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Shikonin derivative formation on the stem of cultured shoots in *Lithospermum erythrorhizon*

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Abstract Shoot cultures of *Lithospermum erythrorhizon*, which are capable of producing red pigments, have been established. The red pigments were formed on the stems of *L. erythrorhizon* shoots cultured both on solid and in liquid media without phytohormones at 25 °C in the dark. Thin-layer chromatography, high-performance liquid chromatography and ^{1}H nuclear magnetic resonance analyses revealed that the red pigments which accumulated on the cultured shoots were shikonin derivatives. The effects of various basal media and phytohormones (indole-3-acetic acid, indole-3-butyric acid and kinetin) on the growth and the formation of shikonin derivatives were investigated. When the shoots were cultured on Murashige and Skoog solid medium, the addition of kinetin remarkably enhanced shikonin derivative accumulation in the shoots. However, these effects of kinetin were not observed in the liquid culture when cultured in Gamborg B5 medium. The maximum content of shikonin derivatives (2.3 % as dry weight, ca. 1.5 mg/100 ml flask) was observed in the shoots cultured in phytohormone-free B5 liquid medium for 5 weeks.

Key words *Lithospermum erythrorhizon* · Shoot cultures · Stem · Shikonin derivatives

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Abbreviations *B5:* Gamborg B5 (Gamborg et al. 1968) · *HF:* Phytohormone-free · *HPLC:* High-performance liquid chromatography · *IAA:* Indole-3-acetic acid · *IBA:* Indole-3-butyric acid · *LS:* Linsmaier and Skoog (1965) · *MS:* Murashige and Skoog (1962) · *1/2 MS:* Half strength Murashige and Skoog (1962) · *NMR:* Nuclear magnetic resonance· *RC:* Root culture (Thomas and Davey 1982) · *SD:* Standard deviation · *TLC:* Thin-layer chromatography · *WP:* Woody plant (Lloyd and McCown 1980)

Introduction

Lithospermum erythrorhizon Sieb. et Zucc. (Boraginaceae) contains shikonin derivatives solely in its roots, which are widely used as a dye and as an ingredient of ointments for external injury such as burns and wounds (Kyogoku et al. 1973; Fujita 1988). Production of shikonin by callus and cell suspension cultures (Deno et al. 1987; Fujita et al. 1983) as well as the shikonin biosynthetic pathway (Yazaki 1997) have been studied. Recently, hairy root cultures, which is a useful system for production of shikonin derivatives, have also been reported (Shimomura et al. 1991). In order to study the propagation and the secondary metabolism of *L. erythrorhizon*, we have tried to establish various tissue cultures, such as shoot and adventitious root cultures. For the establishment of adventitious root cultures, the shoots were cultured on Root culture (RC, Thomas and Davey 1982) and Murashige and Skoog (MS, Murashige and Skoog 1962) solid media with an auxin in the dark. The adventitious roots which differentiated from the stem showed a dark red color. The newly developed shoots grew slowly and red pigment formation was also observed in their stems. The newly developed shoots, when subcultured on solid and in liquid MS media without phytohormones, were stable in producing the red pigments. In this paper we describe the production of shikonin derivatives in the cultured shoots, which

were identified by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and ^{1}H nuclear magnetic resonance (NMR) analyses, and the effects of various media and phytohormones on growth and the pigment formation.

Materials and methods

Plant material

The seeds of *L. erythrorhizon* were dipped in 75 % ethanol for 30 s, rinsed with sterilized water, surface-sterilized in 2 % sodium hypochlorite with Tween 20 (1 drop/40 ml) for 10 min and rinsed with sterilized water three times. The axenic shoots germinated from seeds were maintained on phytohormone-free (HF) Woody plant (WP, Lloyd and McCown 1980) solid medium (3 % sucrose, 0.2 % Gelrite, 30 ml medium/4-cm i.d. tube) at 25 °C under 14 h/daylight for 10 years at Tsukuba Medicinal Plant Research Station, NIHS, Japan.

