

DOSE-RESPONSE CURVES FOR PHYTOCHROME-MEDIATED ANTHOCYANIN SYNTHESIS IN THE MUSTARD SEEDLING (*SINAPIS ALBA* L.)

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SUMMARY

The dose-response curves for the effect of red and green light on the synthesis of anthocyanin in mustard seedlings show a plateau. This plateau is reached with energy doses of 1 (red light) resp. 10 (green light) $\mu\text{W sec cm}^{-2}$. A further increase in the amount of anthocyanin is induced by red or green light doses exceeding $10^3 \mu\text{W sec cm}^{-2}$.

Thus the occurrence of a plateau in the dose-response curve of a phytochrome-mediated process is not restricted to the growth of the *Avena* mesocotyl and coleoptile.

1. INTRODUCTION

The dose-response curve for the inhibition of the first internode of *Avena* seedlings by low-energy doses of red light shows a plateau between 10^{-1} and $10 \mu\text{W sec cm}^{-2}$ at $\lambda = 660 \text{ nm}$ (BLAAUW, BLAAUW-JANSEN & VAN LEEUWEN 1968). If the same phenomenon might be found for another phytochrome-mediated process a further analysis might contribute to the understanding of the mechanism of phytochrome action and not only of the growth mechanism of *Avena* mesocotyls. For this purpose the anthocyanin synthesis in *Sinapis* cotyledons was chosen.

2. MATERIAL AND METHODS

Seeds of *Sinapis alba* were supplied by a local seed store. Control experiments were performed with seed of *Sinapis alba* var. *gisilba* kindly provided by the Botanical Laboratory in Freiburg. 50 seeds were set out on glass slides ($3 \times 18 \text{ cm}$) wrapped in filter paper. The glass slides were placed horizontal in glass trays ($3.5 \times 20 \text{ cm}$), resting with both ends on strips of foam plastic. Tap water was added to each tray sufficient to fill the room between bottom and glass slide. The trays were placed in absolute darkness at 22°C . The seedlings were grown in the dark for 48 hours before they were irradiated.

Light was obtained by filtering the radiation from an incandescent (iodine) lamp with an appropriate system of lenses by a 5 cm layer of water and precision double-band filters (Schott u. Gen. Mainz) or perspex filters. For irradiation with red light of low intensity a double-band filter of 659 nm ("DEPAL") combined with a Calflex filter was used (incident energy $40 \mu\text{W cm}^{-2}$). Red

light of an intensity of $10^3 \mu\text{W cm}^{-2}$ was obtained by the use of a red plexiglass filter combined with a Calflex filter and an infrared mirror (radiation between 600 and 700 nm). For green light the beam was filtered with a precision line filter of $\lambda = 530 \text{ nm}$ combined with a Calflex filter (Balzer, Liechtenstein). Incident energy was $45 \mu\text{W cm}^{-2}$. For far-red light (incident energy $40 \mu\text{W cm}^{-2}$) a double-band filter of 735 nm was used.

24 hours after irradiation the cotyledons and the hypocotyl of the 10 most red coloured plants from each tray were placed in vials with 10 ml 18% propanol-1% HCl. For extraction of the anthocyanin the vials were immersed in boiling water for 2 minutes. The extinction of the extracts was measured in a colorimeter at $\lambda = 530 \text{ nm}$.

Thin-layer chromatography was applied to separate the anthocyanins. 20 seedlings were extracted with 0.75 ml propanolic 1% HCl and a suitable amount of the extract was spotted directly on a cellulose-coated glass-plate. After drying the plate was placed in a jar, the bottom of which was covered by ethylacetate-formic acid- H_2O (70:15:15). After the solvent had travelled c. 10 cm in the cellulose-layer, which took about an hour, the plates were dried and the separated pigments were extracted with ethanolic- or methanolic HCl. The absorption spectra of the extracts were determined.

3. RESULTS

3.1. Effect of green light

Series of seedlings were irradiated for varying lengths of time with $45 \mu\text{W cm}^{-2}$ of $\lambda = 530 \text{ nm}$. *Fig. 1* shows that by the green irradiation anthocyanin synthesis is induced. $10 \mu\text{W sec cm}^{-2}$ suffices to saturate a low level of anthocyanin formation. At doses larger than c. $10^3 \mu\text{W sec cm}^{-2}$ a second rise in the curve becomes evident. Therefore the use of green safelight was avoided in the following experiments.

