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EFFECT OF GIBBERELLIN A_3 ON SHIKONIN PRODUCTION IN *LITHOSPERMUM* CALLUS CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; plant cell cultures; gibberellin; shikonin; naphthoquinone pigment.

Abstract—Gibberellin A_3 was found to strongly inhibit shikonin formation in *Lithospermum erythrorhizon* callus cultures, at a concentration as low as 10^{-7} M, without affecting cell growth. The amount of endogenous GA-like substances in a shikonin-producing strain was shown to be much smaller than that of a variant strain which was incapable of producing shikonin.

INTRODUCTION

The formation of red naphthoquinone pigments (shikonin derivatives) in *Lithospermum erythrorhizon* cell cultures is stimulated by cytokinin [1], cupric ion [2] and an acidic polysaccharide [3], while it is inhibited by auxin [1], ammonium ion [4] and blue light [5].

This paper reports on the inhibitory effect of gibberellin A_3 (GA_3) on shikonin formation in *Lithospermum* callus cultures.

RESULTS AND DISCUSSION

Effect of GA_3 on shikonin formation

Figure 1 shows the effects of GA_3 administered to the culture medium on cell growth and shikonin formation in *Lithospermum* callus cultures (strain M-18). Cell growth was little affected by GA_3 at any of the concentrations (10^{-12}M–10^{-4}M) tested. On the other hand, shikonin formation was partly inhibited by GA_3 even at a concentration as low as 10^{-11} M. Furthermore, the shikonin content decreased roughly in proportion to the concentration of GA_3 from 10^{-11} M to 10^{-7} M; the ID_{50} of GA_3 was estimated to be *ca* 10^{-10} M. Although shikonin production was inhibited by up to 85% of the control at 10^{-7} M, it was never completely inhibited even at 10^{-4} M. Seitz *et al.* [6, 7] have reported a similar inhibitory effect of GA_3 on anthocyanin formation in *Daucus carota* callus cultures. It is well known that secondary metabolism in cultured plant cells is often influenced by auxins or cytokinins, but there are few reports concerning the effect of GA on secondary metabolite formation.

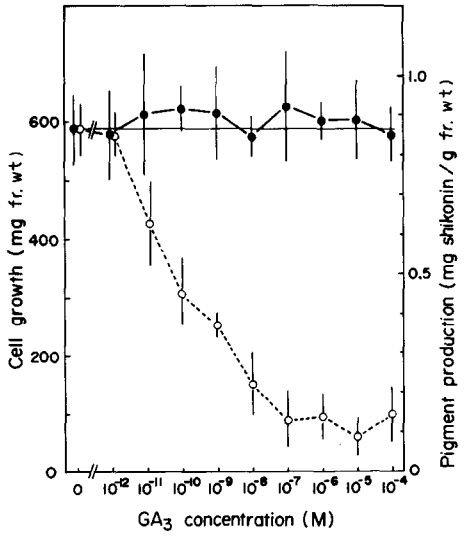

Fig. 1. Effects of GA_3 on cell growth (●——●) and shikonin production (○– – –○) in *L. erythrorhizon* callus cultures (strain M-18). Each value represents the mean ± s.e. of 10 replicates.

Quantitative estimation of endogenous GA

As the experimental results mentioned above suggested that the capability of culture strains to produce shikonin derivatives might be influenced by the endogenous level of GA, the GA activity of the methanol extract from the calli of the shikonin-producing strain M-18 was compared with that from a non-pigment producing variant strain B-17 by a bioassay using rice seedlings [8].

The activities of fractions IV and V of the extracts from 2-week-old cultures, which were presumably due to GA_3, were significantly higher in strain B-17 than in strain M-18 (Fig. 2). The total content of GA_3 in fractions IV and V from strain B-17 was estimated by the equation given in the Experimental to be *ca* 2 ng/g fr. wt of cells. The same amount of GA_3, if administered exogenously to the culture medium, would be expected to cause a 50% inhibition of shikonin production in strain M-18.

