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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 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 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.

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.

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 sub-cultured 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 4×10^5 erg/s·cm^2) 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 H_2O. When necessary, the extract was further diluted with H_2O 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[1] 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 $MgCl_2$, 100 mM NaCl, and 80 mM 2-mercaptoethanol for 1 min in a Waring

[1] Abbreviations: RuBPCase, ribulose bisphosphate carboxylase; NAA, α-naphthaleneacetic acid.

Blendor. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 10,000*g* 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}C]O_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).

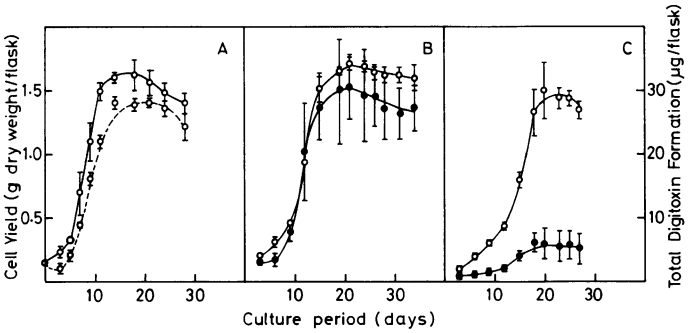

FIG. 1. Growth and digitoxin formation of *D. purpurea* L. cell cultures. About 1.5 g fresh weight of cells were transferred to each flask, containing 100 ml of medium, and cultured in the light or in the dark; then they were harvested at the indicated times. A, Growth curves of undifferentiated cells: green cells (O——O); white cells (O- - -O). B, Growth and digitoxin formation of green, shoot-forming cultures; C, of white, shoot-forming cultures; (O——O), growth; (●——●), digitoxin content. Vertical bars represent SES calculated from six replicates in each stage.

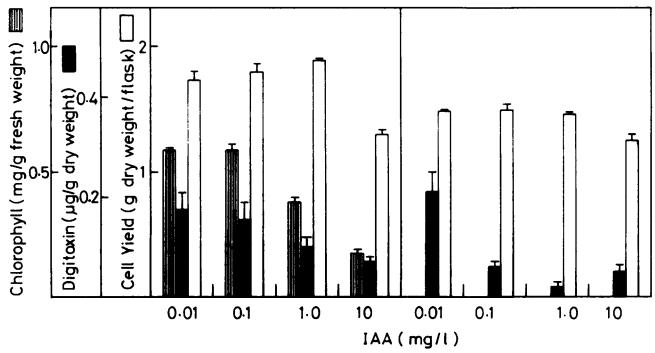

FIG. 2. Effect of IAA on growth, digitoxin content, and Chl content of the undifferentiated cells of *D. purpurea* L. cultured in the light (left) or in the dark (right). About 1.5 g fresh weight of the undifferentiated green cells of *D. purpurea* L. were transferred to medium supplemented with 0.01 to 10 mg/L IAA and cultured for 3 weeks in the light or in the dark. Thin bars represent SES calculated from six replicates.

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 *Digitalis* 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

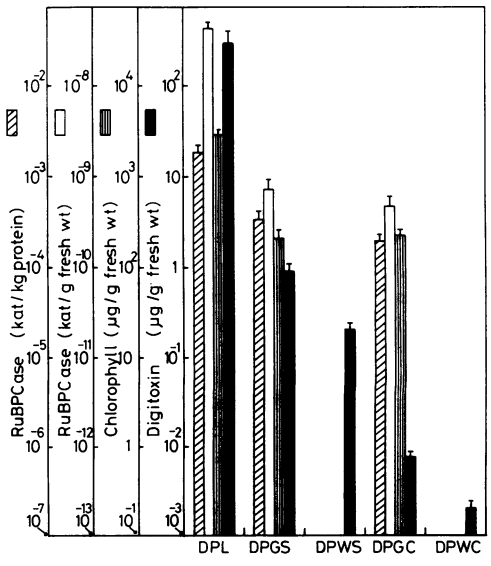

FIG. 3. Digitoxin content, Chl content, and RuBPCase activity of an intact leaf (DPL); of green, shoot-forming cultures (DPGS); of white, shoot-forming cultures (DPWS); and of undifferentiated green cells (DPGC) or undifferentiated white cells (DPWC) of *D. purpurea* L. An intact leaf of a 2-month-old plant and 3-week-old cultures were used in this experiment. The bars are expressed on logarithmic scale. Thin bars represent SES calculated from five replicates.