In addition, the shoots (ca. 2 cm in length) were placed horizontally on solid medium of either RC or MS (containing 0.05 mg/l IBA, 3 % sucrose, 25 ml medium/9-cm i.d. petri dish) and cultured at 25 °C in the dark. The shoots were subcultured on HF-MS solid medium at 25 °C in the dark.

Shoot culture

Shoots derived from RC or MS medium supplemented with 0.05 mg/l indole-3-butyric acid (IBA) and subsequently maintained in HF-MS solid medium produced red pigments in the dark. Those shoots were excised (ca. 2 cm in length) and cultured on solid or in liquid MS, 1/2 MS, Gamborg B5 (B5, Gamborg et al. 1968), RC and WP basal media in the dark. In solid medium, two shoots per petri dish (25 ml medium/9-cm i.d. petri dish, four replicate dishes) were cultured at 25 °C for 5 weeks in the dark. In liquid media, one shoot per flask (10 ml medium/100-ml flask, rotated at 60 rpm, five replicate flasks) was cultured at 25 °C for 5 weeks in the dark. To investigate the effects of phytohormones on shoot growth and red pigment formation, the shoots were cultured on MS solid or in B5 liquid medium containing various concentrations (0.05, 0.1, 0.5 mg/l) of indole-3-acetic acide (IAA) (indole-3-acetic acid), IBA or kinetin for 5 weeks in the dark. All media used for the experiment contained 30 g/l sucrose. They were adjusted to pH 5.7 before autoclaving at 121 °C for 15 min. Solid media were solidified with 0.2 % Gelrite.

Analysis of red pigments formed on the stem of cultured shoots

Lyophilized powder of the cultured shoots was extracted with chloroform by infusion. The chloroform extract was filtered through a cotton plug and dried under a N_2 gas stream. The extract was redissolved in an appropriate volume of methanol. TLC was conducted on Kieselgel 60 F 254 (MERCK) in *n*-hexane-ethyl acetate (4 : 1). After the extract was hydrolyzed with 1 *N* KOH, the solution was neutralized with 1 *N* HCl. The hydrolyzate was analyzed by TLC performed under the same conditions as mentioned above. HPLC analysis; column, TOSOH 120A 4.6 × 250 mm (40 °C); solvent system, acetonitrile-distilled water-triethylamine-acetic acid (70 : 30 : 0.3 : 0.3); flow rate, 1.1 ml/min (Fujita et al. 1983); detection, UV spectra measured from 200 to 700 nm using a photodiode array detector (Waters).

The shoots (9.6 g fresh weight) cultured in B5 liquid medium without phytohormones were extracted with 200 ml of chloroform, and then with 10 ml of 1-pentanol. The combined solution was evaporated under reduced pressure at 40 °C. The extract (108.4 mg) of red pigment from the cultured shoots was subjected to silica gel 60 (MERCK) column chromatography (*n*-hexane/chloroform, 2 : 3) to give three fractions. A fraction containing two major compounds was fractionated by preparative TLC on 0.5-mm-thick silica gel 60 F-254 TLC plates (MERCK) with chloroform. Two bands (R_f 0.6 and 0.3) were eluted separately with chloroform/methanol (2 : 1). The eluants were filtered and evaporated to dryness. The yields of the two compounds were 8.7 and 8.0 mg, respectively. In addition, the shoots (10.2 g fresh weight) cultured on the same method as mentioned above were extracted with 200 ml chloroform and the solution was evaporated to give extract (110.0 mg). The extract was hydrolyzed with 1 *N* KOH, and the solution was neutralized with 1 *N* HCl. The converted compound was extracted with chloroform and the solution was evaporated. The extract was fractionated by silica gel column chromatography (*n* -hexane/ethyl acetate, 8 : 1). The fractions containing shikonin, as detected by TLC examination, were pooled and evaporated. The yield of compound was 6.3 mg. These three compounds thus isolated were analyzed by ^{1}H NMR. ^{1}H NMR was measured at 270 MHz locked to the major deuterium resonance of the solvent (chloroform-*d*).

