Studies on the Production of *Digitalis* Cardenolides by Plant Tissue Culture

II. EFFECT OF LIGHT AND PLANT GROWTH SUBSTANCES ON DIGITOXIN FORMATION BY UNDIFFERENTIATED CELLS AND SHOOT-FORMING CULTURES OF *DIGITALIS PURPUREA* L. GROWN IN LIQUID MEDIA

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ABSTRACT

Undifferentiated, highly chlorophyllous cell cultures; undifferentiated white cell cultures; green, shoot-forming cultures; and white, shoot-forming cultures of *Digitalis purpurea* L. were established and subcultured every 3 weeks in liquid media in the light or in the dark. The digitoxin content, the chlorophyll content, and the ribulose bisphosphate carboxylase activity of these cultures were assayed. The light-grown, green, shoot-forming cultures accumulated considerable amounts of digitoxin (about 20 to 40 micrograms per gram dry weight), and the white, shoot-forming cultures without chloroplasts accumulated about one-third that amount of digitoxin. The chlorophyll content and the ribulose bisphosphate carboxylase activity of the undifferentiated green cells were about the same as they were in the green, shoot-forming cultures, but the digitoxin content of the former was extremely low (about 0.05 to 0.2 microgram per gram dry weight), which is about the same amount as that in undifferentiated white cells without chloroplasts. Thus, it was concluded that the chloroplasts are not essential for the synthesis of digitoxin in *Digitalis* cells. The optimum concentrations of the tested compounds for the accumulation of digitoxin were: benzyladenine, 0.01 to 1 milligram per liter; indoleacetic acid, 0.1 to 1 milligram per liter; α-naphthaleneacetic acid, 0.1 milligram per liter; and 2,4-dichlorophenoxyacetic acid, 0.01 milligram per liter.

*MATERIALS AND METHODS*

Undifferentiated Cell Cultures. Callus was induced from seedlings of *Digitalis purpurea* L. on the basal medium, supplemented with 3 mg/L IAA and 0.8% (w/v) agar. After 30 d, the callus was transferred into the liquid basal medium, supplemented with 1 mg/L IAA, and cultured in the light. Thus, undifferentiated green cell cultures were established and subcultured every 3 weeks. Undifferentiated white cell cultures were obtained by sub-culturing the green cells in the dark.

Shoot-Forming Cultures. The shoot-forming calli of *D. purpurea* L., which had been established in the previous study (5), were transferred into the liquid basal medium, supplemented with 1 mg/L BA and 1 mg/L IAA, and cultured in the light. Thus, green, shoot-forming cultures without root were established and subcultured every 3 weeks. White, shoot-forming cultures were obtained by sub-culturing a portion of the green, shoot-forming cultures in the dark.

Culture Condition. Murashige and Skoog medium (9)—with 1 mg/L thiamin-HCl and without agar, edamin, IAA, and kinetin—was used as the basal medium. Approximately 1.5 g fresh weight of cells were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of liquid medium and cultured in continuous light (fluorescent lamp: about 4 × 10^4 erg/s/cm²) or in the dark at 28°C on a reciprocal shaker (100 strokes/min; 2.0 cm in length).

Measurement of Growth. Fresh weight was measured after removing culture medium by suction filtration. The harvested fresh culture was lyophilized, and its dry weight was determined.

Assay for Digitoxin. Lyophilized cells (0.1—2 g) were homogenized with 50 ml ethanol in a glass homogenizer. The homogenate was heated at 74°C for 4 h and filtered. The filtrate was dried *in vacuo*, and the residue was taken up in 2 ml ethanol and diluted with 18 ml H₂O. When necessary, the extract was further diluted with H₂O to a desirable level. Determination of digitoxin concentration of the extract was done by radioimmunoassays, as described (5).

Assay for Chl. Chl content was determined spectrophotometrically in an 80% (v/v) acetone extract. Chl was extracted by the method of Sunderland (12), and its concentration was calculated using the equation derived by Arnon (1).

Assay for RuBPCase Activity. Five g of cooled fresh cells were homogenized in 10 ml of ice-cold buffer solution containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM MgCl₂, 100 mM NaCl, and 80 mM 2-mercaptoethanol for 1 min in a Waring blendor.

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*Digitalis* cardenolides, especially digitoxin and digoxin, are important in medicine, and cardenolide production of cultured cells of *Digitalis* species has been investigated. Most workers have reported that undifferentiated cultured cells either did not produce cardenolides (2, 4, 6) or contained only trace amounts of cardenolides (8, 11). However, organ-redifferentiating cultures have been reported to accumulate considerable amounts of cardenolides (3, 6, 8). In an earlier report (5), we showed that even the first passage calli of six *Digitalis* species, including root-forming calli, lacked the ability to accumulate cardenolides, but shoot-forming calli accumulated considerable amounts of cardenolides.

