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EFFECTS OF LIGHT ON ANTHRAQUINONE PRODUCTION IN *RHAMNUS PURSHIANA* SUSPENSION CULTURES

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Key Word Index—*Rhamnus purshiana*; Rhamnaceae; Cascara Buckthorn; plant cell culture; light; anthraquinones.

Abstract—In suspension cultures of *Rhamnus purshiana*, the accumulation of anthracene derivatives (i.e. 1,8-dihydroxyanthraquinones, anthrones and/or dianthrones) was increased by a daily photoperiod of 12 hr. In particular the emodin (and to a lesser extent chrysophanol) content of the cultures was significantly raised by a 12 hr light/dark cycle, whereas the levels of physcion were much less affected. The formation of anthra-derivatives, however, was strongly suppressed when the cultures were continuously illuminated.

INTRODUCTION

Besides its effects on plant growth and development, the role of light in the regulation of secondary metabolism can be of great importance [1]. Generally the production of desired secondary compounds by plant cell cultures is markedly increased under controlled illumination conditions [2, 3]. It was however demonstrated that white or blue light almost completely inhibited the formation of shikonin derivatives in callus cultures of *Lithospermum erythrorhizon* [4]. In tissue cultures of *Aloe saponaria*, anthraquinone production is associated with light irradiation, whereas tetrahydroanthracene glucosides, which are found in the subterranean stem of the differentiated plant, are not accumulated under this regime [5]. Tetrahydroanthracene glucosides originally present in dark-grown cultures, disappear upon continuous illumination and are replaced by anthraquinone glucosides such as those of chrysophanol.

Suspension cultures of *Rhamnus purshiana* DC. developed in our laboratory accumulated predominantly glycosides of mainly physcion (and/or its reduced forms) when grown in the dark [6]. The present paper deals with some effects of light on the production of polyketide anthracene derivatives in these suspension cultures.

RESULTS AND DISCUSSION

In the first experiment, suspension cultures of *R. purshiana* were grown either with a daily photoperiod of 12 hr or in the dark to investigate the effect of light on the formation of anthracene derivatives. After 15 days, cell material from both treatments was freeze-dried and subjected to TLC-analysis [7, 8].

In light-grown cultures mainly *O*-glycosides of emodin and physcion were found; in cultures grown in the dark *O*-glycosides of physcion dominated (Table 1). For suspensions exposed to light, a trace of emodin was tentatively identified as *C*- and/or combined *C*- and *O*-glycoside. TLC analysis also showed an increased anthracene derivative content of the light-grown suspension cultures.

In the second experiment suspensions were cultivated with a daily photoperiod of 12 hr or in the dark as described above. Suspensions were taken from both series after 4, 10, 15 and 21 days to determine dry cell mass (g/batch), dry weight content (% of fresh cell mass), anthraquinone content (mg% of dry cell mass) and anthraquinone yield (mg/batch).

The production of anthracene derivatives was increased by a light/dark cycle of 12 hr. After 15 days the anthraquinone yield of light-grown cultures exceeded that

Table 1. Identified anthracene derivatives in freeze dried cell material from suspension cultures of *R. purshiana* grown with a daily photoperiod of 12 hr or in the dark [−, not detectable; (+), trace amounts; +, minor component; + +, major component].

	Aglycones					
	12 hr light/dark			Dark		
	Free	As *O*-glycosides	As *C*-glycosides	Free	As *O*-glycosides	As *C*-glycosides
Chrysophanol	+	+	−	+	−	−
Physcion	+ +	+ +	−	+ +	+ +	−
Emodin	(+)	+ +	(+)	(+)	+	−
Emodinanthrone	+	−	−	(+)	−	−