Quantitative analysis of shikonin

Lyophilized powder of the shoots and the roots (0.1–1 g) was sonicated with 1–2 ml chloroform for 10 min. The extracted solution was filtered and adjusted to 5 or 10 ml using a volumetric flask. An aliquot (50–500 µl) was taken and dried under a N_2 gas stream to yield a shikonin derivative extract. The extract was converted into free shikonin by an appropriate volume of 2.5 % KOH and its absorbance was measured at 620 nm using a DU-70 spectrophotometer (Beckman). To extract the red pigment secreted into the liquid medium, 2 ml of the liquid medium was partitioned with 1 ml chloroform twice, and subsequently with 0.3 ml of 1-pentanol. The pooled solution was treated using the same method as mentioned above to determine the concentration of shikonin derivatives in the liquid medium.

Time course

Two shoots (ca. 2 cm in length) were cultured in B5 liquid medium (10 ml medium/100 ml flask) on a rotary shaker (60 rpm) at 25 °C in the dark and harvested periodically (once a week, 1–6 weeks). After lyophilization, the dry weight and the production of shikonin derivatives were determined.

Results and discussion

Red pigment formed on shoot stem

Axenic shoot cultures which have been subcultured on WP solid medium at 25 °C under illumination were horizontally inoculated on MS or RC solid media containing 0.05 mg/l IBA and cultured in the dark. Both red adventitious roots and new shoots developed during 3 weeks of culture. Under dark conditions, red pigment formation was observed on the stem of the newly developed shoot. This phenomenon was not expected because the callus did not produce the red pigment when cultured on MS or LS liquid medium, and it has been believed that the root is the only organ where shikonin accumulates. The shoots which formed red pigments were able to be subcultured on HF-MS and RC solid media in the dark and the new

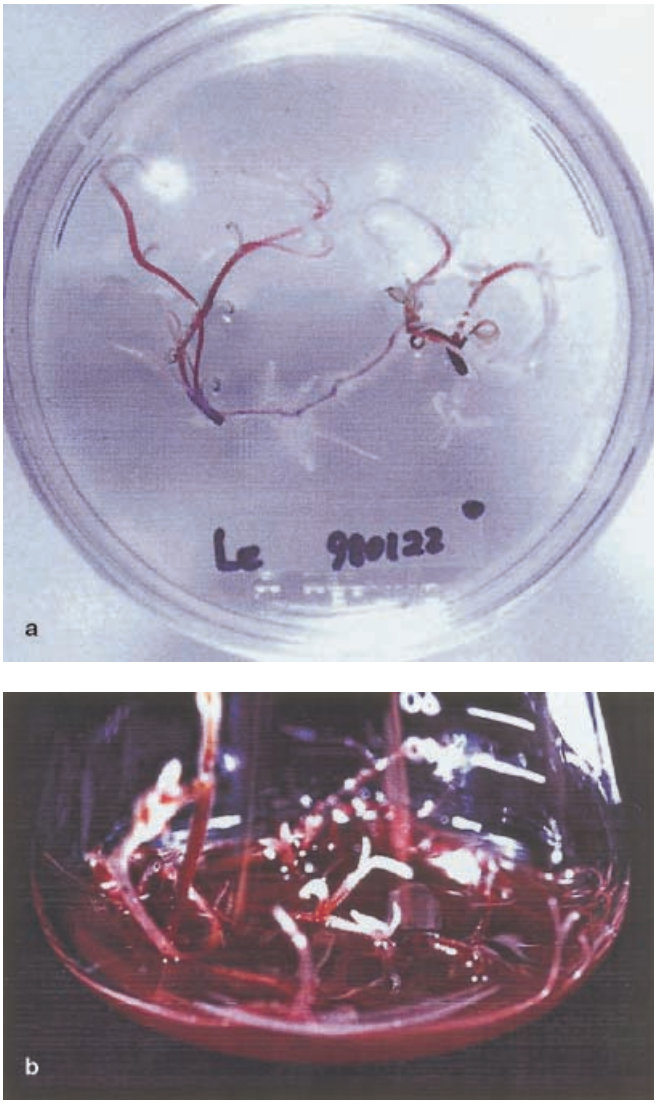


Fig. 1 Red pigment formation on the stem of *Lithospermum erythrorhizon* shoots cultured on MS solid medium (**a**) and in liquid B5 medium (**b**) at 25 °C for 5 weeks in the dark

