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PIGMENT FORMATION IN CALLUS CULTURES OF *LITHOSPERMUM ERYTHRORHIZON*

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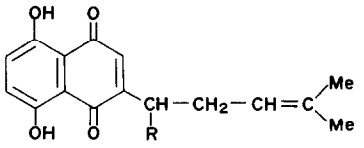
Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; plant tissue culture; 1,4-naphthoquinones; shikonin derivatives; regulation of biosynthesis.

Abstract—Undifferentiated callus tissues of *Lithospermum erythrorhizon* are capable of synthesizing shikonin derivatives, which are normally formed in the cork cells of the roots. Their biosynthesis in cultured cells is controlled by auxin and light. The pigment content increased linearly with time after a lag phase when callus tissues were grown on culture medium containing IAA in the dark, whereas it markedly decreased when 2,4-D was substituted for IAA or when cultures were irradiated with blue light.

INTRODUCTION

RED PIGMENTS in the roots of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) have been used medicinally for wounds, burns or haemorrhoids and also for staining silk. The pigments consist of the 1,4-naphthoquinones: shikonin,[1] acetyl-,[1] β,β-dimethylacryl-,[2] isobutyl-,[2] β-hydroxyisovaleryl-,[3] isovaleryl-,[4] and deoxy-shikonin;[4] and are known to accumulate only in the cork layers of the roots.[5] This localized occurrence of pigments suggests that their syntheses may be induced only under particular conditions associated with the formation of cork cells. However, no studies have so far been made on the factors controlling pigment formation in this plant. In this paper we report for the first time the synthesis of shikonin derivatives in callus cultures and its control by auxin and light.



Shikonin	R = OH
Deoxyshikonin	R = H
Acetylshikonin	R = OCOMe
Isobutylshikonin	R = $OCOCHMe_2$
β,β-Dimethylacrylshikonin	R = $OCOCH{=}CMe_2$
Isovalerylshikonin	R = $OCOCH_2CHMe_2$
β-Hydroxyisovalerylshikonin	R = $OCOCH_2CHMe_2$ (OH)

[1] TAJIMA, T. and KURODA, C. (1922) *Acta Phytochem.* **1**, 43.
[2] MORIMOTO, I., KISHI, T., IKEGAMI, S. and HIRATA, Y. (1965) *Tetrahedron Letters* 4737.
[3] MORIMOTO, I. and HIRATA, Y. (1966) *Tetrahedron Letters* 3677.
[4] KYŌGOKU, K., TERAYAMA, H., TACHI, Y., SUZUKI, T. and KOMATSU, M. (1973) *Japan J. Pharmacog.* **27**, 24.
[5] FUJITA, N. and YOSHIDA, Y. (1937) *Yakugaku Zasshi* **57**, 368.

RESULTS

Induction of pigment synthesis

Callus tissues originally derived from germinating *L. erythrorhizon* seeds cultured on the Linsmaier–Skoog medium[6] containing 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) and 10^{-6} M kinetin in the dark were yellowish white, and failed to produce any red pigments even after successive subculturing. Pigmentation was observed, however, when callus tissues (3-month-old) were transferred to culture medium containing 10^{-6} M 3-indoleacetic acid (IAA) instead of 2,4-D and allowed to grow in the dark. Deep red–purple spots appeared first in the surface of the callus tissues and then inside the tissue in the late culture stage. Cytological studies revealed that the pigments were formed in numerous groups of parenchyma cells distributed almost randomly throughout the unorganized tissue. In these cells, the water-insoluble pigments were mostly located in a great number of unidentified granules or dissolved in oil drops existing in the cytoplasm, but were also partly excreted from the cells to deposit on the outside of cell walls. Nevertheless, only negligible amounts of pigments were released from the cells into culture medium. Some of the cells gave evidence of slight suberization in the staining test with Sudan IV, though they were morphologically indistinguishable from the neighboring, non-suberized parenchyma cells.