3.2. Effect of red light

The relation between red light dose and subsequent anthocyanin formation is not a simple one (*fig. 2*). The induction of anthocyanin synthesis by red light

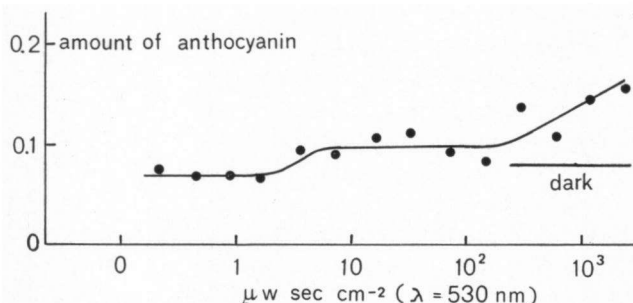


Fig. 1. Dose-response curve for anthocyanin synthesis at $\lambda = 530 \text{ nm}$.

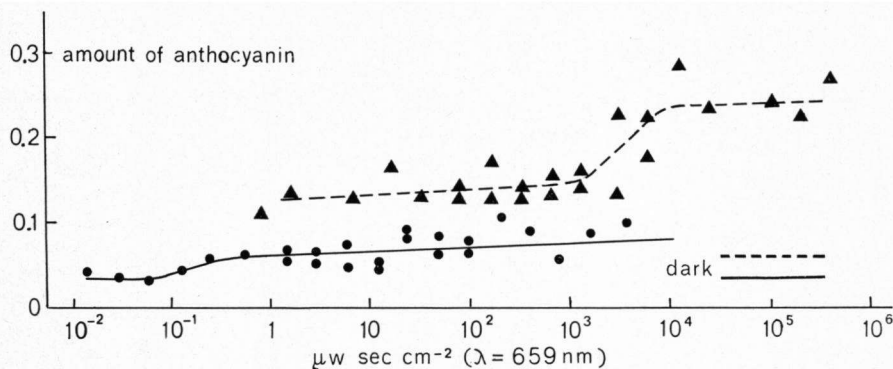


Fig. 2. Dose-response curve for anthocyanin synthesis at $\lambda = 659$ nm (2 experiments).

doses up to $5 \times 10^5 \mu\text{W sec cm}^{-2}$ occurs in two steps. A first plateau is reached with ca $1 \mu\text{W sec cm}^{-2}$, a second increase in anthocyanin synthesis sets in at ca $10^3 \mu\text{W sec cm}^{-2}$ and is completed at ca $10^4 \mu\text{W sec cm}^{-2}$. This second plateau is comparable to the one described by MOHR (1957), the first is not reported in his paper. A control experiment with seedlings of *Sinapis alba* var. *Gisilba* (the variety used in Mohr's experiments) showed the same plateaus as occur with our variety, only the plateaus were shifted to smaller light doses.

As the possibility exists that the rise in the curve is determined by the duration of irradiation and not by the light dose, curves at two intensities were compared. Fig. 3 shows that reciprocity holds for the range of red light doses tested.

These results do not agree with the data of LANGE, SHROPSHIRE & MOHR (1971). These authors performed a test of the reciprocity law with red and far-red light and did find a linear relation between anthocyanin amount and log dose of red light. Possibly their smallest red light dose (c. $200 \mu\text{W sec cm}^{-2}$) was just as large as to miss the low plateau of anthocyanin formation. Another possibility might be that use of green safelight had already established the low plateau.

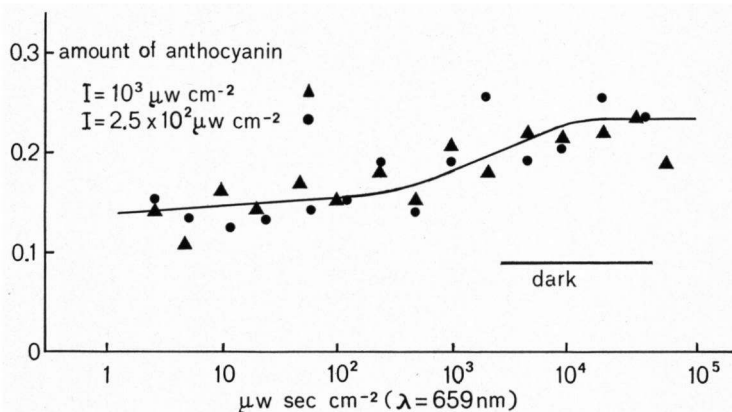


Fig. 3. Test of the reciprocity law with red light.

