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LIGHT AND TEMPERATURE EFFECT ON *EPICOCCUM NIGRUM*

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Abstract—The effect of darkness, and different light intensities, 110 lux, 2000 lux and 2500 lux, and of different temperatures, 24° and 28°, on carotenogenesis, ergosterol synthesis and mycelial growth of *Epicoccum nigrum* grown in submerged culture has been studied. The synthesis of total carotenoids at 24° was inhibited at higher light intensities, but at 28° with higher light intensities the production increased. A regulating effect of light and temperature on the synthesis of the individual carotenoids, β-carotene, γ-carotene, rhodoxanthin and torularhodin, has been observed. The ergosterol synthesis was also influenced by light and temperature but no evidence of a correlation between carotenoid and ergosterol synthesis was found. The mycelial growth at 24° did not depend on the light intensities, but at 28° there was a stimulating influence on the growth by the higher intensities although the level at 24° was never reached.

INTRODUCTION

A NUMBER of factors regulating carotenoid biosynthesis in non-photosynthetic tissues has been reported, of which can be mentioned the effect of light and temperature. The effect of temperature on carotenogenesis in *Phycomyces blakesleeanus*[1] and in *Rhodotorula rubra* and *R. penaus*[2] was restricted only to a quantitatively lower production of the pigments, but in *R. gracilis*, however, qualitative differences were observed.[3]

Lederer[4] observed that light was required for the full pigmentation of *R. rubra* and Garton *et al.*[5] reported that *P. blakesleeanus* synthesizes β-carotene in darkness but that the concentration is doubled after exposure to light. The effect of light on carotenoid formation in *Neurospora crassa* is quite striking as was noted by Went.[6] Cultures grown in darkness are colourless but upon exposure to light become pink within an hour.

Naumann[7] and Schol-Schwarz[8] have communicated general observations on a red pigment extracted from the mycelial material of *Epicoccum nigrum* grown in surface cultures. They observed the influence of light on the synthesis of this red pigment; the cultures grown in darkness showed more pigmentation than those grown in bright daylight.

In previous papers[9, 10] the isolation and identification of four carotenoid pigments from *E. nigrum* were described. In the present investigation a comparative study of the formation of the carotenoids, together with the effect of different light intensities and temperatures on the pigmentation, is reported, while special regard will be paid to the biosynthesis of these pigments compared with the formation of ergosterol.

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RESULTS AND DISCUSSION

Light Effect

During preliminary studies it was found that the red pigments of *Epicoccum nigrum* can be divided into two classes, the lipid-soluble orange–red pigments and the water-soluble wine-red pigments. The lipid-soluble pigments were identified as four known carotenoid pigments,[9,10] while chromatographic separation of the water-soluble pigment extract on cellulose columns showed only one red band of an unidentified compound.

According to Naumann[7] and Schol-Schwarz[8] the red pigmentation of *E. nigrum* is more intense when the fungus is grown in the dark. Therefore the effect of different light intensities

