

PHOTOCONTROL OF ANTHOCYANIN FORMATION IN TURNIP SEEDLINGS

III. THE PHOTORECEPTORS INVOLVED IN THE RESPONSES TO PROLONGED IRRADIATION

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Summary. The substitution of far-red for the first six hours of a prolonged irradiation with red light resulted in a large increase in anthocyanin yield, which was greater than the combined yields from far-red and red when the two treatments were given separately. When intermittent far-red irradiation was followed by a single short exposure to red, a considerable amount of anthocyanin was formed, although each treatment given separately had little effect. Four hours continuous far-red alone yielded some anthocyanin and also resulted in a further large increase in the effect of a short red treatment; this terminal red effect was fully reversible by a subsequent brief exposure to far-red. It is concluded that at least two photochemical reactions are involved in the responses to red and far-red, the first leading to the formation of substrate(s) used in the second reaction.

When red light preceded exposure to the far-red/red irradiation sequence, the far-red enhancement effect was almost entirely lost and the anthocyanin yield approached that in red light. The effect of the red pre-irradiation treatment is attributed to destruction of phytochrome and it is suggested that phytochrome is the only pigment mediating anthocyanin synthesis in red and far-red. A possible interpretation is that the "high-energy" reaction in far-red and the low energy red/far-red reversible reaction are mediated by different forms of phytochrome.

The substitution of blue for the first six hours of a prolonged irradiation with red light also resulted in a synergistic increase in anthocyanin yield; the enhancement effect of blue light was, however, not prevented by prior exposure to red. It is concluded that phytochrome is not the only pigment mediating the reactions occurring in blue light. The synergism between blue and red suggests that the "high-energy" reaction in blue light may lead to the production of substrates for phytochrome action.

I. Introduction

At least two photoreceptors have been assumed to take part in the control of anthocyanin synthesis and in many morphogenetic responses of plants to light. One is the low energy red/far-red reversible reaction of phytochrome and the other is dependent on prolonged exposures to light of higher intensities (the "high-energy" reaction). When long exposures are used the action spectrum for anthocyanin synthesis frequently has peaks in far-red (730 nm) and in blue (e.g. turnip, *Sinapis alba*), but the long wave-length peak may be absent (as in *Sorghum*, tomato, *Helianthus*) or shifted to shorter wave-lengths (e.g. 690 nm in apple). The relationship between the two light reactions is still obscure. EVANS et al.

(1965) have proposed that the "high energy" reaction supplies a substrate for phytochrome action, and a similar model was presented by LANE and KASPERBAUER (1965) for the control of twining in dodder. MOHR (1964) considered that the two reactions independently affected the same metabolic site, but recently has suggested a closer relationship between them, the far-red peak of the "high-energy" reaction itself depending on phytochrome (BERTSCH und MOHR, 1965).

In turnip, the far-red peak of the "high-energy" reaction also depends on phytochrome, at least in part (GRILL and VINCE, 1965); the effect of several hours of far-red or blue light can, however, be shown to be increased by a terminal exposure to red, and this is reversed by far-red. The object of the experiments presented here was, therefore, to investigate further the relationship between phytochrome and the "high-energy" reaction in the control of anthocyanin synthesis in turnip seedlings.

II. Materials and Methods

Seedlings of *Brassica rapa* L. "Red Globe" were grown in the dark for 48-60 hours before transfer to the lighted cabinets used for the irradiation treatments; anthocyanin extracts were made 48 hours after the beginning of the irradiation treatment. The lighted cabinets and the methods of growing the seedlings and extracting and measuring anthocyanin have been described previously (GRILL, 1965; GRILL and VINCE, 1965).

The temperature was either maintained at 25°C throughout or, in the high temperature treatments, the thermostat setting was raised to 35°C four to five hours before the beginning of the light treatment giving two to three hours at 35°C. In one experiment (Table 1) the temperature was raised overnight giving about nine hours at high temperature. All light treatments were carried out at 25°C.

There were two sources of seed (Thompson and Morgan, Ltd. and Sutton and Sons Ltd.) and some quantitative differences were observed. The Thompson and Morgan strain gave a higher yield of anthocyanin, and also gave a large response to a terminal exposure to red light; following blue light the Sutton strain only gave a response to a terminal red light treatment when pre-treated with high-temperature (GRILL, 1965). Except for one experiment (Table 1), the Thompson and Morgan strain was used and a high temperature pre-treatment was usually given in order to increase the response to red light.