shoots which were subcultured on the same media produced the red pigment (Fig. 1a). The addition of NH_4^+ to White's medium (1963) generally caused an inhibition of shikonin derivative production in cell suspension cultures of *L. erythrorhizon* (Fujita et al. 1981). However, the shoots cultured on MS solid medium, which contained a high concentration of NH_4^+, produced shikonin derivatives. This result was different from that reported in cell suspension cultures (Yazaki et al. 1987).

To confirm the stable formation of the red pigment on the shoot stems cultured in liquid medium, the shoots were cultured in HF-MS liquid medium (10 ml medium/100-ml flask) on a rotary shaker (60 rpm) in the dark. The shoots cultured in MS liquid medium also formed red pigments and some red pigments were released into the medium.

Analysis of red pigments

Since the shikonin formation by shoot cultures had not previously been reported, it was important to confirm whether the red pigments produced on the shoot stems were shikonin derivatives. Red pigments extracted from shoot cultures were compared to those of intact roots grown in the field. After the chloroform extract was dissolved in methanol, TLC analysis was performed. Extracts of red pigment from shoot stems and roots cultured both on solid and liquid media, from pigments released into liquid medium, and from field-grown roots had three spots at the same R_f values, showing no spot corresponding to the shikonin standard (data not shown). This result suggests that the pattern of shikonin derivatives in the shoot stem is almost the same as that of the field-grown roots. Subsequently, each extract was hydrolyzed with 1 *N* KOH, followed by neutralization with 1 *N* HCl, in which the shikonin derivatives were converted into free shikonin. The treated solution was analyzed by TLC, and each sample gave a single red spot with the same R_f value as that of shikonin. The extracts of red pigment prepared from the cultured shoots and the field-grown roots were further analyzed by an HPLC system equipped with a photodiode array detector. Each sample showed five peaks and the HPLC profile of the shoot cultures was similar to that of the field-grown roots (Fig. 2). Five peaks detected by HPLC showed the same UV spectrum as shikonin (data not shown). In addition, two major compounds isolated from the extract of shoot cultures were identified as acetylshikonin and β-hydroxyisovalerylshikonin by ^1H NMR spectrum analysis (Kyogoku et al. 1973). Furthermore shikonin derivatives extracted from shoot cultures were treated with 1 *N* KOH and 1 *N* HCl solution, and the converted compound was extracted using chloroform and purified using silica gel column chromatography. The ^1H NMR data for the compound showing the same R_f value as shikonin analyzed by TLC, were identical with those of standard shikonin. These results suggest that stems of shoots cultured in the dark have the biosynthetic capability of shikonin derivatives which respond negatively to light, since the shoots started to accumulate the red pigment when cultured in the dark.

Effects of basal medium on shoot growth and shikonin derivative formation

To investigate the shoot culture conditions on growth and shikonin derivative formation, the shoots were cultured on various basal solid (MS, 1/2 MS, B5, RC and WP) or in liquid [10 ml (the same kinds as the solid media)/100-ml flask, rotated at 60 rpm] media at 25 °C for 5 weeks in the dark. The shoots cultured in liquid medium showed better growth and shikonin

