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SELECTION OF *BUPLEURUM FALCATUM* CALLUS LINE PRODUCING ANTHOCYANINS IN DARKNESS

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ABSTRACT.—A callus line (BfDR) of *Bupleurum falcatum* producing anthocyanins in the dark was obtained through repeated selection. The callus accumulated more anthocyanin pigments than the original red callus line (BfLR) maintained in circadian light/dark cycles. The average anthocyanin contents in BfDR and BfLR callus lines were 0.10% and 0.05%, respectively, on the basis of fresh weight of cells.

The production of anthocyanins by plant tissue culture has been studied by various workers with many kinds of plant species, mainly because their formation is easily detected with the naked eye (1-3). Most experiments were done under continuous light or in diurnal light/dark cycles and only a few in the dark (4-6). There have also been a few reports on the intended selection of cell lines that produce anthocyanins in continuous darkness (7-9). Tissue cultures producing an apparently light-dependent secondary metabolite such as an anthocyanin could be used as materials for the study on regulatory role of light in metabolic pathways. From a practical point of view, especially in a large-scale production of metabolites by plant cell culture, dark culture conditions will be more desirable to light-irradiated ones because of the simplicity of a culture apparatus and low operating cost, providing that there is no, or little, difference in metabolite productivity between the two culture conditions.

As previously reported (10), *Bupleurum falcatum* L. (Umbelliferae) callus produced anthocyanin pigments under light irradiation. Two pigments were isolated from the callus and identified as glucosides of malvidin. No anthocyanin pigments, however, were formed in the same callus line subcultured in continuous darkness without intentional selection for the pigment productivity. This paper describes the selection of a high anthocyanin-producing callus line of *B. falcatum* grown in continuous darkness. The results were compared with those from the original callus, which had been maintained in cycling light.

RESULTS

As the surface of the original stock callus, BfLR, was homogeneously red, further selection for higher anthocyanin content by means of the naked eye was impossible. Accordingly, intentional selection of more densely colored portions of callus at every subculture was not attempted for the BfLR callus culture which was kept in cycling light. The anthocyanin content fluctuated between a little less than 0.02% and 0.09% during the experiment period of five years, averaging 0.048%.

We attempted the selection of a callus line which is able to accumulate anthocyanin pigments in continuous dark culture conditions. A part of the red stock callus (BfLR) that had been maintained in the light was transferred on to fresh agar medium and subcultured periodically in the dark. At passage one, the red callus tissues were decolored gradually and became white. During the following two passages, they remained white. At passage four, however, the pigment production appeared in discrete, small groups of cells. At the next passage, the cultures were divided into two lines, i.e., one (line BfDR) was selected for the pigment content at every passage, the other (line BfD) remained nonselected. Figure 1 shows changes in the anthocyanin contents of both lines at the following passages. The nonselected line BfD was white or faint pink, and its anthocyanin content was always less than 0.02% during the experiment period of three

years, after which it was abandoned. The anthocyanin content of the selected line BfDR increased sharply 14 months after the artificial selection was started. It fluctuated between 0.05% and 0.14% after March 1982. The average anthocyanin content (0.102%) of BfDR in 1984 was significantly ($p<0.01$) higher than that of BfLR (0.052%).

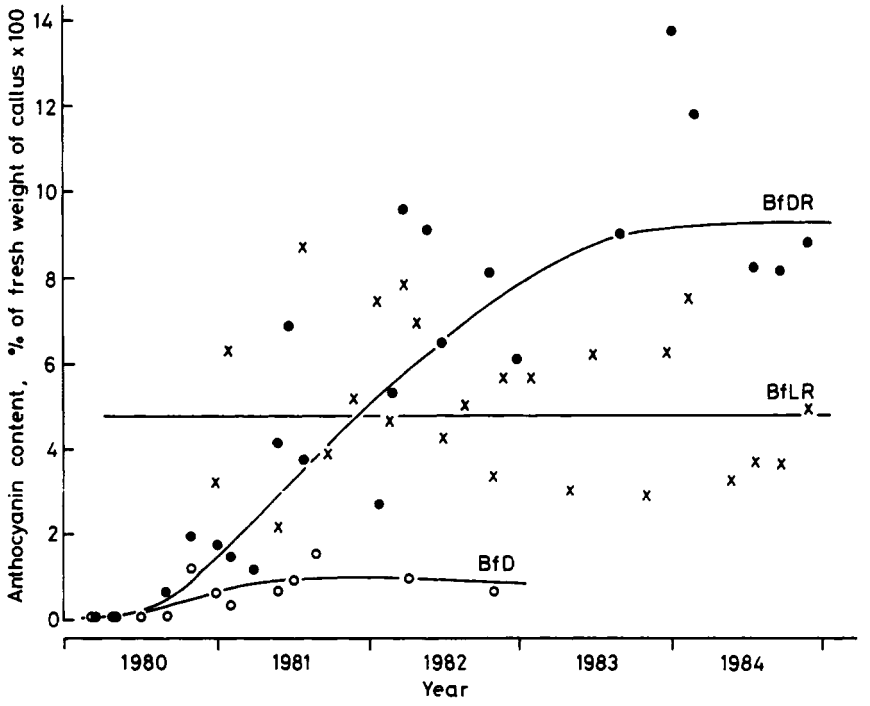

FIGURE 1. Change in anthocyanin content of three lines of *Bupleurum falcatum* callus cultures. Callus tissues of BfD and BfDR were grown in continuous darkness and those of BfLR in cycling light.