In the present study, we have established four liquid-cultured cell lines of *Digitalis purpurea* L., i.e., undifferentiated green cells; undifferentiated white cells; green, shoot-forming cultures; and white, shoot-forming cultures. These cell lines were used to study the effect of light on the expression of cardenolide-production. Furthermore, we have investigated the effect of several plant growth substances on growth and digitoxin formation of the green, shoot-forming cultures, which accumulated the highest amounts of digitoxin in the four cell lines.
Blendor. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 10,000g for 30 min. The supernatant was used as the enzyme solution. RuBPCase (EC 4.1.1.39) was assayed at 30°C by measuring the incorporation of $^{14}$CO$_2$ into acid-stable compounds in reaction mixtures (pH 7.8) containing the following compounds: D-ribulose 1,5-bisphosphate, 0.25 μM; NaH$^{14}$CO$_3$ (0.36 μCi), 10 μM; MgCl$_2$, 5 μM; EDTA, 0.03 μM; GSH, 3 μM; and 0.1 ml of enzyme solution; total volume, 0.42 ml (10). The reaction was started by adding D-ribulose 1,5-bisphosphate and was stopped by adding 0.2 ml HCOOH; then the reaction mixture was evaporated to dryness. The acid-stable $^{14}$C-product was dissolved in 0.5 ml of water and added to 10 ml scintillator (Instagel, Packard Instruments Company, Downers Grove, IL), and the radioactivity was counted with a liquid scintillation counter. The protein content in the enzyme preparation was determined by the method of Lowry et al. (7).

RESULTS

Undifferentiated Cells. Figure 1A shows the growth curves of undifferentiated green cells and undifferentiated white cells. The green cells grew slightly faster than did the white cells, and both kinds of cells reached their maxima 2 weeks after initiation of the cultures. Effects of IAA concentration on growth, digitoxin content, and Chl content of the undifferentiated cells are shown in Figure 2. Organ-redifferentiation was not observed for any concentration of IAA. The Chl content of the green cells cultured in medium containing 1 mg/L IAA (about 350 to 400 μg/g fresh weight) is high for cultured cells as compared with that of scotch broom cells or tobacco cells (70 to 120 μg/g fresh weight), which were reported to grow photoautotrophically (15). The Chl and digitoxin contents of the light-grown cells cultured in medium with low IAA concentrations (0.01 and 0.1 mg/L) were higher than were those in the cells cultured in medium with high IAA-concentrations (1 and 10 mg/L). This was also the case for the digitoxin content of dark-grown cells without Chl. However, even the highest digitoxin content of the undifferentiated cells (0.21 μg/g dry weight) was very low in comparison with that of the shoot-forming cultures (40 μg/g dry weight).

Shoot-Forming Cultures. Green, shoot-forming cultures and white, shoot-forming cultures of _D. purpurea_ L. were established in liquid medium. These cultures consisted of shoots and undifferentiated cells without roots. Figure 1 (B and C) shows the growth curves and the time courses of digitoxin accumulation in these cultures. The amount of digitoxin per flask increased in parallel with growth and reached a maximum 3 weeks after initiation of the culture. The light-grown cultures accumulated higher amounts of digitoxin than did the dark-grown cultures. In both cases, the amount of digitoxin in the culture medium was less than 1 μg per flask.

Is the Chloroplast Concerned with the Synthesis of Digitoxin? The main site of cardenolide storage and formation in _Digitatis_ is known to be in the leaves, especially in the mesophyll cells (14). The distinctive feature of the mesophyll cells is chloroplasts which carry out the entire photosynthetic process.

To investigate whether the chloroplast was involved in digitoxin formation, the digitoxin content, Chl content, and RuBPCase activity in an intact leaf were determined (Fig. 3). The same parameters were measured in the four cell lines of _D. purpurea_ L. (Fig. 3). In all of these parameters, leaf was much higher than were the green, shoot-forming cultures, which were the highest among the four cell lines. Comparing the green, shoot-forming cultures with the undifferentiated green cells, the Chl content and RuBPCase activity of both were about the same. However, there
supplemented with:

Thin bars represent low. On extremely growth was contained in the undifferentiated cultures, although they were repressed in cultures, whereas those of Garve et al. (3), who showed that medium containing 0.1 mg/L NAA plus 2.0 mg/L BA was the most effective and that medium containing 1.0 mg/L 2,4-D plus 0.02 mg/L kinetin was repressive for both differentiation and digitoxin formation in long-term culture of D. lanata. They did not test IAA.

The most effective concentrations of IAA for green, shoot-forming cultures were 10- to 100-fold greater than those for undifferentiated cells. This difference may depend on the presence (Fig. 4) or absence (Fig. 2) of BA in the medium, inasmuch as it is generally observed that the ratio of auxin to cytokinin determines cell differentiation.

How expression of the cardenolide-biosynthesis system is connected with differentiation of cells into leaves is an interesting problem yet to be solved.

LITERATURE CITED


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