased; the carbon to nitrogen ratio in cells may be an important regulating factor for shikonin derivative production, as reported for tannin synthesis in *Acer* suspension cultures [7].

Ascorbic acid

Other than those mentioned below, there are few reports on the effects of vitamins on secondary metabolism in cultured plant cells. Matsumoto *et al.* [6] reported that anthocyanin formation in *Populis* suspension cultures exposed to light was promoted by riboflavin, which they suggested might function as a photoreceptor. *Morinda* suspension cultures were reported to have an absolute requirement for *myo*-inositol for both growth and anthraquinone production [4].

Table 4 shows that the apparent stimulatory effect of ascorbic acid on growth and shikonin derivative formation in *Lithospermum* callus cultures. Since ascorbic acid participates in redox systems in cells, the positive effect of ascorbic acid on shikonin derivative formation may be due to the activation of redox systems present in the biosynthetic pathway of shikonin derivatives.

Chemical precursors

Schmidt and Zenk [8] reported that DL-phenylalanine, *trans*-cinnamic acid, *p*-hydroxybenzoic acid and DL-mevalonic acid fed to *Plagiobothrys arizonicus* were incorporated into alkannin, an optical isomer of shikonin, and that alkannin was synthesized by prenylation of *p*-hydroxybenzoic acid with two molecules of mevalonic acid. Recently, Inouye *et al.* [9] demonstrated that shikonin is synthesized in *Lithospermum* cultured callus via *p*-hydroxybenzoic acid and mevalonic acid as is alkannin.

Table 5 shows that L-phenylalanine stimulated shikonin derivative formation markedly when added during the fourth week of culture. However, 4 phenolic acids

Table 5. Effects of L-phenylalanine* on growth and shikonin derivative formation

Conc of L-phenylalanine (M)	Fr. wt of callus (g/flask)	Content of shikonin derivatives (μg/g fr. wt)
0	8.6†	37†
10^{-5}	9.2	58
10^{-4}	10.3	126
10^{-3}	10.8	136

* Added to the culture medium in two equal portions 28 and 33 days after initiation of culture, † Mean of 4 replicates.

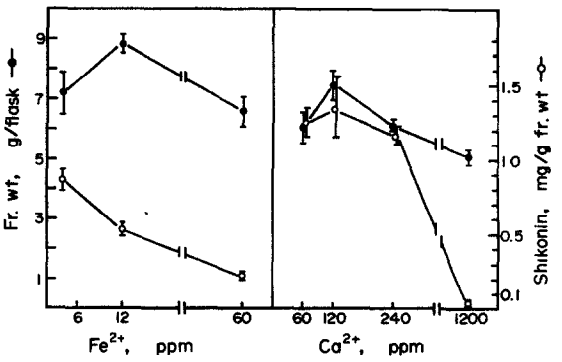

Fig. 5. Effects of Fe^{2+} and Ca^{2+} on growth and shikonin derivative formation.

(*trans*-cinnamic, *p*-coumaric, benzoic and *p*-hydroxybenzoic), all of which are synthesized from phenylalanine, were highly toxic to cell growth and inhibited shikonin derivative formation at concentrations above 10^{-5} M. Furthermore, geraniol (at 10^{-5} M and 10^{-4} M), a conjugate of two molecules of mevalonic acid, had no effect on shikonin derivative formation.

These results suggest that free phenylalanine plays an important role in regulating shikonin derivative formation and may be a key intermediate in the biosynthesis of shikonin derivatives. It may be that when the levels of auxin and N are high, phenylalanine is converted to protein; but after auxin and N are exhausted and protein synthesis has ceased, phenylalanine is converted to shikonin.

Inorganic ions

Itokawa *et al.* [10] reported that dried roots of *L. erythrorhizon* contained high amounts of Fe (338 ppm). It is well known that *L. erythrorhizon* is usually, if not always, found growing on calcareous soil. Therefore, the effects of iron as $FeSO_4$ and calcium as $CaCl_2$ on shikonin derivative formation were tested. Surprisingly, both ions inhibited shikonin derivative formation at high concentrations (Fig. 5). At 12 ppm, Fe stimulated growth but inhibited shikonin derivative formation. According to these results, the high concentration of Fe found in dried roots or of Ca in the environment are unlikely to play a role in shikonin derivative formation.

EXPERIMENTAL

Cultured callus. Lithospermum cultured callus were induced from seedlings of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) in 1971 and subcultured on Linsmaier–Skoog's agar medium [5] at transfer intervals of one month.

Culture medium and culture conditions. Basal medium containing 1% agar, 3% sucrose, 10^{-6} M IAA and 10^{-5} M kinetin was used. The initial pH of the medium was adjusted to 6 with M NaOH soln. Sterilization at 120° and 1 kg/cm² for 20 min. Certain compounds (urea, streptomycin sulphate, ascorbic acid, L-phenylalnine, phenolic acid and geraniol) were sterilized by membrane filter (0.45 μm) apparatus. Small pieces of stock callus were inoculated on 40 ml of media in 100 ml flasks and cultured at 25° in the dark for 6 weeks.

Determination of the content of shikonin derivatives in callus. Fresh callus tissues were homogenized in a mortar containing $CHCl_3$. After standing for at least one day in the dark, the homogenate was filtered. The filtrate was dried with $MgSO_4$, filtered and evaporated to 5 ml *in vacuo*. A 0.01–0.05 ml aliquot

of this test soln was evaporated to dryness. Then, 5 ml of 2.5% KOH soln was added and shaken vigorously for 10 min. The A of the blue soln produced was measured at 622 nm. Content of shikonin derivatives was calculated as mg shikonin.

Estimation of protein content in callus. Fresh callus tissues were homogenized in a mortar containing Tris–HCl buffer (pH 7.1) and the homogenate centrifuged at 3000 rpm for 20 min. An equal vol. of 14% TCA soln was added to an aliquot of the supernatant. The ppt. formed was dissolved in M NaOH soln and assayed for protein content by the method of ref. [11].

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