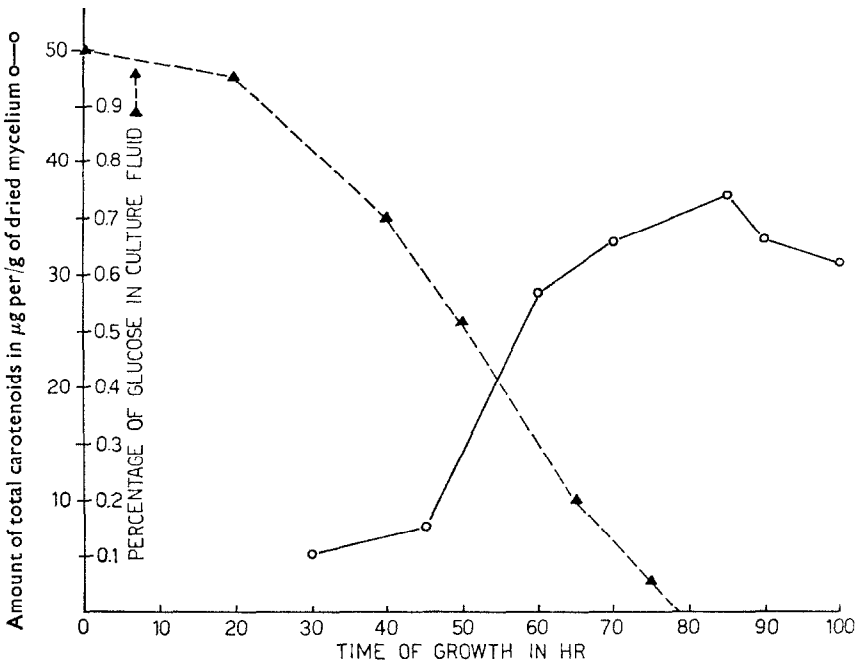

FIG. 1. THE GLUCOSE UPTAKE AND THE PRODUCTION OF TOTAL CAROTENOIDS IN *Epicoccum nigrum* VS. TIME OF GROWTH AT 24° UNDER ILLUMINATION AT 110 lux.

on the formation of the four carotenoid pigments was studied by growing the fungus in shake cultures in darkness and under illumination at 110, 2000 and 2500 lux. For standardization of the growth period several culture conditions were studied. It was observed that glucose uptake had finished after 75–85 hr of growth, which coincided with the highest levels of carotenoid content per gram of dry weight of the mycelium (Fig. 1). Continued growth led to a decrease in total carotenoids and also a qualitative shift towards the more oxygenated carotenoids rhodoxanthin and torularhodin. Therefore, when the glucose was found to be absent from the culture fluid the carotenoid pigments were extracted from the mycelial material. Light did show a very small effect on the growth of the mycelium expressed as the dry weight per 100 ml of culture fluid. The highest light intensities only slightly accelerated the uptake of glucose over 2–3 hr.

The total amount of carotenoids produced in darkness was some 5 per cent higher per

gram of dry weight, than under our normal growth conditions at 110 lux, about 7 per cent higher than under illumination at 2000 lux and about 25 per cent higher than at 2500 lux (Table 1).

TABLE 1. EFFECT OF DIFFERENT LIGHT INTENSITIES ON CAROTENOGENESIS, ERGOSTEROL SYNTHESIS AND MYCELIAL GROWTH IN SUBMERGED CULTURE OF *Epicoccum nigrum* AT 24°

Light intensity (lux)	Dry weight (mg per 100 ml culture fluid)	Amount of carotenoids in μg		Amount of ergosterol in mg	
		Per 100 ml culture fluid	Per g dry weight	Per 100 ml culture fluid	Per g dry weight
Darkness	414±14	14·8±1·2	37·9±2·4	6·19±0·42	16·77±1·12
110	432±18	14·5±0·9	36·2±1·9	5·55±0·39	11·74±1·02
2000	441±20	11·8±0·9	35·4±1·5	4·66±0·24	10·47±0·89
2500	438±18	11·7±0·6	29·0±1·1	3·38±0·12	8·45±0·61

The results are the mean of ten experiments (±S.E.M.).

This suggests that light causes the inhibition of the production of the four carotenoids. The percentage of these pigments differed only slightly under the different light intensities with a tendency towards the more highly oxygenated carotenoids.

Kuhn and Brockmann[11] have put forward the hypothesis that rhodoxanthin, one of the pigments of *E. nigrum*, might be formed from zeaxanthin (3,3'-dihydroxy-β-carotene) via a double-bond rearrangement through dihydro-rhodoxanthin. The biosynthesis of torularhodin was studied by Simpson *et al.*[13] in *Rhodotorula gracilis*. They found that torularhodin can be regarded as an oxidation product of torulene which is derived directly from γ-carotene (Fig. 2).