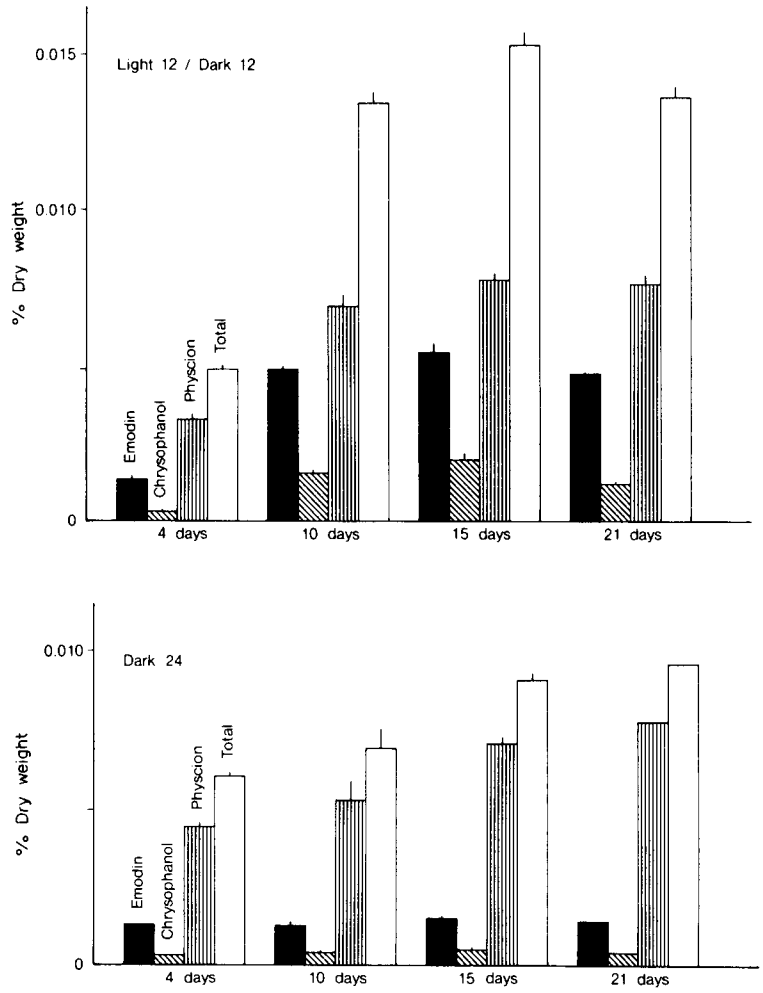
of cultures grown in the dark by a factor of 1.6. The dry cell mass of suspensions cultivated with light was slightly decreased; the dry weight content of light- and dark-grown cultures determined after 15 days was 2.5 and 2.1% respectively.

In cultures grown with light, the accumulation pattern of anthraquinones had markedly changed (Figs 1, 2). It was clearly demonstrated that the emodin (and to a lesser extent chrysophanol) content was significantly raised by a daily photoperiod of 12 hr (emodin: $p<0.001$, $p<0.005$, $p<0.001$ and chrysophanol: $p<0.005$, $p<0.02$, $p<0.002$, calculated for quantitative determinations after 10, 15 and 21 days respectively), whereas in comparison with dark-grown cultures levels of physcion were shown to be much less affected. When the above experiments were repeated, similar results were obtained. These results established that increased levels of predominantly emodin (and/or possibly its reduced forms) contributed to the raised total anthraquinone yield, when suspension cultures of *R. purshiana* were daily exposed to light for 12 hr.

When it had been established that anthra-derivative production was positively influenced by a 12 hr light/dark cycle, another experiment was performed to investigate the effect of continuous illumination. Suspension cultures of *R. purshiana* were grown either with continuous light or in the dark. After a passage of 17 days both series were subjected to an analytical evaluation as described above. Interestingly, however, it was found that production of anthracene derivates was strongly suppressed when suspensions were illuminated continuously. The total anthraquinone yield of illuminated cultures was only one fourth of the amount determined for dark-grown cultures. Dry cell mass production of suspensions grown with constant illumination was slightly decreased, which had also been observed for cultures subjected to a 12 hr light/dark cycle. The dry weight content of light- and dark-grown suspensions in this experiment was 2.6 and 2.0% respectively.

Although anthracene derivative production was significantly inhibited by continuous illumination, the relative amounts of emodin corresponded with those found in cultures of the previous experiment (Fig. 3). In suspensions daily exposed to light for 12 or 24 hr and in cultures grown in the dark respectively 36 and 18% of the total anthra-derivative content was represented by emodin (including its reduced forms) (Figs 1–3).

It has been shown by several authors that the spectral composition of the light source can be of great influence on the synthesis of secondary compounds [4, 9, 10]. Although the spectral emission characteristics of the two



Figs 1, 2. Total and individual anthraquinones present in suspension cultures of *R. purshiana* grown with a daily photoperiod of 12 hr or in the dark respectively, determined after 4, 10, 15 and 21 days of a single passage.