III. Experimental Results

1. *The Effect of High Intensity Blue or Far-Red Light Given Before Transfer to Continuous Red*

Seedlings were treated over a 48-hour period. One group received 6 hours of far-red, blue, or red light followed by darkness for the remaining 42 hours; the other group received 6 hours of far-red, red, blue or darkness followed by 42 hours of red light. The results (Table 1) show that substitution of blue or far-red during the first 6 hours resulted in a considerably increased anthocyanin yield when compared with 48 hours of red light; furthermore the measured yield from far-red, or blue, fol-

Table 1. *The Effect of a Pre-Treatment with Blue or Far-Red Given Before Transfer to Continuous Red Light*

Seedlings were 48 hours old and grown in water (W) or phenylalanine (P), and were either kept at 25° C throughout or (H.T.) were transferred to high temperature overnight before starting the light treatments; plants receiving high temperature +42 hours of red light remained for 6 hours in darkness at 25° C before transfer to continuous red.

Light treatments		Anthocyanin yield after 48 hrs (absorbance at 525 nm, increase over dark control)				
During first six hours	During subsequent forty-two hours		From six hours pre-treatment	From forty-two hours red (preceded by 6 hours dark)	Total	
			(a)	(b)	(i) Calculated (a + b)	(ii) Measured
Red	Red	H.T.P.	0.258	0.234	0.492	0.477 *
		H.T.W.	0.136	0.112	0.248	0.242 *
		P.	0.064	0.188	0.252	0.188 *
		W.	0.084	0.115	0.199	0.110 *
		Mean	0.135	0.162	0.298	0.254
Far-red	Red	H.T.P.	0.232		0.466	0.807 *
		H.T.W.	0.137		0.249	0.447 *
		P.	0.169		0.357	0.587 *
		W.	0.107		0.222	0.356 *
		Mean	0.161		0.323	0.549
Blue	Red	H.T.P.	0.207		0.441	0.732 *
		H.T.W.	0.122		0.234	0.437 *
		P.	0.134		0.322	0.501 *
		W.	0.123		0.238	0.366 *
		Mean	0.146		0.309	0.509

Significant difference at $P=0.05$ for means* in column (ii) = 0.115.

lowed by red was greater than the sum of the yields when the treatments were given separately.

The increases resulting from the pre-treatments were approximately the same with far-red and blue; the percentage increase was not much affected by the addition of phenylalanine, but was smaller when high temperature was given before the light treatments.

As the exposure to red light was very long in relation to the duration of far-red or blue, it seems likely that the red-promoted phytochrome reaction was saturated; these results, therefore, appear to rule out the suggestion that far-red and blue act only by maintaining a low level of P_{fr} phytochrome over a long period of time. The observed synergism between far-red (or blue), and red also makes it unlikely that the reactions leading to anthocyanin synthesis in the former wavebands are independent of those occurring in red light, and affords some support for the suggestion that in far-red and blue substrates for phytochrome action are

produced. MEIJER and ENGELSMA (1965) have also shown that a relatively short irradiation with blue or far-red light increases the effectiveness of a subsequent exposure to continuous red light in the inhibition of growth in length of gherkin hypocotyls.

The yield from 48 hours of red light is rather less than the combined yields from 6 and 42 hours, as would be expected if the reaction is virtually saturated after a few hours of red light. In the treatments without high temperature the yields after 42 and 48 hours red are the same. The rather large differences that occurred when the high temperature treatment was given appear to have resulted from a loss of some of the high temperature effect during the 6 hours of darkness at 25°C before transfer to 42 hours of red light; in the 48-hour treatment plants were transferred directly from high temperature to red light and the expected increase in yield resulted.

2. The Effect of Far-Red

The hypothesis that two inter-dependent steps are required for anthocyanin synthesis was investigated in two further experiments by shortening the total duration of exposure to red and far-red light. In the first of these, brief exposures to red, to far-red, or to red followed by far-red were given before and after a main light period of six hours in far-red (Figure 1). All treatments followed a previous exposure to high temperature to increase the response to a brief terminal red treatment. The results show that the amount of anthocyanin produced after six hours of far-red was approximately doubled by a short terminal exposure to red (note that the absorbance value in darkness is always about 0.120 and does not represent anthocyanin (SIEGELMAN and HENDRICKS, 1957); this effect of red was almost completely reversed by subsequent far-red. A pre-irradiation sequence with red light, however, depressed anthocyanin yield slightly and this depression by red was reversed in the red/far-red pre-treatment. Pre-irradiation with far-red shots alone increased the total amount of anthocyanin formed.