Therefore it is interesting to note that β-carotene and rhodoxanthin form 70–80 per cent of the total pigment in *E. nigrum* under all four conditions, and the ratio β-carotene+rhodoxanthin:γ-carotene remains unchanged at 9:1. In other fungi, however, light has also a quantitative effect on carotenoid synthesis; Mase *et al.*[12] reported an increase of about 20 per cent in the yield of the mycelium and a decrease of about 90 per cent in total carotenoids when dark-grown *Penicillium sclerotiorium* was compared with light-grown cultures, and in *R. gracilis* the ratio α-carotene+β-carotene:torulene changed from 1·67:1 in dark-grown cultures to 2·29:1 in the light.[13] Chu *et al.*[14] observed that *Choanephora cucurbitarum*, from which only β-carotene was isolated, contained more of this pigment per gram of dry weight in darkness than in light-grown cultures.

Although it is quite difficult to deduce from the data obtained what the biochemical relationship is between the four carotenoid pigments from *E. nigrum*, it is clear that light can act as an inhibitor of carotenogenesis in this fungus, or as a destroyer of synthesized pigments. It is our view that the action of light is due to an inhibition of carotenogenesis; firstly, because we have not observed an effect on one pigment only but on the total; and second, it is known that β-carotene is rather more light-sensitive than the other three pigments, so if there were a destructive effect on the carotenoids produced one might expect a greater

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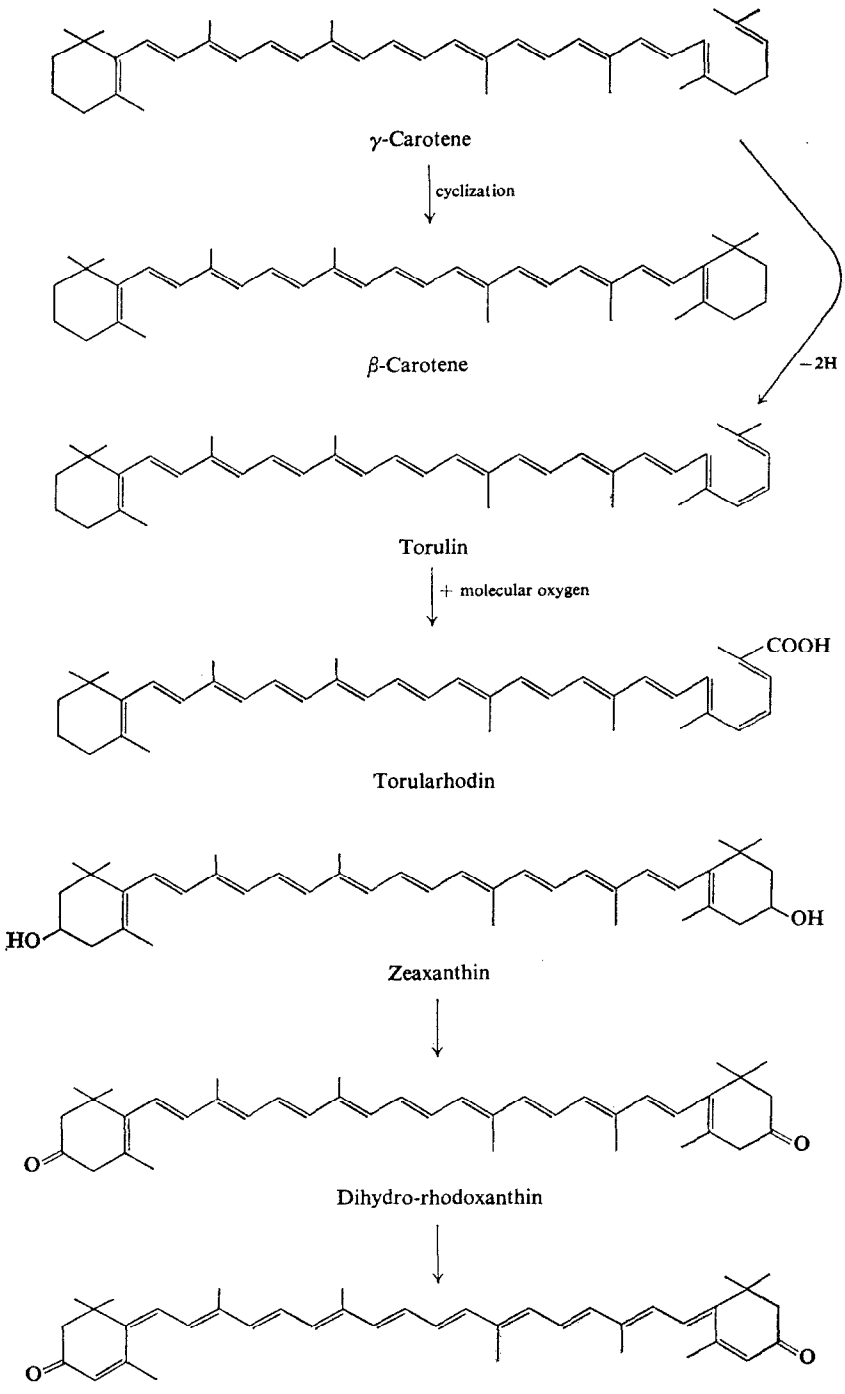