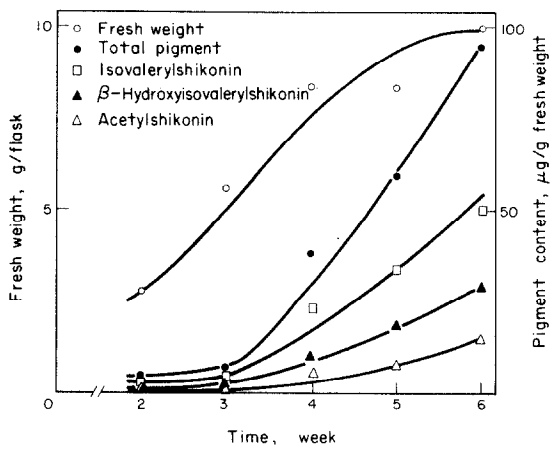


FIG. 1. THE TIME-COURSE OF PIGMENT PRODUCTION IN THE CALLUS CULTURES (11-MONTH-OLD) GROWN ON THE BASAL MEDIUM CONTAINING 10^{-6} M IAA AND 10^{-5} M KINETIN IN THE DARK AT 25°.

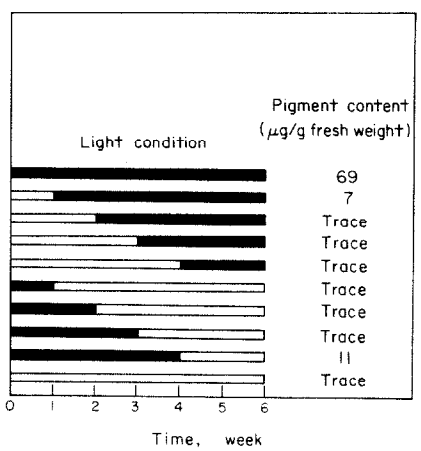


FIG. 2. THE RELATIONSHIP BETWEEN THE TIME OF LIGHT EXPOSURE AND THE PIGMENT PRODUCTION IN THE CALLUS CULTURES (17-MONTH-OLD) GROWN ON THE BASAL MEDIUM CONTAINING 10^{-6} M IAA AND 10^{-5} M KINETIN.
Light condition: continuous illumination (5300 lx) from white fluorescent lamps.

Analyses of the red pigments extracted from callus cultures confirmed the presence of acetyl-, β-hydroxyisovaleryl-, and isovaleryl-shikonin. In addition, a trace red pigment was found to correspond to deoxyshikonin. By contrast, shikonin, β,β-dimethylacryl-, and isobutyl-shikonin, which are present in the root, could not be detected in the callus. These findings indicate that cultured cells can synthesize at least four of the shikonin derivatives contained in the whole plant.

[6] LINSMAIER, E. M. and SKOOG, F. (1965) *Physiol. Plant* **18**, 100.

The time-course of pigment production in callus cultures grown in the dark on the IAA-medium is illustrated in Fig. 1. The lag in pigment synthesis that extends over 3 weeks after initiating culture is followed by a linear increase in pigment content during the last 3 weeks, though the rate of increase varies according to the nature of the compound. Further, callus cultures kept on forming pigments until the 7th week of culture in spite of the cessation of cell growth after the 5th week.

Effect of auxin

Culture experiments were conducted in order to clarify the functional difference between the natural auxin IAA and the synthetic auxin 2,4-D in the hormonal regulation of pigment synthesis in dark-grown cultures (Table 1). The stock callus, which had been maintained on the medium containing IAA (5×10^{-6} M) prior to the experiment, produced 35 μg of pigments per g of the fresh tissue in a 4-week culture when transferred to the same medium, while the substitution of 2,4-D (10^{-6} M) for IAA (5×10^{-6} M) resulted in a decrease of 57% in pigment content. In addition, colourless cultures, which had been maintained on the medium with 2,4-D for 11 months since the induction of callus, became pigmented when IAA was substituted for 2,4-D. This demonstrates clearly that the genetic potential for pigment synthesis is not lost during the long-term culturing in the presence of 2,4-D, and that its expression is dependent on the kind of auxin supplied to the culture medium.