Although the incubation of the dark-grown callus cultures was performed in complete darkness, they were exposed to light irradiation from lamps in a laminar flow cabinet and in the room for a short period during transfer to fresh culture media. In order to evaluate the effect of this irradiation on the anthocyanin production of BfDR callus, they were transferred in the dark except for light from the flame of a gas burner used for sterilizing transfer loops. This operation was carried out for three consecutive passages. The callus cultures at each passage produced as much anthocyanin as those transferred normally (data not shown). Therefore, the special precautions against light were not heeded during the routine transfer operation thereafter.

Protoplasts obtained from both BfLR and BfDR callus were graded according to the intensity of pigmentation. The data of experiments No. 1 and No. 2 in Table 1 show that each percentage of white, pink, red, and deep red cells in BfDR callus was higher than the corresponding one in BfLR, and that the reverse is the case for light pink cells. The anthocyanin content of line BfDR is about twice as high as that of BfLR.

Figure 2 shows the time course of growth and anthocyanin accumulation in both lines of BfLR and BfDR. The BfLR calli were grown in cycling light, and the BfDR in continuous darkness. Growth of both lines continued until week 8. Although anthocyanin contents of both lines fluctuated during the culture period, clear peaks of total anthocyanin were observed at week 7 and 8 for BfLR and BfDR, respectively. The latter was more than twice as high as the former.

TABLE 1. Frequency Distribution of Red-color Intensity of the Protoplasts Obtained from Two-Month-Old Callus Tissues Grown in Circadian Light/Dark Cycles (Line BfLR) or in Continuous Darkness (Line BfDR)

Exp. No.[a]	Callus line	Intensity of pigmentation					Anthocyanin content (%×100)
		white	light pink	pink	red	deep red	
1	BfLR	35.1%	45.4	14.2	4.8	0.5	4.13±1.00[b]
	BfDR	37.7	37.5	16.8	6.8	1.2	8.82±0.84
2	BfLR	45.7	45.4	7.0	1.8	0	4.05±1.04
	BfDR	52.5	28.1	11.9	6.5	1.0	8.29±2.23

[a]The experiment was repeated twice at the consecutive passages (September and November 1984).
[b]Each figure is the mean ± standard deviation from five replicates.

DISCUSSION

Generally anthocyanin synthesis is greatly enhanced on exposure to light not only in intact plants (11) but also in cultured plant cells (1). In some cases, however, a certain amount of anthocyanins was found in nonirradiated materials, e.g., seedlings of red cabbage (12, 13), sunflower cotyledons (14), internodes of sorghum (15), seedlings of *Vigna radiata* (16), cultured cells of carrot (4, 5, 7), *Happlopappus gracilis* callus tissues (17), and grape cell suspension cultures (9). Although intentional selection of cell lines capable of producing anthocyanins in the dark was recently performed with grape suspension culture by Yamakawa *et al.* (9), the details of the selection process were not shown. Figure 1 clearly shows that artificial selection of deep-colored cell clumps of callus tissues at each subculture is effective in establishing such a cell line of dark-grown *B. falcatum* callus cultures. This finding and the above-mentioned examples demonstrate that anthocyanins are synthesized in plant cells without light irradiation to some extent.