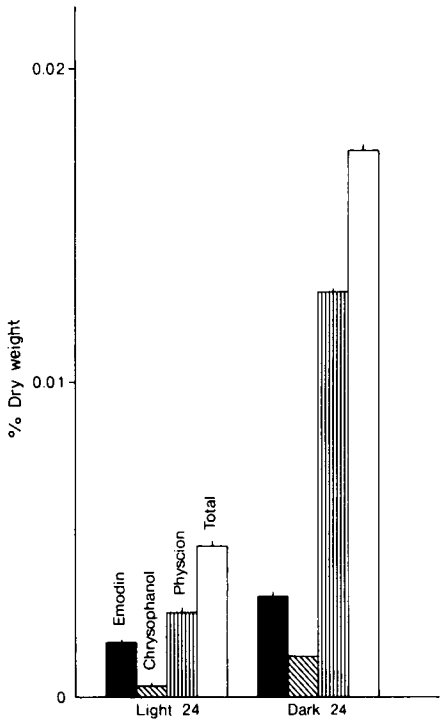


Fig. 3. Contents of total and individual anthraquinones of suspension cultures of *R. purshiana* grown with continuous light or in the dark after a passage of 17 days.

light sources used in our experiments were slightly different (continuous light—3200 K; light/dark cycle—3600 K), it does not seem likely that this difference in spectral composition was the cause of the suppressed anthra-derivative production in continuous light. So for the present, it has been shown by our results that among other factors, an alternation of light and dark periods in a daily cycle is of significant importance for an optimal production of polyketide anthracene derivatives in suspension cultures of *R. purshiana*. It is likely that further studies on the influence of alternative periodicities in light/dark cycles, and the use of light of specific wavelengths, might lead to further optimisation.

EXPERIMENTAL

Plant cell culture methods. Callus of *R. purshiana* subcultured for 970 days was used to initiate suspension cultures. Suspensions were grown in 250 ml Erlenmeyer flasks on gyratory shakers at 110 r.p.m. at 25°, using the same medium as described for the callus cultures [8], apart from the addition of 1 mg/l NAA and the omission of agar and kinetin. After subcultivation for 165 (I), 330 (III) or 365 (II) days, several batches were mixed to serve as starting material. Mixed suspension material was added in 30 ml portions to Erlenmeyer flasks containing 60 ml of culture medium. Except for their illumination, the batch cultures were grown under uniform conditions.

I. Batch cultures were grown either with a daily photoperiod of 12 hr (cool-white fluorescent light, 6000 l ×, 3600 K) or in the dark. After a single passage of 15 days, cell material from both series was freeze-dried and subsequently subjected to TLC-analysis.

II. Batch cultures were cultivated either with a daily photoperiod of 12 hr (cool-white fluorescent light, 6000 l ×, 3600 K) or in the dark. After 4, 10, 15 and 21 days of the passage, batches from both series were harvested to determine dry cell mass, dry weight content and anthraquinone content.

III. Batch cultures were grown either with continuous illumination (mixture of cool-white fluorescent and incandescent light, 2000 l ×, 3200 K) or in the dark. After a single passage of 17 days, dry cell mass and dry weight content were determined for both series and cell material was subjected to quantitative HPLC-analysis.

Extraction procedures. Freeze-dried cell material (0.5 g) from the suspension cultures was extracted with 100 ml Et_2O by refluxing for 1 hr. The Et_2O soln was dried (Na_2SO_4) and the solvent evapd under red. pres. (Free aglycones). After extraction of the cell material with Et_2O, the marc was extracted with 100 ml MeOH under reflux for 1 hr. MeOH was removed from the extract under red. pres. at 50° (Glycosides). To the fraction containing glycosides, 50 ml 1 M H_2SO_4 was added and hydrolysis was performed by boiling for 1 hr. After cooling the mixture was exhaustively extracted with $CHCl_3$. The extract was dried (Na_2SO_4) and the solvent was removed (fraction of aglycones found as *O*-glycosides).

After removal of aglycones found as *O*-glycosides, 1.2 g of $FeCl_3$ was added to the H_2O soln and the mixture was refluxed for 1 hr. The mixture was then cooled and extracted with $CHCl_3$. The extract was treated according to the procedure for aglycones found as *O*-glycosides (fraction of aglycones found as *C*-glycosides and/or combined *C*- and *O*-glycosides).

Identification of 1,8-*dihydroxyanthracene aglycones* was performed as previously described [7, 8].

Quantitative determination of anthracene derivatives. Anthracene derivatives were converted into the corresponding anthraquinone mixtures and determined quantitatively by HPLC according to the procedures reported by us before [11]. Quantitative determinations were performed in duplicate. The determined values are expressed as a mean ± s.d. and were evaluated by the Student's *t*-test.

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