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Studies on the Production of Anthraquinone Derivatives by Tissue Culture of *Rubia* Species

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Abstract□The tissue culture of *Rubia cordifolia* var. *pratensis* and *R. akane* were performed to enhance the biosynthesis of anthraquinone pigments under various conditions. The production of alizarin and purpurin in the callus was separately analysed and was quantitatively compared. The pigment biosynthesis was more active in the callus from *R. cordifolia* var. *pratensis* than from *R. akane*. The addition of α-ketoglutaric acid, a biosynthetic precursor of anthraquinones, enhanced the production of alizarin and purpurin remarkably.

Keywords□*Rubia cordifolia* var. *pratensis, Rubia akane,* tissue culture, alizarin, purpurin, natural dyestuffs, phytohormone

The plants of Rubia species have been important resources of dyestuffs in Europe (*Rubia tinctorium* L.)[1] and Asia (*R. akane Nakai*).[2] The roots of the plants have been used as emmenagogue, hemostatics and antipyretics.[3] Recently, an antitumor activity was found from a non-pigment fraction of *R. cordifolia* L.[4]

The main components of *Rubia* spp. have been reported to be alizarin, ruberythric acid, purpurin, xanthopurpurin, munjistin and pseudopurpurin.[5] Several other new anthraquinone glycosides have also been identified recently.[6,7] Anthraqunones full of color variety in the plants are very attractive as the pigment source bur their small quantities in the plant have limited their practical uses. Suzuki *et al.* reported the formation of anthraquinones in the cultured cells of *R. cordifolia* var. Mungista Miq.(=*R. akane*), the effects of physical conditions and the medium on the production of anthraquinones and the proliferation of callus.[8-10] However, they assayed only the total amount of anthraquinones, but each compound was not seperately analysed. Moreover, the production of pigment in the callus was not enough for practical use.

The author has carried out the tissue culture of *R. cordifolia* var. *pratensis* and *R. akane* in order to improve the production of each anthraquinone pigment under various conditions, and assayed separately the anthraquinone pigments.

The idea was rooted in the fact that the anthraquinones localize in the newly multiplied cells of the callus and then it was assumed that the higher activity of cell division could lead to the higher production of the pigments. Therefore, the higher productive cell-line was expected from *R. cordifolia* var. *pratensis* which has more leaves on the node than other *Rubia* species.[11]

The effects of the biosynthetic precursors on anthraquinones were also experimented in this study. It has been known that the addition of precursors could enhance the production of secondary metabolites in the callus.[12,13] The productions of alizarin and purpurin in the callus from *R. cordifolia* var. *pratensis* were remarkably enhanced by the addition of α-ketoglutaric acid to the medium.

EXPERIMENTAL METHODS

Materials and induction of callus

The calluses were derived from the leaves, nodes, rhizomes and roots of *Rubia akane* L. and *R. cordifolia* var. *pratensis* Max. which were collected from May to August, 1988. The young leaves were cut into 5 × 5mm after thorough cleaning, strilized in 70% ethanol for 10 sec and then four times repeatedly in 0.5% sodium hypochlorite for 10 sec, and rinsed five times with sterile water. The young parts of nodes were cut into 2 mm thick, the scales were removed, and sterilized in the same way as the leaves. The sterilized samples were transfered to the media in clean bench. The calluses were induced after 3 weeks of incubation at 25 °C in the dark. The small pieces of calluses were transferred

to the same media again and then subcultured three times at three-week intervals.

Cultivation of calluses

The modified Murashige and Skoog medium[14] was supplemented with 3g of malt extract for the basal medium. Various quantities of auxins and kinetin were then added to the medium and the pH was adjusted to 5.6-6.0.[15] Each 10 m*l* of the medium were alloted in the 25 × 15 mm test tubes after addition of agar and then autoclaved for 15 min at 121 °C. To compare the callus proliferation and pigment biosynthesis of *Rubia akane* and *R. cordifolia* var. *pratensis*, 5 ppm of kinetin was added to the medium which was supplemented with 2 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) or α-naphthaleneacetic acid (NAA). The comparision was also made at three pH ranges (5.65, 5.80, 5.95), and α-ketoglutaric acid (10 mg/*l*, 100 mg/*l*) or shikimic acid (500 mg/*l*) was added separately before the incubation in the dark for 3-4 weeks at 25 °C.

Analysis of pigments

The quantitative analysis of anthraquinones produced in the callus was done by spectrophotometry and HPLC. The absorbances of total yellow pigments, purpurin and alizarin at 433, 516 and 572 nm were measured by a UV-visible spectrophotometer, Gilford system 6000, and their amounts were calculated by reading on the calibration curves of the standard compounds. The production of alizarin in the callus was assayed also by using HPLC under the following conditions; Spherisorb S 50 DC column, UV-detector (280 nm), $CH_3CN/H_2O/HOAc$ (60:39.9:0.1) as mobile phase.