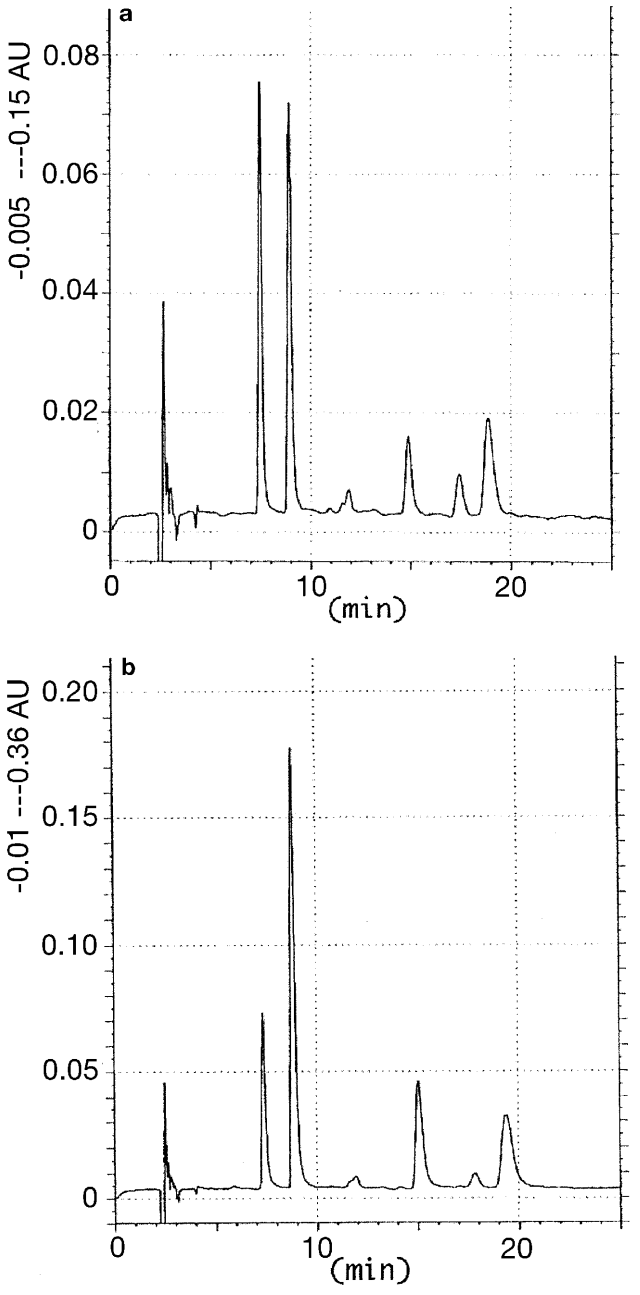


Fig. 2a,b HPLC analysis of shikonin derivatives extracted from *L. erythrorhizon* shoot cultures and field-grown roots. **a** Shoot cultured in MS liquid medium at 25 °C for 5 weeks in the dark. **b** Root of plant cultivated for 3 months in a field

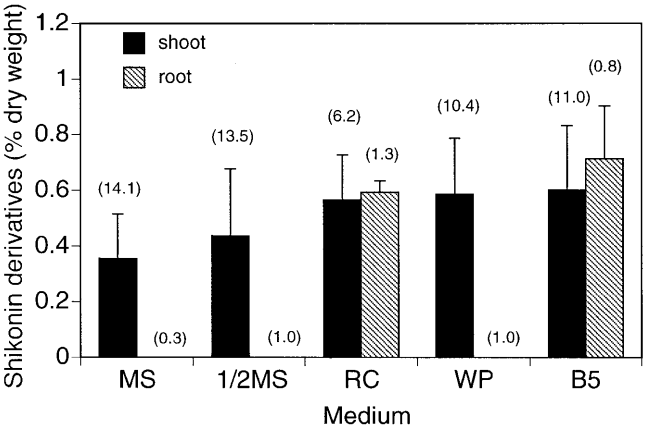


Fig. 3 Effects of various solid media on growth and shikonin derivative formation. Shoots were cultured on solid medium at 25 °C for 5 weeks in the dark. The numbers in parentheses above columns show dry weight (mg). *Error bar* SD ($n = 8$)