FIG. 2. THE BIOSYNTHETIC PATHWAY FROM γ-CAROTENE TO β-CAROTENE AND TORULARHODIN ACCORDING TO SIMPSON *et al.* AND THE HYPOTHETIC SYNTHESIS OF RHODOXANTHIN FROM ZEAXANTHIN ACCORDING TO KUHN *et al.*

decrease of β-carotene at higher light-intensities, and this was not observed. Thirdly, we have also followed the formation of the carotenoids up to the end of the growth period and we have found that the production of these pigments increased until glucose uptake was complete, suggesting that the synthesis depended on the rate of glucose uptake and not on the light intensity.

As the biosynthetic pathways of carotenoids and sterols share a number of common steps in the early stages, up to farnesyl pyrophosphate, it was important to see if light has any effect on the nature and amount of the sterol present in *E. nigrum*, identified as ergosterol. A direct effect of the light was observed giving a decrease in ergosterol from 16 mg/g dry weight in darkness to half this value at 2500 lux. The ratio carotenoids: ergosterol changed from 1:450 in total darkness to 1:300 at 2500 lux, demonstrating a more pronounced effect of the light on ergosterol synthesis than on carotenoid synthesis, but again it is not possible to say where the light interferes in the sterol biosynthesis.

Temperature Effect

The effect of a higher temperature on carotenogenesis in *E. nigrum* was studied. According to Naumann the optimal temperature range for the red pigmentation of this fungus in surface cultures is 21–26°. Therefore we chose a growth temperature of 28° and studied also the effect of different light intensities.

The first observation was that glucose uptake was not influenced by the higher temperature or different light intensities. However, it was observed that the mycelial growth was lower at this temperature than at 24°. This effect could be counteracted by higher light intensities but the level of growth at 24° was never reached. The effect of the different light intensities on carotogenesis at 28° was exactly the opposite of the effect at 24°. While at 24° there was a clear inhibitory effect, we observed at 28° a considerable increase of total carotenoid production at higher intensities (Table 2). The comparison of the quantity of carotenoids produced at the same light intensity but at different temperatures showed that at 24° the synthesis in darkness, at 110 lux and at 2000 lux is higher than at 28°, but at 2500 lux there is a much higher production at 28° than at 24°. It is interesting to note that at 24° the highest level is reached in darkness, whereas at 28° it is the highest light intensity which gives the greatest increase. The conclusion may be drawn that there is a competitive correlation between the inhibitive effect of light and temperature inhibition. Very similar quantitative data were obtained after the separation of the carotenoids synthesized at 24° in darkness, and at 28° at 2500 lux.