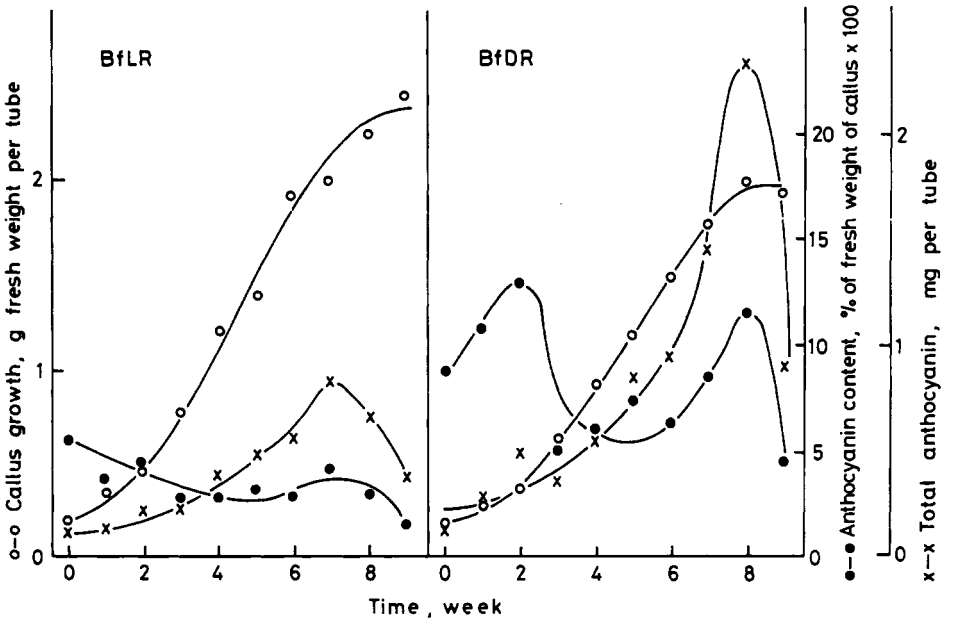

FIGURE 2. Time course of growth and anthocyanin accumulation in BfLR and BfDR callus lines of *Bupleurum falcatum* grown under light and dark conditions, respectively. Each point represents the mean of five replicates.

Higher anthocyanin contents of BfDR than BfLR seem to reflect the higher total percentage of pink, red, and deep red cells (Table 1). Unexpectedly, the percentage of white cells in macerated cells of BfDR is similar to (Exp. 1) or even higher than (Exp. 2) that of BfLR, although BfDR has a higher anthocyanin content than BfLR. In spite of the homogeneously red appearance of BfLR and BfDR callus tisssues, unpigmented cells occupied approximately a third to a half of total cells comprising each callus tissue. The relatively higher percentage of white and light pink cells in those two selected callus lines suggests the possibility that callus lines with much higher anthocyanin contents might be established through the selection by cell cloning and quantitative evaluation of pigments in each clone.

EXPERIMENTAL

TISSUE CULTURE.—The callus tissues (line BfLR) used here were established from the leaf of *B. falcatum* in 1971 by Tomita and his co-workers (10) and have been maintained in cycling light for more than 10 years. These were friable and homogeneously deep red in appearance as a result of repeated selection of highly pigmented portions of callus tissues at every passage. The BfLR callus was grown at $25\pm3°$ under 16 h/day of light from two cool-white fluorescent tubes (40 W) maintained at a distance of 20 cm from the cultures. The BfDR and BfD cultures were incubated in continuous darkness at $25\pm1°$. Subcultures were performed at intervals of 2 months. Linsmaier-Skoog (18) medium supplemented with 10^{-6} M 2,4-dichlorophenoxyacetic acid and 10^{-6} M kinetin was used in the present study. For the experiments, a small piece of callus (ca. 0.1 g fresh weight) was inoculated in each test tube (18×180 mm) containing 10 ml of agar medium.

SELECTION METHOD.—The callus line, BfDR, was raised from the callus line, BfLR, as follows. In 1981, red segments of the BfLR callus were transferred on to fresh agar medium. As the callus cultures recovered the capability to produce anthocyanins after a few passages in the dark conditions, more densely pigmented parts of callus tissues were selected at every subculture thereafter. A callus line nonselected for pigment formation and maintained in continuous darkness was coded as BfD.

GRADING CELLS ACCORDING TO THE INTENSITY OF PIGMENTATION.—Callus tissues (0.5 g) grown for 2 months were suspended in 10 ml of enzyme solution, pH 5.5, containing 1 g sorbitol, 1 mg $CaCl_2\cdot2H_2O$, 1 mg $NaH_2PO_4\cdot H_2O$, 100 mg Cellulase Onozuka R-10, 50 mg Driselase, and 50 mg Macerozyme R-10 in a 50-ml Erlenmeyer flask and kept for 10 min under reduced pressure (40 mm Hg). The mixture was incubated at 37° for 2 h and shaken at 60 reciprocations per min with a 78 mm stroke. The suspensions thus obtained were counted at a magnification of ×100 on a cell counting slide with a depth of 1.0 mm. Ten random fields were observed per preparation. Five slides were prepared from each of five callus tissues. All the protoplasts in 250 fields in total were observed for each callus line and graded according to the intensity of pigmentation: white, light pink, pink, red, and deep red. The experiment was repeated twice.

QUANTITATIVE ANALYSIS OF ANTHOCYANINS.—Callus tissues were homogenized with 1% HCl in MeOH and extracted for 1 h at room temperature. After filtration, the absorbance of clear solution obtained by centrifugation at $1100\times g$ for 10 min was measured at 535 nm. Anthocyanin content was calculated as the percentage of malvidin monoglucoside based on fresh weight of cells according to the molecular extinction coefficient (log $\epsilon=4.47$) for malvidin 3-monoglucoside (19).

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