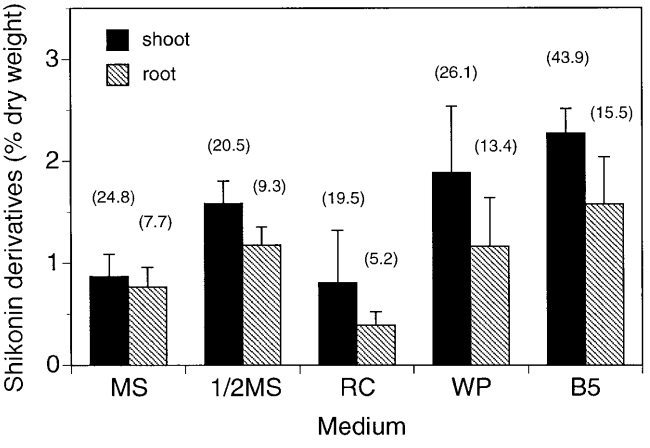
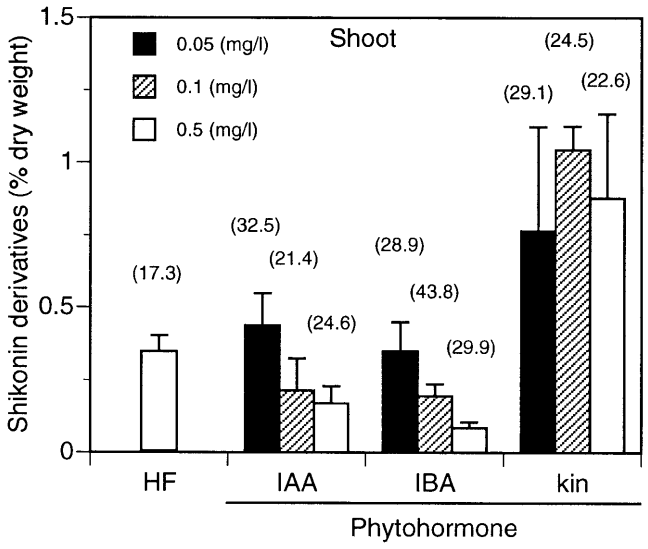


Fig. 4 Effects of various liquid media on growth and shikonin derivative formation. Shoots were cultured in liquid medium (10 ml medium/100-ml flask) on a rotary shaker (60 rpm) at 25 °C for 5 weeks in the dark. *Parenthesis* Dry weight (mg). *Error bar* SD ($n = 5$)

derivative formation than those cultured on solid medium (Figs. 3, 4). The shoots cultured on MS and WP solid and liquid media grew well and proliferated many new shoots, whereas the shoots cultured on solid and in liquid B5 media did not elongate and proliferate well. However, best growth (in terms of dry weight) was observed in B5 liquid medium at 25 °C in the dark, and the shoots cultured under these conditions showed the highest shikonin derivatives content (Fig. 4). In contrast, on B5 solid medium, the cultured shoots did not grow well and their apexes turned brown before the end of the culture period. It is well known that inhibition of shikonin derivative production is caused by NH_4^+ in cell suspension cultures (Fujita et al. 1981). In fact, the hairy roots, transformed with *Agrobacterium rhizogenes* strain 15834, and cultured in RC liquid medium showed best growth and a high production of shikonin derivatives (ca. 1 % as dry weight; Shimomura et al. 1991). Therefore, a high shikonin derivative formation was expected from the shoot culture on RC medium which contained no NH_4^+ as the nitrogen source. However, the shoots cultured on RC medium showed poor growth and a low level of shikonin derivative formation.