TABLE 1. EFFECTS OF IAA (5×10^{-6} M) AND 2,4-D (10^{-6} M) ON PIGMENT FORMATION IN THE CALLUS CULTURES (11-MONTH-OLD) GROWN ON THE BASAL MEDIUM CONTAINING 10^{-5} M KINETIN IN THE DARK FOR 4 WEEKS AT 25°

Kind of auxin supplied to		Fr. wt of callus	Pigment content (μg/g fr. wt)		
Pre-culture	Test-culture	(g/flask)	3*	6	7
IAA	IAA	4·9	7	22	6
	2,4-D	3·7	3	9	3
2,4-D	IAA	5·1	5	19	7
	2,4-D	4·8	Trace	Trace	Trace

* **3**—acetylshikonin; **6**—isovalerylshikonin; **7**—β-hydroxyisovalerylshikonin.

Table 2 shows the relationship between the auxin concentration in culture medium and the pigment content in the callus harvested 6 weeks after inoculation. Although 2,4-D strongly inhibited the synthesis of pigments even at a low concentration of 5×10^{-8} M, IAA was rather stimulatory at 10^{-6} M. There was no obvious correlation between the amount of growth and the pigment content.

Effect of light

In addition to auxin, light also is an important factor controlling pigment formation in *Lithospermum* cultures. It has been observed repeatedly that the pigment synthesis is repressed by irradiating cultures with white light. For example, callus tissues (12-month-old) grown with IAA (5×10^{-6} M) and kinetin (10^{-5} M) under illumination (12 hr/day) from white fluorescent lamps (5300 lx) produced practically no pigments during 6 weeks of culture, while the yields of pigment synthesis in the control cultures grown in the dark were 10, 71, and 12 μg/g fresh wt for acetyl-, isovaleryl- and β-hydroxyisovaleryl-shikonin, respectively. Furthermore, tests conducted under continuous irradiation with monochromatic light revealed that the marked inhibition of pigment synthesis is caused by

blue light (450 lx) but not by red or green (Table 3). Thus the blue region (380–560 nm, with peaks at 410 and 440 nm) of light plays an important role in the control of pigment formation.

TABLE 2. EFFECTS OF AUXINS ON GROWTH AND PIGMENT FORMATION IN THE CALLUS CULTURES (14-MONTH-OLD) GROWN ON THE BASAL MEDIUM CONTAINING 10^{-5} M KINETIN IN THE DARK FOR 6 WEEKS AT 25°

Concn (M)	IAA Fr. wt*	IAA Pigment content†	2,4-D Fr. wt	2,4-D Pigment content
0	8·5	53	—	—
5×10^{-8}	10·2	32	11·3	Trace
10^{-7}	9·6	42	15·7	4
10^{-6}	10·2	78	11·6	Trace
10^{-5}	12·6	52	6·8	Trace
10^{-4}	17·1	26	1·1	Trace

* g/flask.

† μg/g Fresh wt (the total of acetylshikonin, isovalerylshikonin and β-hydroxyisovalerylshikonin).

Figure 2 shows the total pigment content of cultures exposed to white light for various lengths of time at different stages of culture. Unexpectedly, an irradiation during only the 1st week after inoculation led to a decrease of 90% in the final pigment content compared to the dark-grown control; inhibition was almost complete when cultures were exposed to light for more than 2 weeks prior to the dark period. Thus the effect of light becomes clearly visible even when the cultures have been irradiated only at the early culture stage preceding pigment synthesis. Despite its detrimental effect on pigment formation, light had little influence on the growth of callus.

TABLE 3. EFFECTS OF MONOCHROMIC LIGHTS ON PIGMENT FORMATION IN THE CALLUS CULTURES (15-MONTH-OLD) GROWN ON THE BASAL MEDIUM CONTAINING 5×10^{-6} M IAA AND 10^{-5} M KINETIN FOR 6 WEEKS AT 25°

Mono-chromic light*	Wave length (nm)	Fr. wt (g/flask)	Pigment content (μg/g fr. wt) 3†	6	7
Red	610–710	5·9	5	25	7
Green	490–590	4·9	9	26	8
Blue	380–560	8·0	Trace	Trace	Trace

* The cultures were continuously illuminated throughout the culture period.

