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VARIATION IN PIGMENT PRODUCTION IN *LITHOSPERMUM ERYTHRORHIZON* CALLUS CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; plant tissue culture; variation; synthesis of shikonin derivatives.

Abstract—Different strains of callus cultures of *Lithospermum erythrorhizon* showed wide variations in the production of shikonin derivatives. From these cultures, two high pigment-producing strains, whose content of shikonin derivatives are stable and similar to that of intact plant root, have been established by repeated selection.

INTRODUCTION

Secondary metabolite production by plant tissue and cell cultures, except for those producing certain anthraquinones [1, 2], ginseng saponins [3] and ubiquinone has generally been disappointingly low compared with whole plants (for review see [5]). In addition, the biosynthetic potential of cultured cells has been observed to change during subculturing. Since the biochemical capabilities of cultured cells have been shown to vary widely among strains or cell clones, a possible remedy for these problems might be the selection of stable and high-producing strains by plating or cloning techniques.

Previously, we reported that the production of shikonin derivatives in callus cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) was regulated by environmental factors such as light, plant growth regulators [6] and nutritional factors [7]. Shikonin derivatives have been reported to have antimicrobial [8], anti-inflammatory [9] and anti-tumor [10] activities and thus to be potentially important compounds for medicinal use. Furthermore, since shikonin derivatives are red pigments, it is easy to select callus tissues containing high amount of shikonin derivatives with the naked eye. The present study was undertaken to examine the possibilities of establishing culture strains showing a stable and high capacity for the production of shikonin derivatives.

RESULTS

Variation in pigment content

Callus tissues were induced separately from 45 tissue segments prepared from the seedlings of *L. erythrorhizon*. Among 10 subcultures obtained from each original callus, tissues showing either a higher growth rate or different pigmentation (red, yellow or white) were selected and used as inocula for the next generation. A total of 12 strains showing different properties were obtained by consecutive selection at the 4th, 5th and 6th subculture generations.

Callus tissues of these selected strains were subcultured for 9 months before they were characterized quantitatively for pigment content. The results (Table 1) show that strains M1233 and M1332 produced comparatively high amount of shikonin derivatives, whereas strains M123, M231, M292 and M386 produced only trace amounts. Furthermore, strains from M0, M1, M2 and M3 contained less acetylshikonin than did other pigment-producing strains. Thus, there are striking differences in the content of shikonin derivatives in the various strains.

Stability of pigment production in culture strains

In order to examine the stability of the production of shikonin derivatives, 8 culture strains were assayed three times during 6-month period. No selection was made for these strains during this time.

Except for strains M123, M231 and M386, which produced virtually no shikonin derivatives, the content of these compounds in other strains varied markedly with generation time without showing any definite tendency. On the other hand, the relative proportions of different shikonin derivatives remained relatively constant on successive subcultures.

Isolation of high pigment-producing strains

During the experiments mentioned above, a section of red callus was found in the low-producing strain, M231, after 19 months. From this section, strain M231a was established by four selections at 1-month intervals. The content of shikonin derivatives, initially *ca* 300 μg/g fr. wt, was by successive selection increased to 500–600 μg/g fr. wt which corresponds to the content of shikonin derivatives of intact roots of *L. erythrorhizon*. As shown in Table 2, callus tissues of the most productive strain of M231a showed a pigment composition similar to that found in the roots of intact plants, but contained hardly any shikonin itself. Furthermore, the biosynthetic potential for pigment production of strain M18, whose content of total shikonin derivatives was *ca* 50 μg/g fr. wt at primary selection, was increased by repeated selection to attain a pigment content as high as 1000 μg/g fr. wt (Fig. 1). Interestingly, this strain lost its ability to produce acetylshikonin for 20 months

Table 1. Variation of the content of shikonin derivatives in various culture strains (13 months old)

Original culture	Derived culture strains	Fr. wt of callus* (g/flask)	Content of shikonin derivatives (μg/g fr wt)*			
			DMAS†	AS‡	HIVS§	Total
M0	M14	8.9	5	Trace	4	9
	M18	13.8	33	Trace	24	57
M1	M123	7.8	Trace	Trace	Trace	—
M2	M231	14.0	Trace	Trace	Trace	—
	M292	11.2	Trace	Trace	Trace	—
M3	M386	10.4	Trace	Trace	Trace	—
M12	M1233	6.4	51	18	14	83
M13	M1323	6.6	15	3	4	22
	M1332	7.0	52	10	11	73
M23	M2312	7.8	28	11	5	44
M24	M2421	8.9	32	5	7	44
M33	M3315	11.8	9	4	2	15

* Mean of 4 replicates.
† Mixture of isobutyl-, isovaleryl-, α-methyl-*n*-butyl- and β,β-dimethylacryl-shikonin and calculated as β,β-dimethylacrylshikonin.
‡ Acetylshikonin.
§ β-Hydroxyisovalerylshikonin.

Table 2. Comparison of the contents of shikonin derivatives between callus tissues (M231a)* and mature roots

Compound	Pigment content (% of dry wt)	
	Callus	Root
Deoxyshikonin	0.02†	0.02†
Shikonin	Trace	0.15
Acetylshikonin	0.12	0.26
β,β-Dimethylacrylshikonin‡	0.34	0.46
β-Hydroxyisovalerylshikonin	0.09	0.27
Total	0.57	1.16

* 10 months after isolation from the original strain M231.
† Calculated from yields of crude crystals.
‡ Mixture of isobutyl-, isovaleryl-, α-methyl-*n*-butyl- and β,β-dimethylacryl-shikonin and calculated as β,β-dimethylacrylshikonin.

after establishing the strain but regained this ability later.