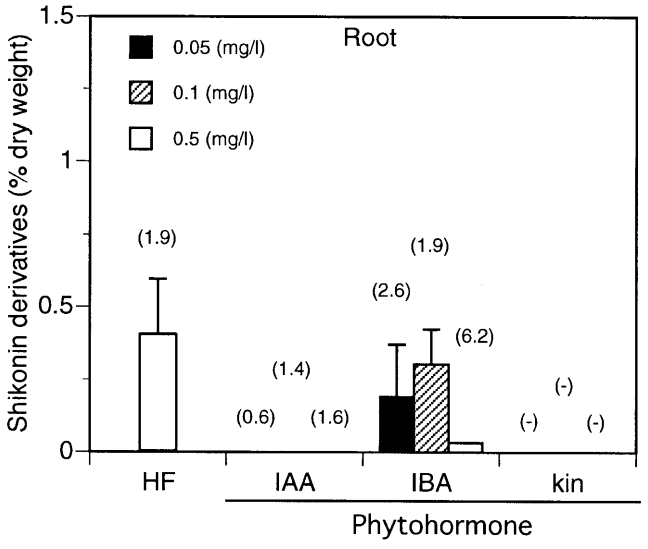
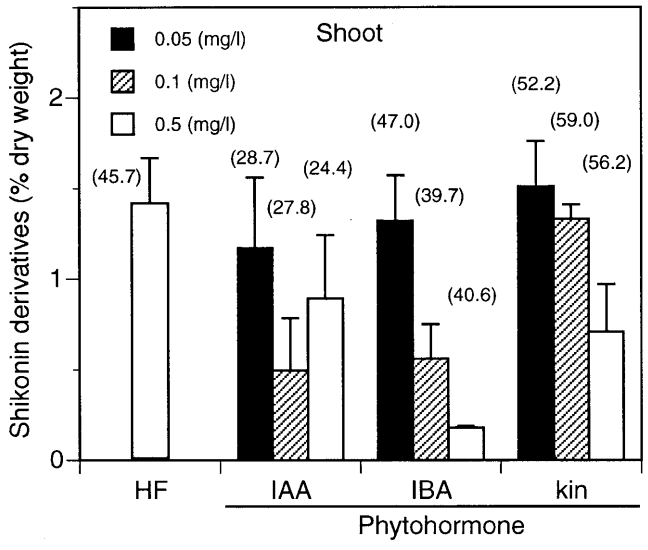


Fig. 5 Effects of phytohormone on growth and shikonin derivative formation in shoot cultures on MS solid medium. Shoots were cultured on solid medium with or without phytohormone at 25 °C for 5 weeks in the dark. *Parenthesis* Dry weight (mg). *Error bar* SD ($n = 6$). (–) Not analyzed (roots were not formed)



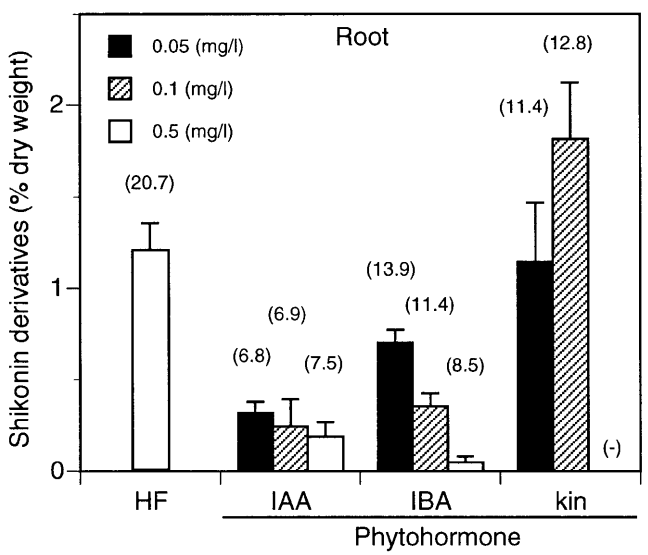


Fig. 6 Effects of phytohormone on growth and shikonin derivative formation in shoot cultures in B5 liquid medium. Shoots were cultured in liquid medium with or without phytohormone on a rotary shaker (60 rpm) at 25 °C for 5 weeks in the dark. *Parenthesis* Dry weight (mg). *Error bar* SD ($n = 5$). (–) Not analyzed (roots were not formed)