RESULTS AND DISCUSSION

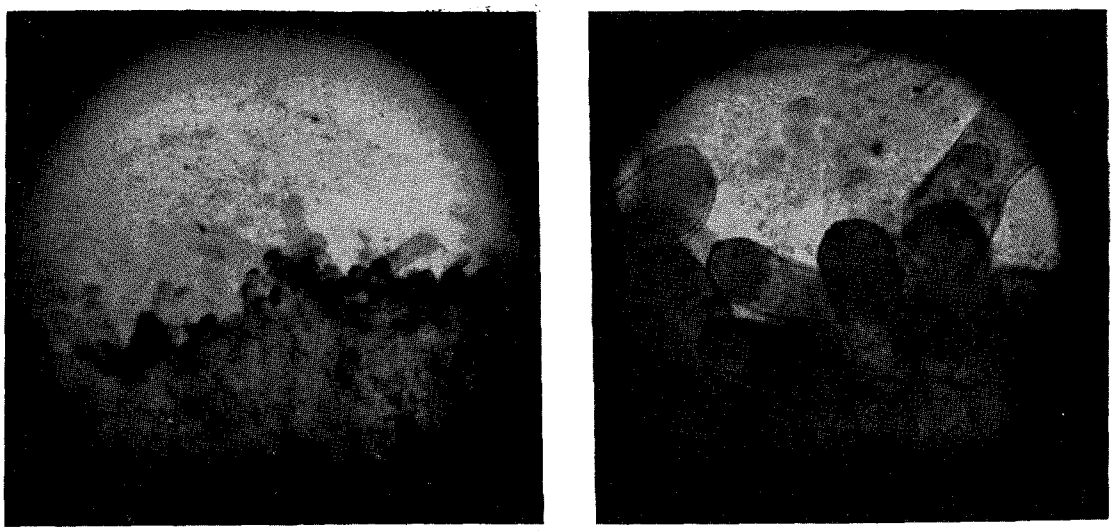
Localization of pigments in the callus

Only two of the fourty samples from the rhizomes showed the callus formation, while the calluses were extensively induced from the leaves. The color of callus mass was initially yellow and gradually changed to reddish brown. As shown in Fig. 1, the pigment biosynthesis was distinct in the newly multiplied cells (Fig. 1, A) mainly in the nucleus area (Fig. 1, B). It suggests that the formation of anthraquinones in the cells of *Rubia* species might be closely related to the activity of cell division and to the increase in the callus weight.

Separate determination of alizarin and purpurin produced in the callus

As shown in Fig. 1, the absorbance peaks of alizarin (433 nm) and purpurin (516 nm) were partially overlapped when measured in ethanol solution. On the other hand, the absorption spectra of purpurin ethanol solution (516 nm) and alizarin NaOH solution (572 nm) were negligibly overlapped. On the basis of the fact, alizarin and purpurin could be separately analysed by spectrophotometry. The method was compared with HPLC to confirm the reliability in the same samples.

The HPLC peak of alizarin in ethanol was



A B

Fig. 1. Microscopic examination of the callus induced from *R. cordifolia* var. *pratensis*. A, 100x; B, 600x.

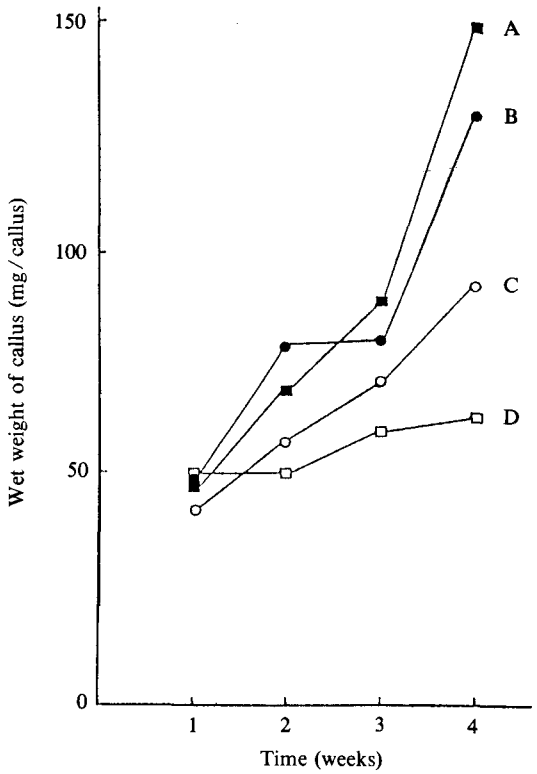


Fig. 2. Effects of 2,4-dichlorophenoxyacetic acid (2,4-D) α-naphthaleneacetic acid (NAA) on growth of callus induced from *R. akane.* and *R. cordifolia* var. *pratensis.*

A,*R. akene* (NAA); B,*R. cordifolia* var. *pratensis* (NAA); C,*R. akane* (2,4-D); D,*R.cordifolia* var. *pratensis* (2,4-D).