TABLE 2. EFFECT OF DIFFERENT LIGHT INTENSITIES ON CAROTENOGENESIS, ERGOSTEROL SYNTHESIS AND MYCELIAL GROWTH IN SUBMERGED CULTURE OF *Epicoccum nigrum* AT 28°

Light intensity (lux)	Dry weight (mg per 100 ml culture fluid)	Amount of carotenoids in μg		Amount of ergosterol in mg	
		Per 100 ml culture fluid	Per g dry weight	Per 100 ml culture fluid	Per g dry weight
Darkness	347±11	7·8±0·5	23·4±1·2	2·16±0·14	5·92±0·38
110	352±10	9·3±0·5	30·8±1·0	0·98±0·09	3·04±0·17
2000	381±14	9·9±0·6	26·6±1·0	1·88±0·11	4·79±0·21
2500	399±11	16·3±0·9	41·1±2·5	6·21±0·51	15·18±1·32

The results are the mean of ten experiments (±S.E.M.).

TABLE 3. EFFECT OF DIFFERENT LIGHT INTENSITIES ON THE SYNTHESIS OF CAROTENOIDS PRODUCED BY *Epicoccum nigrum* GROWN IN SUBMERGED CULTURE AT 24° AND 28°

Carotenoid	Absorption spectrum (nm) in hexane	Temperature of growth, 24° Amount of separated carotenoids in % of total				Temperature of growth, 28° Amount of separated carotenoids in % of total			
		Darkness	110 lux	2000 lux	2500 lux	Darkness	110 lux	2000 lux	2500 lux
β-Carotene	(426), 453, 482	55·2±5·1	44·2±3·1	61·2±5·4	50·4±4·0	38·2±2·2	37·8±1·9	38·9±2·1	56·8±4·9
γ-Carotene	438, 463, 495	9·1±0·6	8·3±0·7	7·3±1·1	10·3±0·9	6·0±0·8	12·1±0·9	13·5±1·1	5·1±0·3
Rhodoxanthin	458, 489, 524	25·4±1·9	35·1±2·8	21·7±2·0	31·3±2·2	45·8±2·6	38·2±2·1	32·9±3·6	23·8±1·8
Torularhodin	468, 502, 538	10·3±0·7	12·4±1·0	9·8±0·6	8·0±0·4	10·0±0·9	11·9±0·8	14·7±1·1	14·3±1·5

The results expressed in per cent of total carotenoids are the mean of ten different experiments (±S.E.M.).

The separation and quantitative determination of the carotenoids have shown that at 28° β-carotene and rhodoxanthin also formed 70–80 per cent of total carotenoids. The ratio β-carotene + rhodoxanthin : γ-carotene varied between 16 : 1 at 2500 lux and 5·5 : 1 at 2000 lux.

However, if the quantities of the individual carotenoids at 24° are compared with those at 28° at the same light intensities, especially under darkness and at 110 lux, it can be seen that the quantity of rhodoxanthin increased at the expense of β-carotene and γ-carotene, while the quantity of torularhodin remained the same (Table 3). This may indicate that there is a channelling effect of temperature on the synthesis of the carotenoids derived from γ-carotene with a preference for the β-carotene–rhodoxanthin pathway under these conditions; Simpson *et al.*[3] also observed a channelling effect of the temperature on carotenogenesis in their studies of the formation of β-carotene and torularhodin from γ-carotene by *R. glutinis* grown at 5° and 25°. Our results can be interpreted as an indication that rhodoxanthin is formed from β-carotene and γ-carotene. Since γ-carotene has been regarded as a precursor of β-carotene, it is logical to propose that rhodoxanthin originates from β-carotene. This data confirm the hypothesis of Kuhn and Brockmann[11] based on the qualitative presence of β-carotene, zeaxanthin and rhodoxanthin in *Taxus baccata*. In none of our experiments were carotenoids other than these four isolated, so that it may be concluded that the probable biosynthetic pathway of the carotenoids produced by *E. nigrum* will involve the formation from γ-carotene of torularhodin or of β-carotene and rhodoxanthin.