Earlier [6], we reported that pigment production in *L. erythrorhizon* callus cultures was inhibited by either the application of 2,4-dichlorophenoxyacetic acid (2,4-D) or light. The effects of light and auxins on the pigment production in a high pigment-producing strain M18 were therefore tested. Callus tissues of M18 grown in the presence of IAA (10^{-6} M) and kinetin (10^{-5} M) under illumination by white fluorescent lamps (5000 lx) produced only 18 ± 2 μg/g fr. wt. With 2,4-D (10^{-6} M) and kinetin (10^{-5} M), pigment formation in strain M18 was only 7% of this figure (*ca* 1.2 μg/g). These results suggest that the regulatory mechanism of the biosynthesis of shikonin derivatives in high pigment-producing strains is not different from that in low pigment-producing strains.

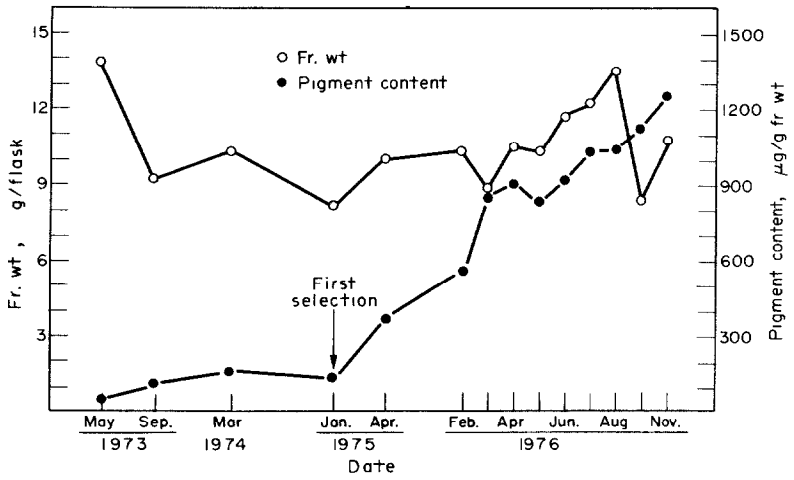

Fig. 1 The content of shikonin derivatives of culture strain M18 during subculturing.

DISCUSSION

Clonal variations in the content of several other metabolites have been reported. Blakely and Steward [11] reported that strains isolated from cell cultures of *Haplopappus gracilis* showed differences in amino acid content. Venketeswaren [12] isolated cell clones showing higher chlorophyll and carotenoid contents than the original suspension culture of tobacco. Davey *et al.* [13] reported that three cell clones of *Atropa belladonna*, isolated by a cell plating method, differed from one another in various properties such as growth rate, nutrient requirement and chlorophyll content. Secondary metabolite production may also vary as shown by Alfermann and Reinhard [14] who isolated both anthocyanin-producing and anthocyanin-free strains from callus cultures of *Daucus carota*. Sugano *et al.* [15] reported that of the strains derived from a red carrot, one produced primarily β-carotene and the other lycopene. Alfermann *et al.* [16] isolated six cell lines from carrot callus cultures of which only three could produce anthocyanins in the dark. Tabata and Hiraoka [17] succeeded in isolating a culture strain containing high amount of nicotine from single-cell clones on *Nicotiana rustica*. From cultured carrot cells treated with a mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, four variant cell lines whose pigmentation properties differed from stock callus tissues were isolated by Nishi *et al.* [18]. Gathercole and Street [19] reported that a *p*-fluorophenylalanine-resistant cell line isolated from the suspension culture of *Acer pseudoplatanus* had a higher content of phenols and a higher PAL activity. However, it has not been clarified whether or not the variations reported arise from genetic mutations.

The content of shikonin derivatives in callus cultures varies from almost 0 to 1000 μg/g fr. wt of tissues. This suggest that *Lithospermum* callus tissues are composed of heterogeneous cells varying in their ability to produce shikonin derivatives.

The results in this paper also suggest that selection of cultured cells is very effective in improving the production potentials of cultured cells, even though it is unknown whether variation of pigment production in these strains is due to a gene mutation or even extrachromosomal variation as seen in plastids of higher plants or plasmids of bacteria. Since culture strains of *Lithospermum* callus tissues are not of single-cell origin, many heterogeneous cells are likely to be contained in any single culture strain. Thus, it may be possible to isolate single-cell clones having higher pigment synthesizing capabilities by the cell plating method.

EXPERIMENTAL

Plant material and culture method. Callus tissues derived from seedlings of *Lithospermum erythrorhizon* Sieb. et Zucc. Boraginaceae) were subcultured on Linsmaier–Skoog s medium [20] containing 1% agar, 3% sucrose, 10^{-6} M IAA and 10^{-5} M kinetin. For culture experiments, small pieces of stock callus were inoculated on the same medium (40 ml) in 100 ml flasks and cultured in the dark at 25° for 6 weeks.

Estimation of the content of shikonin derivatives of callus tissues. Extraction and quantitative assay of shikonin derivatives were carried out according to refs [6, 7].

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