† **3**—acetylshikonin; **6**—isovalerylshikonin; **7**—β-hydroxyisovalerylshikonin.

DISCUSSION

Though shikonin derivatives normally accumulate only in the cork cells of the roots in the plant, no differentiation of such special cells occurred in the callus, where the pigments were formed in the parenchyma cells. The formation of morphologically distinct cork cells is apparently not essential for the induction of pigment synthesis. Presumably, the occurrence of suberization observed in some of the pigmented cells cultured on the IAA-medium is not necessarily connected with pigment synthesis, since suberized cells are found also in the non-pigmented tissues grown on the 2,4-D-medium. In the roots,

however, the synthesis of suberin might be associated more intimately with that of shikonin derivatives, so that the pigments produced would have a high affinity for and would eventually be adsorbed on the suberized cell walls. This view seems to be consistent with our anatomical observation[7] on the roots of *Lithospermum officinale* seedlings, in which shikonin derivatives are first produced in the epidermal cells of the root tips and later accumulate in the cork cells after the epidermis has been stripped off from the thickening root.

The inhibition of pigment synthesis by 2,4-D in the present material is comparable to that of nicotine,[8] polyphenol[9] or anthocyanin[10] synthesis by 2,4-D or NAA in various plant cell cultures. Striking differences between 2,4-D and IAA in the control of secondary metabolite formation in cultured cells have been described for the syntheses of nicotine in *Nicotiana tabacum*,[11] coumestrol in *Phaseolus aureus*,[12] and digitolutein in *Digitalis lanata*,[13] all of which are markedly stimulated by substitution of IAA for 2,4-D. The reason for this phenomenon, however, has not been clarified.

The inhibitory effect of light on pigment formation in *Lithospermum* cultures is interesting in connection with the fact that the pigments are confined to the underground part of the plant. Actually, results of preliminary experiments have shown that the syntheses of shikonin derivatives in both the young roots and the excised root segments are highly inhibited by exposing the tissues to light (12 hr/day) from white fluorescent lamps. Thus, this is in sharp contrast to the stimulatory effects of light on the syntheses of anthocyanins,[9,14] carotenoids,[15] and polyphenols[9] in various tissue cultures. Although a characteristic alteration of the pattern of chemical composition between light- and dark-grown callus has been reported for sesquiterpene lactones in *Andrographis paniculata*[16] and the essential oil in *Ruta graveolens* cultures,[17] no significant change in the qualitative pattern of constituents was found in *Lithospermum* cultures, since all pigment syntheses ceased in the light. It is significant that blue light specifically inhibits the synthesis of pigments in the present material, whereas those of carotenoids[18] and betacyanin[19] have been reported to be blue-light dependent. The stimulatory effect of blue light on anthocyanin formation in *Populus* suspension cultures was also markedly superior to other kinds of monochromic (green and red) light.[20] These differences in response to light seems to suggest the existence of a unique control mechanism for synthesizing shikonin derivatives.