In contrast to strain B-17, fractions IV and V of strain M-18 showed no GA activity but slightly inhibited the elongation of the leaf sheath in comparison with the control. These results seem to suggest that GA could be one of the important endogenous regulators in the

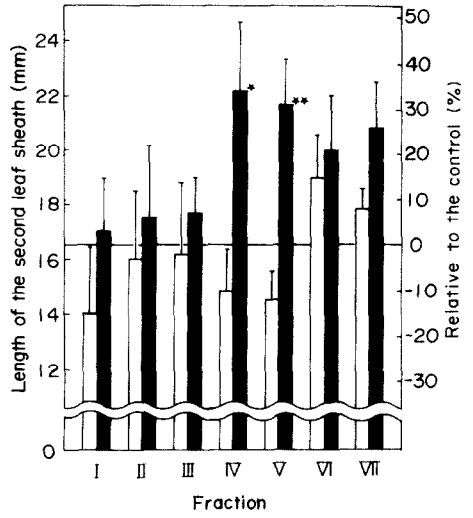

Fig. 2. Effects of fractions I–VII of the callus extracts from the pigment-producing strain M-18 (□) and the apigmented strain B-17 (■) on the elongation of the second leaf sheaths of the seedlings of a dwarf rice variety Tan-ginbozu. The length of the second leaf sheath of the control plants was 16.5 ± 1.8 mm. Each value is the mean ± s.e. of seven seedlings. * and **: Significantly different from the corresponding fraction of strain M-18 at 5% and 1%, respectively.

biosynthesis of shikonin derivatives in *Lithospermum* cells.

EXPERIMENTAL

Plant material and culture method. The callus cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. were originally derived from germinating seeds in 1971 [1]. A red, shikonin-producing strain M-18 and a white, variant strain B-17 incapable of producing shikonin, which were isolated from the original callus culture by selection, have been maintained on Linsmaier–Skoog's agar medium [9] containing 10^{-6} M IAA and 10^{-5} M kinetin for 10 years at 25° in the dark.

In testing the effect of gibberellic acid (GA_3), the aq. soln (0.5 ml) of GA_3 was added aseptically through a porous membrane filter (Sartorius, mesh size: 0.45 μm) to the warm, unsolidified agar medium (9.5 ml) in test tubes (18 × 180 mm). Callus tissues (100 mg fr. wt) were inoculated onto the solid agar medium and cultured for 4 weeks under the same conditions as described above before measurements.

Quantitative analysis of shikonin. The extraction and quantitative analysis of shikonin derivatives in the cells were carried out according to the method described earlier [1].

Bioassy of gibberellin activity. Fresh cells (50 g) which had been cultured on the above-mentioned medium (40 ml) in 100 ml Erlenmeyer flasks for 2 weeks in the dark at 25° were extracted (× 2) with MeOH at 4° for 1 week. The extracts were combined and concd *in vacuo* to give an aq. concentrate, which was adjusted to pH 3 with 1 M HCl and then extracted (× 3) with EtOAc. The EtOAc extract (*ca* 100 mg) was subjected to prep. PC (Whatmann 3 MM, 46 × 57 cm, EtOAc–EtOH–ligroin–H_2O, 1:1:1:1, upper phase). The paper chromatogram was divided equally into seven sections (fractions I–VII, in order of increasing R_f values). It was ascertained that, under the same chromatographic conditions, a standard sample of GA_3 (Tokyo Chemical Industry) gave an R_f (0.65) corresponding to fraction V when visualized by spraying with an aq. soln of $KMnO_4$. Each fraction was eluted with 2 × 50 ml EtOH at 4°. The eluate was concd *in vacuo* and dissolved in 50 μl Me_2CO–H_2O (1:1). An aliquot (2 μl) of the soln was applied to each of seven seedlings (2 days after germination) of a dwarf variety (Tan-ginbozu) of rice according to the method of ref. [8]. The GA activity was expressed in terms of the percent increase in the length of second leaf sheath treated with the extract over that of the control seedlings which were treated only with the solvent (Me_2CO–H_2O) during the 3 day experimental period. The quantity of GA_3 was calculated by the following equation: $\log 2Y = 0.0247X - 0.507$, where Y is the quantity (ng) of GA_3 per 1 g fr. wt of the cells and X is the percent increase in the length of the second leaf sheath. This equation proved to be applicable when X values were between 20 and 90.

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