Effects of phytohormones on growth and shikonin derivative formation

Since the shoots showed good growth on MS solid medium (Fig. 3), the medium was used for investigating the effects of phytohormones. The shoots were cultured on MS solid medium supplemented with IAA, IBA or kinetin (0.05, 0.1, 0.5 mg/l) for 5 weeks in the dark. Among the phytohormones tested, the shoots proliferated well on the medium containing 0.05 and 0.1 mg/l kinetin (Fig. 5). Although the shoots cultured on MS solid media containing auxin were relatively heavy in dry weight, large quantities of callus tissues were formed on the shoot explants and they did not generate new shoots. The shoots cultured on MS solid medium containing 0.1 mg/l kinetin showed higher shikonin derivative content than those cultured on the medium with auxin or without phytohormone (Fig. 5). A few roots were observed on shoots cultured on MS solid medium containing auxin but no roots were formed on the medium with kinetin (Fig. 5).

The best growth and the best shikonin derivative formation were obtained in B5 liquid medium. Therefore, B5 basal medium was used for studying the effects of phytohormones in liquid culture (Fig. 6). The shoots cultured in B5 liquid medium containing 0.05 and 0.1 mg/l of kinetin showed good growth.

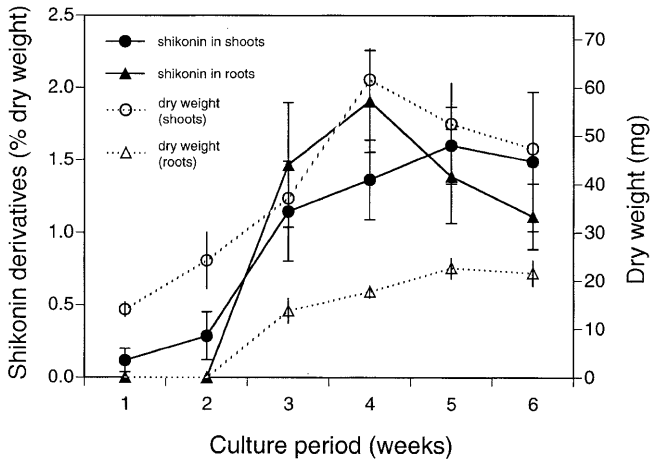


Fig. 7 Time course of growth and shikonin derivative formation in shoot culture. Shoots were cultured in HF-B5 liquid medium (10 ml medium/100-ml flask) on a rotary shaker (60 rpm) at 25 °C for 6 weeks in the dark

However, the levels of shikonin derivatives in these shoots were almost the same as those cultured in the phytohormone-free culture medium (Fig. 6). The marked increase in the content of shikonin derivatives in the shoots which had been cultured on MS solid medium containing 0.1 mg/l kinetin was not observed in B5 liquid medium with the addition of kinetin.

Time course of growth and shikonin derivative formation

Two shoots were cultured in HF-B5 liquid medium on a rotary shaker at 25 °C in the dark. The dry weight of the shoots increased up to 4 weeks of culture, then decreased slightly toward the end of culture (Fig. 7). The best root growth (in terms of dry weight) was obtained at week 5 (Fig. 7).

The content of shikonin derivatives in the shoots and the roots rose rapidly at week 3. The highest content of shikonin derivatives in the shoots (1.5 % as dry weight) was observed at the end (week 6) of the culture period and the best content in the roots was obtained at week 4 (1.9 % as dry weight). The highest amount of total shikonin derivatives (1.38 mg/flask) was obtained at week 5 (Fig. 7).

In this study, the highest shikonin content was obtained in B5 liquid medium without phytohormone. This culture system, in which shikonin derivatives accumulate on the shoot stems, may offer an alternative model system for biosynthetic studies of shikonin derivatives in *L. erythrorhizon*. We are further investigating in detail shoot culture conditions to elucidate the regulation mechanism of shikonin biosynthesis in the shoot, and isolation of gene(s) related to the biosynthesis of shikonin derivatives is also in progress.

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