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EFFECT OF LIGHT ON CARDENOLIDE PRODUCTION BY *DIGITALIS LANATA* TISSUE CULTURES

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Key Word Index—*Digitalis lanata*; Scrophulariaceae; tissue culture; cardenolide production; light; chlorophyll; carotene; SAN 9789.

Abstract—The influence of light on the accumulation of cardenolides by *Digitalis lanata* S-1 was studied. Optimal radiant flux for chlorophyll accumulation was 10 W/m^2 between 350 and 750 nm with a spectral energy distribution according to Thorn daylight special CM/56. Different regions of this spectrum were examined using fluorescent tubes. The highest cardenolide and chlorophyll contents were obtained using light in the blue region. Light from the yellow region had little or no effect. The digoxin content did not vary within the examined light conditions. SAN 9789 inhibited cardenolide formation in the light but stimulated its accumulation in darkness. A new strain derived from *D. lanata* S-1 did not produce chlorophylls. It accumulated cardenolides in the dark but not in the light. A medium containing 0.2 mg benzyladenine/l. stimulated cardenolide accumulation by strain S-1 in the light and by strain S-2 in the dark.

INTRODUCTION

Due to high production costs and a large selling volume several attempts have been made to find new techniques for the production of cardenolides. The most promising alternative source is the fermentative production by plant tissue cultures of *Digitalis* species. A breakthrough in this field was obtained by Nover *et al.* [1] by the development of a stable, cardenolide-producing strain of *Digitalis lanata*. However, the need for light for the production of cardenolides by this strain is a complication for the large scale fermentation. The present paper demonstrates cardenolide accumulation in the dark.

RESULTS

Strain S-1

The effect of light upon the cardenolide production by tissue cultures of *D. lanata* strain S-1 was studied. Optimal irradiance for chlorophyll accumulation, viability and growth was obtained at 10.0 watts of white light/m^2. The growth rate was highest in green and white light. Low growth rates as well as low digitoxin* and chlorophyll contents were obtained in yellow light and in darkness (Fig. 1). With increasing chlorophyll content there was an increase in the digitoxin content (Fig. 2). The variation of the digitoxin content with the chlorophyll content in the cultures was also observed when phytohormones other than benzyladenine (BA) were used (Fig. 3). No correlation could be found between the digoxin and the chlorophyll content. There was no significant difference in the digoxin content between cells grown in light of different spectral distributions or in darkness (Fig. 1).

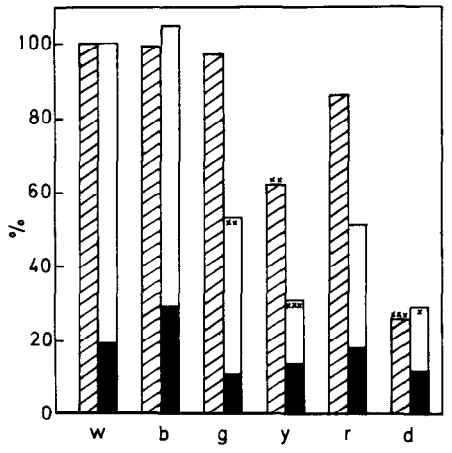

Fig. 1. Chlorophyll, digitoxin and digoxin contents of strain S-1 cultured under different light conditions. The mean value for digitoxin content in white light was 108 μg/g dry wt. ▨, Chlorophyll content expressed as % of the chlorophyll content in white light; □, digitoxin content and, ■, digoxin content, expressed as % of total cardenolides (digitoxin and digoxin) in white light. w, white; b, blue; g, green; y, yellow; r, red; d, darkness. Statistical analysis was made by *t*-test: *P*-values for difference from cultures in white light are: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. *P*-values for the cardenolides refer to the digitoxin part. There was no significant difference between the digoxin values.

Strain S-2

Strain S-2 was a new stable phenotype isolated from strain S-1. It did not produce chlorophylls or carotenes. The carotene precursor phytoene was accumulated. When cultured in the light minor amounts of cardenolides were produced. In the dark, there was a considerable increase

*Digitoxin and digoxin refer to equivalents of these two compounds (see Experimental).

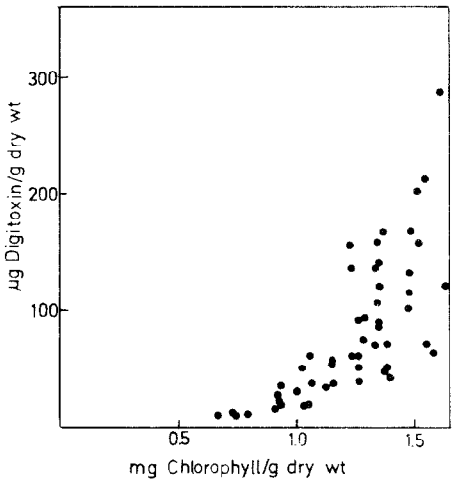

Fig. 2. Digitoxin content in strain S-1 grown on medium A as a function of the chlorophyll content. Each point represents the digitoxin and chlorophyll contents of a single culture.