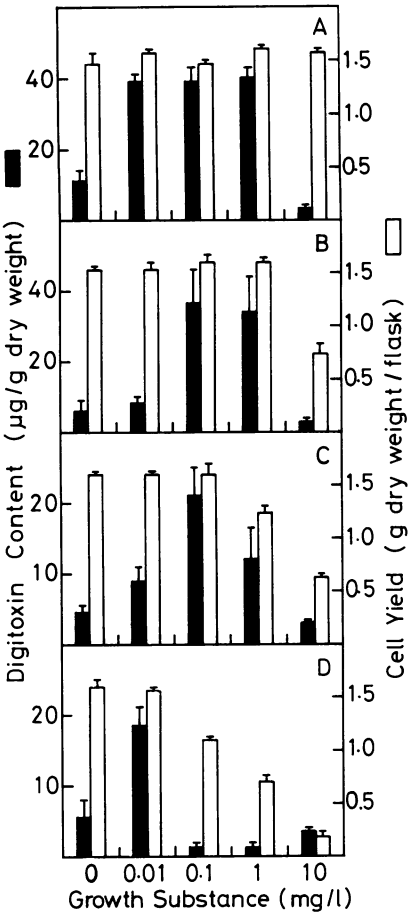

FIG. 4. Effects of plant growth substances on growth and digitoxin content of green, shoot-forming cultures of *D purpurea* L. cultured in the light. The green, shoot-forming cultures were transferred to medium supplemented with: A, 1 mg/L IAA and 0 to 10 mg/L BA; B, 0.1 mg/L BA and 0 to 10 mg/L IAA; C, 0.1 mg/L BA and 0 to 10 mg/L NAA; D, 0.1 mg/L BA and 0 to 10 mg/L 2,4-D. Then, they were cultured for 3 weeks. Thin bars represent SEs calculated from six replicates.

was a great difference in their digitoxin content. The green, shoot-forming cultures contained considerable amounts of digitoxin, while the digitoxin content in the undifferentiated green cells was extremely low. On the other hand, white, shoot-forming cultures contained about one-third the digitoxin of the green, shoot-forming cultures, although they did not contain Chl or RuBPCase. These results suggest that photosynthetically active chloroplasts are not essential for the synthesis of digitoxin in *Digitalis* cells.

Effects of Plant Growth Substances. Effects of BA, IAA, NAA, and 2,4-D on growth and digitoxin formation of the green shoot-forming cultures were examined. The digitoxin formation, as well as the shoot differentiation, was stimulated by BA in the tested range from 0.01 to 1 mg/L and suppressed by 10 mg/L, whereas growth was not affected significantly by BA (Fig. 4A).

Figure 4B shows that, from 0.1 to 1 mg/L, IAA was suitable for digitoxin formation, and 10 mg/L IAA repressed both growth and digitoxin formation. Figure 4C shows that 0.1 mg/L NAA was optimal for digitoxin formation, and 1 mg/L or more NAA repressed both growth and digitoxin formation. Figure 4D shows that digitoxin formation was stimulated by 0.01 mg/L 2,4-D and was markedly repressed by higher concentrations, whereas growth was repressed stepwise by serially 10-fold higher concentrations, up to 10 mg/L. As for the auxins, the concentration required for optimum response was highest for IAA and lowest for 2,4-D, in accord with the order of relative activity generally observed for these auxins. In these experiments, degree of shoot differentiation was in parallel with degree of digitoxin formation. That is, shoot differentiation was most stimulated by the concentrations of these substances which were optimal for digitoxin formation and repressed by the concentrations which repressed digitoxin formation.

DISCUSSION

Undifferentiated cells of *Digitalis* have been reported to accumulate only extremely low amounts, if any, of cardenolide (2–6, 11); there are no reports dealing with cardenolide-production by undifferentiated cells possessing chloroplasts. The main site of cardenolide storage and synthesis in *Digitalis* is the leaves (14), so that the development of the cardenolide biosynthesis system seems to be associated with their differentiation and is not prominent in undifferentiated cells as suspension cultures or in differentiated cells in roots. Voigt *et al.* reported that, in the cells of *D. purpurea* leaf tissue, most of the cardenolide glycosides present were localized in the mitochondrial and chloroplast fractions (13). If the chloroplast is involved in cardenolide synthesis, then it can be speculated that undifferentiated cells with chloroplasts should synthesize cardenolides.

In the present study, we established highly chlorophyllous, undifferentiated cells of *D. purpurea* L., the Chl content and RuBPCase activity of which were about the same as those of the green, shoot-forming cultures. However, even the highest digitoxin content of the undifferentiated green cells (0.18 μg/g dry weight) was much lower than that of the green, shoot-forming cultures (40 μg/g dry weight) and about the same as that of the dark-grown, undifferentiated white cells (0.21 μg/g dry weight); however, in the subculturing condition (IAA, 1 mg/L), the green cells contained about 6 times more digitoxin than did the white cells (Fig. 2). On the other hand, the dark-grown, white, shoot-forming cultures without chloroplasts accumulated about one-third as much digitoxin as did the light-grown, green, shoot-forming cultures (Fig. 3). These results suggest that the chloroplast is not essential for the synthesis of digitoxin. The stimulatory effect of light on digitoxin formation by shoot-forming cultures seems to allow the cells to be closer to the status of leaf cells. However, it is possible that the proplastids, which would develop into chloroplasts if illuminated, contain the cardenolide-biosynthesis system. Furthermore, it can be speculated that, when the cells differentiate to form shoots, the system in the proplastid is expressed regardless of light conditions.

Our results relative to the effects of plant growth substances are compatible with 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.

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