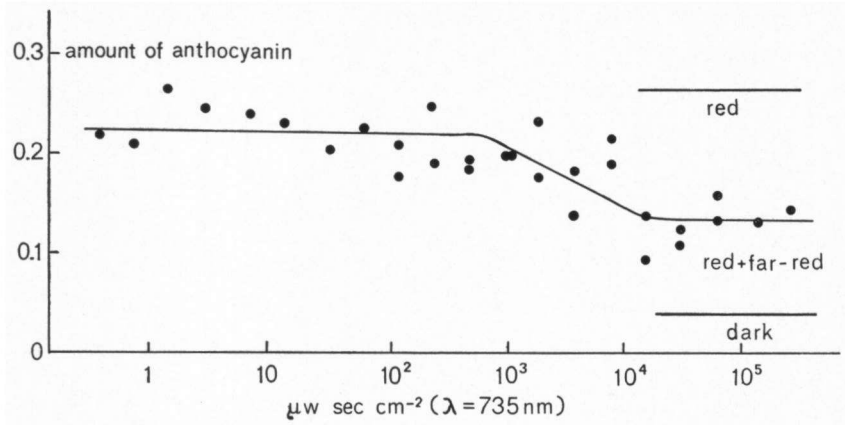


Fig. 4. Far-red reversal of red light effect.

3.3. The effect of far-red light

The induction of anthocyanin synthesis by red light is partially reversible by far-red irradiation (fig. 4). The reversion is saturated with far-red doses of c. $10^4 \mu\text{W sec cm}^{-2}$. When given alone far-red light induces anthocyanin synthesis (fig. 5) with a saturating dose at c. $10^3 \mu\text{W sec cm}^{-2}$. A second rise in the curve could not be observed, neither when the irradiation time was extended till 30 minutes, nor when the intensity was increased to $2 \times 10^3 \mu\text{W cm}^{-2}$ by the use of perspex filters instead of an interference filter.

3.4. Analysis of anthocyanins

It might be imagined that the anthocyanins synthesized on the lower plateau are different from the anthocyanins formed after irradiation with larger light doses, with a different threshold-requirement for the synthesis of the anthocyanins of the lower and those of the higher plateau. Analysis of the anthocyanins shows however that on the lower plateau the same anthocyanins are synthesized as on the higher plateau be it in different proportions.

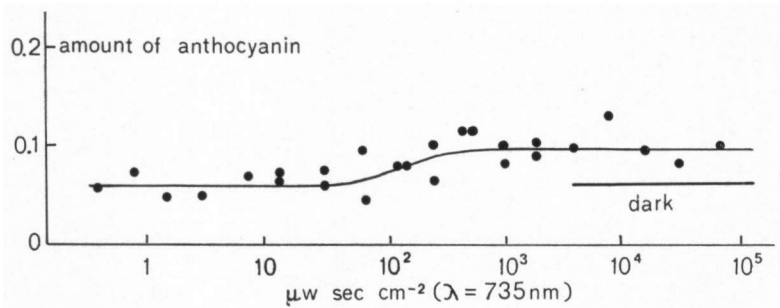


Fig. 5. Dose-response curve for anthocyanin synthesis at $\lambda = 735 \text{ nm}$.

According to HAVELANGE & SCHUMACHER (1966) an anthocyanin extract from *Sinapis* cotyledons can be separated into 5 components by thin layer chromatography. The aglycon of the five pigments is cyanidin. This is glycosidized with two or three glucose molecules. Four components are believed to be acylated with sinapic acid in various degrees; one component is not acylated.

In our chromatograms three anthocyanin spots can be observed at Rf 0.08, Rf 0.15 and Rf 0.40. Chromatograms were developed of extracts from seedlings which were irradiated with 10^2 and $10^6 \mu\text{W sec}^{-1} \text{cm}^{-2}$ ($\lambda = 660 \text{ nm}$) respectively. These doses of red light induce anthocyanin formation on the lower or the higher plateau respectively (cf. *fig. 1*). Chromatograms from these two plateaus show the same spots, but the spots differ in significance as follows:

Lower plateau: $\text{Rf} 0.40 = \text{Rf} 0.15 > \text{Rf} 0.08$.