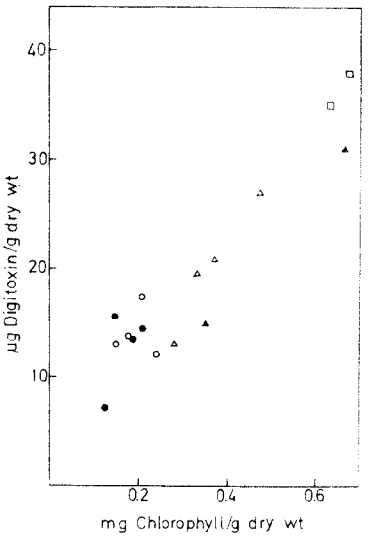

Fig. 3. Digitoxin content in strain S-1 grown on media with different phytohormones. ○, 2,4-dichlorophenoxyacetic acid; ●, 3,5-dichlorophenoxyacetic acid; △, α-(2-chlorophenoxy) isobutyric acid; ▲, 4-chlorophenoxyacetic acid; □, no phytohormone. 0.5, 1.0 and 5.0 mg/l of each hormone was used.

in cardenolide production (Table 1). The increased production appeared in medium A (BA) but not in medium B (2,4-dichlorophenoxyacetic acid).

Inhibition of carotene synthesis in strain S-1

Carotene synthesis can be inhibited by addition of SAN 9789 to the cultures [2]. In this case, the inhibition leads to phytoene accumulation. Exposure to light of the SAN-treated cultures results in photobleaching and inhibition of chlorophyll accumulation. Under these conditions digitoxin production was inhibited. However, when grown in darkness on medium A and in the presence of SAN 9789, digitoxin production was stimulated. The carotene content was still low and the chlorophyll content fell to a low level due to the lack of stimulation by light (Table 1).

DISCUSSION

The synthesis of digitoxin by tissue cultures of *D. lanata* prepared according to Garve *et al.* [3] varies with light of different spectral distribution. A spectral distribution that stimulated the accumulation of cardenolides but not that of chlorophyll has not been found. Yellow light (500–650 nm) is without effect on chlorophyll and digitoxin accumulation. Our observation that white light can be substituted by a small amount of blue light can be used for reduction of the irradiation costs. By contrast to the accumulation of digitoxin equivalents the content of digoxin equivalents remained unchanged within the examined light conditions. The hydroxylation of digitoxin to digoxin in strains which lack *de novo* production of digitoxin is also light independent [4].

We propose that the estimation of the chlorophyll content in light grown cultures can be used for screening purposes, as the frequency of high producing colonies is higher in cultures rich in chlorophyll. Similar results were obtained by Luckner *et al.* [5]. As light dependent processes are complicated to perform on a large scale, we have tried to exchange the light mediated stimulation for a stimulation through different plant hormones. These attempts have not been successful. The relationship between chlorophyll content and the digitoxin content is maintained (Fig. 3).

In *Digitalis*, there is a close relationship between the light stimulated chlorophyll synthesis and digitoxin synthesis. But, as we have shown with strain S-2 and with strain S-1 treated with SAN 9789, light and chlorophyll are not indispensable for cardenolide synthesis. It is likely

Table 1. Influence of light conditions and SAN 9789 on growth and the production of pigments and digitoxin by *D. lanata* strains S-1 and S-2 grown in medium A

Strain	Light conditions	SAN 9789 (mg/l)	Chlorophyll (mg/g dry wt)	Digitoxin (µg/g dry wt)	β-Carotene (µg/g dry wt)	Phytoene (µg/g dry wt)	Growth (mg dry wt)
S-1	Light	0	0.32	10	31	<60*	231
	Light	50	0.20	6	<5	198	188
	Darkness	0	0.16	4	<5	<80*	294
	Darkness	50	0.20	129	<5	132	157
S-2	Light	0	<0.003	1	<5	58	48
	Darkness	0	<0.003	45	<5	68	247

*Overestimated due to presence of unidentified substance with overlapping of UV spectrum.

that the stimulation of the respective biosynthetic pathways are parallel phenomena, independent of each other. Green cultures without cardenolide accumulation have been reported by e.g. Reinhard *et al.* [6] and Kartnig *et al.* [7, 8]. Cardenolide synthesis in strain S-2, which lacks chlorophyll and carotene, is stimulated by benzyladenine in the dark. This is an effector which favours both chlorophyll and cardenolide synthesis in strain S-1 when grown in the light.