The biochemical nature of the light-induced inhibition is not yet clear, but the

[7] MIZUKAMI, H., TABATA, M. and KONOSHIMA, M. unpublished work.
[8] TABATA, M., YAMAMOTO, H., HIRAOKA, N., MARUMOTO, Y. and KONOSHIMA, M. (1971) *Phytochemistry* **10**, 723.
[9] DAVIES, M. E. (1972) *Planta* **104**, 50.
[10] GAMBORG, O. L., CONSTABEL, F., LARUE, T. A. G., MILLER, R. A. and STECK, W. (1971) *Les Cultures des Tissus de Plantes*, pp. 335–343, Le Centre National de la Recherche Scientifique, Paris.
[11] FURUYA, T., KOJIMA, H. and SYONO, K. (1971) *Phytochemistry* **10**, 1529.
[12] BERLIN, J. and BARTZ, W. (1971) *Planta* **98**, 300.
[13] FURUYA, T. (1970) *Farumashia* **6**, 676.
[14] ALFERMANN, W. and REINHARD, E. (1971) *Experientia* **27**, 353.
[15] GODNEV, T. N., AKULOVICH, N. K., ORLOVSKAYA, K. I. and DOMASH, V. I. (1966) *Dokl. Akad. Nauk SSSR* **169**, 692.
[16] BUTCHER, D. N. and CONNOLLY, J. D. (1971) *J. Exp. Botany* **22**, 314.
[17] CORDUAN, G. and REINHARD, E. (1972) *Phytochemistry* **11**, 917.
[18] BRANDT, A. B. (1958) *Biofizika* **3**, 659.
[19] KOEHLER, K. H. (1972) *Phytochemistry* **11**, 133.
[20] MATSUMOTO, T., NISHIDA, K. and NOGUCHI, M. (1973) *Agric. Biol. Chem.* **37**, 561.

possibility that the pigments may be photochemically decomposed as soon as they are formed in the cells exposed to light is unlikely, since the biosynthesis of these compounds having maximal absorbance at 520 nm is not inhibited by green light (490–590 nm with a peak at 520–530 nm). Moreover, this assumption contradicts the fact that pigment synthesis in the dark period is inhibited by a pre-treatment of the culture with light. Thus it is more reasonable to assume that light would act to repress either the induction of a common precursor or the conversion of an intermediate involved in the synthetic pathways.

EXPERIMENTAL

Plant material and method of culture. The callus tissues used were originally derived from germinating seeds of *Lithospermum erythrorhizon* Sieb. et Zucc. cultured in test tubes on Linsmaier and Skoog's basal agar medium[6] containing 10^{-6} M 2,4-D and 10^{-6} M kinetin. These tissues were transferred to the same basal medium containing either 10^{-6} M 2,4-D or 10^{-6} M IAA in addition to 10^{-5} M kinetin every 4 weeks. All the stock cultures were maintained at 25° in the dark. Small pieces of the stock callus were inoculated on various media in 100-ml flasks. Fluorescent lamps were used for irradiating cultures with white (5300 lx), red (620 lx), green (340 lx) or blue (450 lx) light.

Extraction and identification of shikonin derivatives. Fresh callus tissues harvested after a 6-week culture on the culture medium containing 10^{-6} M IAA and 10^{-5} M kinetin in the dark were homogenized in a mixer with $CHCl_3$. The filtrate of the homogenate was washed with water, and the $CHCl_3$ layer containing red pigments was dried, filtered, and evaporated *in vacuo*. The pigments were separated by both column chromatography and preparative TLC (silica gel G; solvent: $CHCl_3$). Two of the four red pigments isolated were identified as acetylshikonin and β-hydroxyisovalerylshikonin by TLC and IR spectral comparison with authentic samples. The third pigment corresponded to deoxyshikonin in its chromatographic behavior on TLC, but its quantity was too small to be subjected to further analysis. The fourth component was thought to be a mixture of β,β-dimethylacryl-, isobutyl- and isovaleryl-shikonin since these are inseparable by TLC, according to Kyōgoku *et al.*,[4] who worked on the material from intact roots of *L. erythrorhizon*. However, GLC analysis[4] of the fatty acid fraction that afforded on 2% KOH hydrolysis of the pigment from the callus tissues revealed the presence of only isovaleric acid. Thus the fourth component contained isovalerylshikonin, but lacked both β,β-dimethylacrylshikonin and isobutylshikonin.

Quantitative estimation of shikonin derivatives. Shikonin derivatives in the $CHCl_3$ extract of callus cultures prepared by the above procedure were separated by TLC, scraped off from the plate individually, and then extracted with $CHCl_3$. The quantity of each compound was determined spectrophotometrically by measuring the absorption at 520 nm. The content of pigment was expressed as μg per g of fresh callus. As for the pigment corresponding to isovalerylshikonin, its content was estimated as that of β,β-dimethylacrylshikonin, since the former has never been isolated in the pure state.

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