Higher plateau: $\text{Rf} 0.08 = \text{Rf} 0.15 > \text{Rf} 0.40$.

The absorption spectra of these three pigments were determined. Some spectral properties are summarized in *table 1*.

Assuming that we are dealing with cyanidins glycosylated with glucose and acylated with sinapic acid the anthocyanin with Rf 0.40 might be acylated with 3 sinapic acid molecules ($E_{322}/E_{520} = 9$) and might be glycosylated in the 3- and the 5-OH position (absorption maximum in the visible shifted to shorter wavelengths, no shoulder between 410 and 440 nm, low short wave maximum). Probably the anthocyanin with Rf 0.15 contains one sinapic acid molecule and has a free 5-OH group. The anthocyanin of Rf 0.08 appears to be not acylated ($E_{322}/E_{520} = 1$) and to have a free 5-OH group (shoulder between 410 and 440 nm), cf. GEISSMAN (1962) and STROH, SEIDEL & SARODNICK (1965).

We can conclude that the P_{FR} level in the plants has an effect on the composition of the anthocyanins. On the lower plateau of anthocyanin synthesis an anthocyanin acylated with presumably 3 molecules of sinapic acid forms a relatively large part of the anthocyanins, but on the higher plateau of anthocyanin formation an unacylated anthocyanin takes its place. The occurrence of two steps in the induction of anthocyanin formation can, however, not be ascribed to a threshold regulation of the synthesis of one or more of the anthocyanin components.

Table 1. Spectral properties of the *Sinapis*-anthocyanins.

Rf	λ max in eth-ol-HCl nm	E 322/E 520	Shoulder between nm
0.08	540	265	c. 1
0.15	540	260	c. 3
0.40	530	275	c. 9

5. DISCUSSION

LANGE et al. (1971) concluded that in the synthesis of anthocyanin by *Sinapis* P_{FR} exerts two functions: it regulates the build-up of a potential for biosynthesis of anthocyanin and it mediates the anthocyanin synthesis itself.

In the case of *Fagopyrum esculentum* red and far-red irradiation induce insignificant amounts of anthocyanin, ascribed to the action of a small amount of already derepressed genes (SCHERF & ZENK 1967). The red irradiation has to be preceded by a large dose of blue or white light to induce the synthesis of fairly large amounts of anthocyanin. The authors attribute the effect of this high-energy light dose to the derepression of more genes, but the presence of P_{FR} is thought to be necessary for the derepressed genes to induce enzyme- and anthocyanin synthesis. Combining the findings of LANGE et al. and SCHERF et al. with the outcome of our experiments we suggest that on the lower plateau of anthocyanin formation P_{FR} effectuates the low potential for biosynthesis of anthocyanin that is present in etiolated mustard seedlings, whereas a higher P_{FR}/P_{tot} ratio has to be established to enhance this potential (c.q. enzyme synthesis?) and to raise the anthocyanin production to the second plateau. The P_{FR}/P_{tot} ratio established by far-red irradiation does not suffice to enhance the potential for anthocyanin synthesis of the etiolated seedlings. The difference in height of the lower plateaus brought about by red resp far-red irradiation (fig. 6) shows that even the lower plateau of anthocyanin synthesis can not be saturated by far-red light.

Apparently the plateau occurring in the dose-response curves for the growth-inhibition of the *Avena* mesocotyl and for the growth-promotion of the *Avena* coleoptile (BLAAUW et al. 1968) are not specific for the growth of the *Avena* seedling. Perhaps they are a general feature of the dose-response curves for the action of phytochrome in positive and negative photoresponses.

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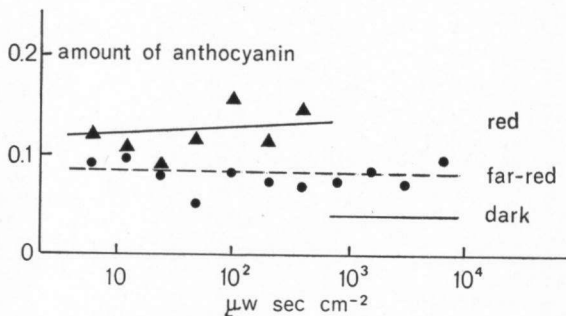


Fig. 6. Height of the lower plateaus in the dose-response curves for red and far-red light.

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