It is interesting to note the similarities between strain S-1 treated with SAN 9789 and strain S-2. Cultured in darkness they lack carotene, accumulate phytoene and have a stimulated digitoxin synthesis (Table 1). In all cases, the highest values of cardenolide production were obtained with phytohormones that favour differentiation of the tissue. This is consistent with the hypothesis of Hagimori *et al.* [9], who state that morphological differentiation, and not the formation of chlorophyll or chloroplasts, is the important factor for obtaining cardenolide production. Cardenolide production as a result of morphological differentiation has also been discussed by Garve *et al.* [3] and Luckner *et al.* [10].

EXPERIMENTAL

Cultures and medium. D. lanata strain S-1 was obtained from anther filaments according to Garve *et al.* [3]. The seeds were obtained from Hammenhög, Sweden. The strain was maintained on a revised Murashige–Skoog medium [3] and kept on a rotatory shaker at 175 rpm, with a stroke length of 25 mm, at 20° and 12 + 12 hr light and dark periods. The experiments were performed in the same medium containing 0.2 mg benzyladenine/l. (medium A) in 250-ml flasks with 100 ml medium. The inocula were previously grown in white light. The cell material was grown as aggregates with a diameter of up to 4 mm. After 20 days they were harvested using suction filtration and the cell material was lyophilized. The yield was about 9 g wet wt/100 ml medium (760 mg dry wt). *D. lanata* strain S-2 was isolated from strain S-1, when grown on a medium containing 0.1 mg NAA and 2.0 mg BA/l. Strain S-2 was stable for two years. Medium A and medium B (1 mg 2.4-D and 0.02 mg kinetin/l.) were used in the experiments. Other conditions were the same as for strain S-1.

Light sources. Fluorescent tubes were obtained from the Swedish division of Thorn Electrical Industries Ltd., Stockholm. The light was filtered through 5 mm polycarbonate (Rohm and Haas, Philadelphia). The incident light on the surface of the cultures from the different light sources is reported in Figs 4 and 5. According to these figures they are referred to as white, blue, green, yellow and red within the text. Integrated values for the irradiation in the visible region are 10.0 W/m² for white, 1.3 W/m² for blue, 2.9 W/m² for green, 2.8 W/m² for yellow and 2.1 W/m² for red light.

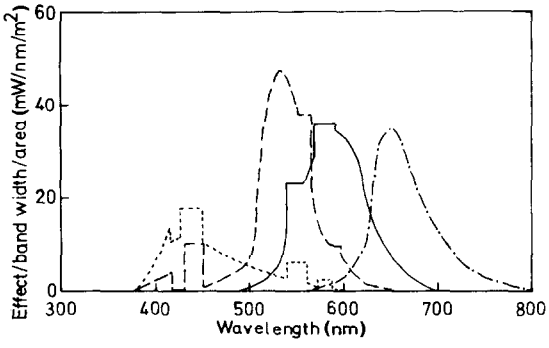

Fig. 4. Spectral distribution of the incident light at the surface of the culture. (------), blue light; (– – –), green light; (——), yellow light; (—.—.), red light.

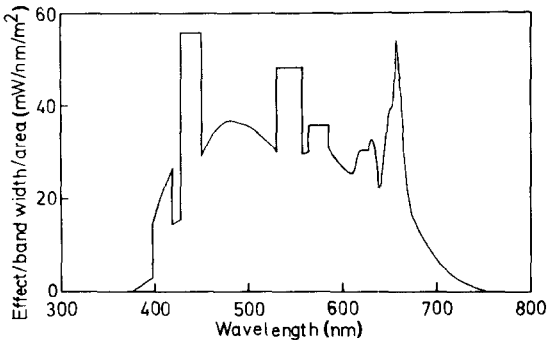

Fig. 5. Spectral distribution of the incident white light at the surface of the culture. Courtesy of The Swedish Division of Thorn Electrical Co Ltd.

Extraction. The dry cell material was extracted with EtOH (10 mg/ml) in an ultrasonic bath for 20 min.

Cardenolides in the cell extracts. These were determined using the RIA method according to Vogel [11]. Digitoxin and digoxin in the text mean digitoxin and digoxin equivalents, respectively, because of cross reactivity with related cardenolides. Antisera against digitoxin and digoxin and tritium labelled digitoxin and digoxin were gifts from colleagues at The Institute of Plant Physiology in Halle, GDR.

Chlorophyll in the cell extracts. This was determined using the method of Wintermans *et al.* [12]. It was checked that the bleaching of chlorophyll by digitoxin reported by Jonas [13] did not affect our results.

Carotene and phytoene. These were determined from their absorption spectra in hexane extracts.

Viability. This was determined according to Johne *et al.* [14].

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