The synthesis of ergosterol, which was inhibited at 24° by increased light intensities, behaved differently. In darkness a net synthesis of this sterol was observed, which decreased at 110 lux, but at 2000 lux the quantity increased per gram of dry weight. The highest level was reached at 2500 lux. From a comparison of the results it is interesting to note that the total synthesis of carotenoids and of ergosterol were at their highest levels at 24° in darkness, and at 28° at 2500 lux, again demonstrating the competitive effect of light and temperature on the enzyme reactions.

MATERIALS AND METHODS

Strain

The strain used was *Epicoccum nigrum* Link, 5-I-3, received from Professor E. Küster, Department of Industrial Microbiology, University of Dublin, Irish Republic.

Preparation of the Culture

Erlenmeyer flasks (500 ml), containing 100 ml of culture fluid, consisting of 1 per cent glucose and 0·5 per cent yeast autolysate in distilled water at pH 6·8–7·0, were inoculated with 0·5 ml of the culture fluid from a mother flask. The flasks were incubated on a rotatory shaker at 220 rev/min in a thermostatic room in which the illumination could be regulated. The illumination was supplied by a series of fluorescent tubes of 15 W "daylight" type at about 40 cm from the shelf of the shaker.

Extraction of the Mycelium

The mycelium was harvested by filtration on a Buchner filter and was extracted three times with acetone in a Waring Blendor for 2 min. The combined acetone extracts were diluted with the same volume of sat. NaCl solution, followed by extraction with hexane till the hexane layer remained colourless. The total extract was dried (Na_2SO_4) and the u.v. and visible light spectra determined. The hexane solution was concentrated in the dark at

30° (N_2) to a few ml and allowed to stand for 48 hr at −20°, after which the sterol precipitate was filtered off.

The total extract was chromatographed on a cellulose column (W. & R. Balston Ltd., Maidstone, Kent; standard grade) (30 cm × 1·5 cm per 30 μg of carotenoids). Two fractions were separated; fraction I was eluted with solvent-system hexane–acetone (99:1, v/v) and fraction II with hexane–acetone (9:1, v/v).

Fraction I was rechromatographed on a MgO (E. Merck A.-G., Darmstadt, Germany): Celite (Johns-Manville, New York, N.Y., U.S.A.; grade 503) (2:1, w/w) column. The column was developed with a gradient of acetone in hexane up to 10 per cent (v/v). Three pigment fractions, P_1, P_2 and P_3, were obtained. After washing with water, drying and concentrating *in vacuo*, the three fractions were purified on alumina columns (E. Merck A.-G., Darmstadt, Germany; Brockmann grade I) with hexane containing respectively 1, 2 and 8 per cent (v/v) of acetone. Pigment fraction P_1 was identified as β-carotene, P_2 as γ-carotene and P_3 as rhodoxanthin, as described previously by Foppen and Gribanovski-Sassu.[10]

Fraction II was re-chromatographed on a cellulose column with hexane–acetone (92:8, v/v) as eluent giving only one pigment fraction, which previously was identified as torularhodin (Gribanovski-Sassu and Foppen,[9]).

Quantitative Determinations

The dry weight of the mycelium was determined by drying the filtered material to constant weight in an oven at 105°. The amount of ergosterol was determined directly from the u.v. absorption spectrum, using a calculated $E^{1\%}_{1cm}$ value of 290 at 282 nm. The quantity of the total carotenoids was measured at 460 nm with an $E^{1\%}_{1cm}=2500$ and the quantity of the separated carotenoids was calculated using the $E^{1\%}_{1cm}$ values given by Goodwin.[15]

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