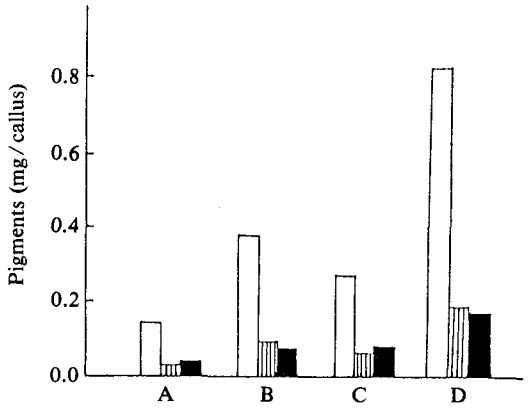


Fig. 3. Production of pigments in the callus after incubation four weeks.

A,*R. akane* (2,4-D); B,*R. cordifolia* var. *pratensis* (2,4-D); C,*R. akane* (NAA); D, *R. cordifolia* var. *pratensis* (NAA). □, total yellow pigments; ▥, alizarin; ■, purpurin.

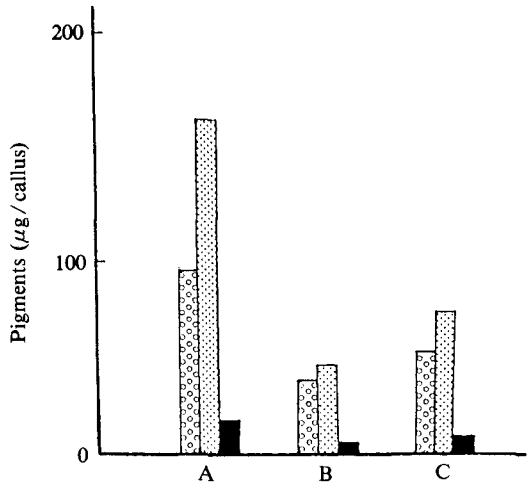


Fig. 4. Effects of culturing temperature on production of pigments in calluses.

A, total yellow pigments B, purpurin; C, alizarin. ▩, 20 °C; ▨, 25 °C; ■, 30 °C.

shown at the retention time of 6.092 min. The results from the spectrophotometry were 4.6% higher than those from HPLC measurement in the same sample. However, the spectophotometry was more convenient than HPLC as the process of hydrolysis and extraction were required for the measurement of HPLC.

The proliferation and pigment production of callus under various culturing conditions

The time course of callus formation is shown in Fig. 2. The increase in the wet weight of calluses was significant after three weeks of incubation. The supplementation of the medium with α-naphthaleneacetic acid (NAA) showed the faster callus proliferation than that with 2,4-dichlorphenoxyacetic acid (2,4-D).

The pigment synthesis was more active in the callus from *R. cordifolia* var. *pratensis* than from *R. akane* in the case of the supplementation of NAA. Fig. 3 shows that the yellow pigments, as well as purpurin and alizarin were much higher in the callus from *R. cordifolia* var. *pratensis.* These results were obtained in the pH range of 5.65 to 5.95 (data not shown). Suzuki *et al.* also found that the supplement of NAA increased the formation of anthraquinones in the callus of *R. cordifolia* L.[9]

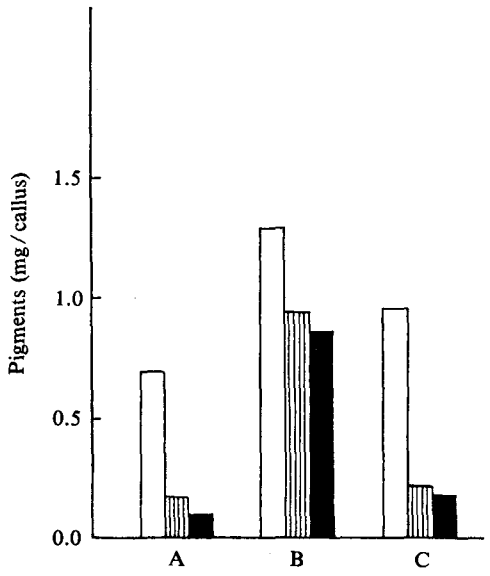


Fig. 5. Effects of precursors on production of pigments in the callus from *R. cordifolia* var. *pratensis* after incubation four weeks.

A, on standard medium; B, on medium containing α-ketoglutaric acid (100 mg/*l*); C, on medium containing shikimic acid (500 mg/*l*). □, contents of total yellow pigments; ▥, contents of alizarin; ■, contents of purpurin.

The culture temperature is an another important factor in tissue culture.[16] The highest amounts of total yellow pigments, purpurin and alizarin were produced at 25 °C and the formation of the pigments was significantly inhibited at 30 °C as shown in Fig. 4.

The effect of supplementation with α-ketoglutaric acid or shikimic acid, known as the precursor of anthraquinone biosynthesis, was investigated as shown in Fig. 5.

Especially, the production of anthraquinones was greatly increased by the addition of α-ketoglutaric acid, suggesting that α-ketoglutaric acid highly involved in the biosynthesis of anthraquinones. The amount of alizarin and purpurin produced in the callus from *R. cordifolia* var. *pratensis* was here 0.94 and 0.86 mg per one test tube. This was more than three times as much as the pigments in the callus cultured without supplementation of α-ketoglutaric acid.

ACKNOWLEDGEMENT

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