As total anthocyanin yield was found to be increased by a pre-treatment with far-red shots, a comparison was made between the effects of intermittent and continuous exposures to far-red with, and without, a terminal red treatment. During a four hour pre-treatment period seedlings received continuous far-red, or 8 or 16 exposures to 5 minutes of far-red; they were then placed in the dark immediately or after one, or more, exposures to red light. (The first exposure to red without preceding far-red was made at the end of the high temperature treatment to avoid any loss of its effect, and subsequent exposures were given at hourly intervals). The results (Fig. 2) show that, while far-red shots had little effect on anthocyanin synthesis when given alone, they resulted in a

manyfold increase in the effect of a single exposure to red light; this increase was in proportion to the logarithm of the total far-red dose given beforehand. Only in continuous far-red, however, was the anthocyanin

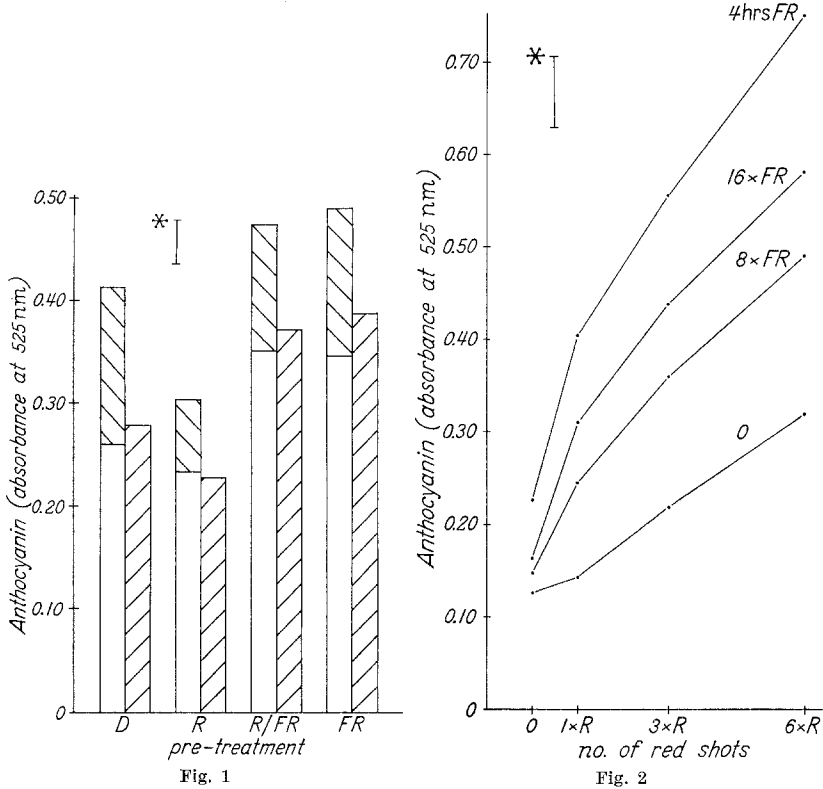


Fig. 1. The effect of short exposures to red and far-red light given before and after continuous far-red. All seedlings received high temperature before beginning the irradiation sequences. The pre-treatments were given over a period of 4 hours, during which plants received four equally spaced shots of 5 min red (R), 5 min red followed by 5 min far-red (R/FR), or 5 min far-red (FR). The pre-treatment was followed by 6 hours of continuous far-red, after which plants were transferred to darkness immediately, or after 10 min red or 10 min red followed by 10 min far-red. Plants receiving no light pre-treatment (D) remained for a further 4 hours in darkness at 35° C before transfer to 6 hours of far-red. Seedlings were 57 hours old and grown in 5×10^{-3} M. phenylalanine. * Significant difference at $P = 0.05$. □ terminated by dark. ▨ terminated by 10 min R; ▩ terminated by 10 min R/10 min FR

Fig. 2. The effect of pre-treatments with far-red on anthocyanin yield after subsequent intermittent exposures to red light. All seedlings were given high temperature before beginning the irradiation sequences. The far-red pre-treatment, which was given over four hours, consisted of continuous light (4 hr FR), or 8 (8xFR) or 16 (16xFR) equally spaced 5 minute shots; plants receiving no far-red pre-treatment (0) were transferred from high temperature immediately into the intermittent red light treatments, which were given as one 5-minute shot every hour. Seedlings were 60 hours old and grown in 5×10^{-3} M. phenylalanine. * Significant difference at $P = 0.05$

yield appreciable in the absence of a subsequent exposure to red light. These results also support the suggestion that the first light step (in far-red) supplies substrates for phytochrome action and it is interesting that

this reaction can proceed with intermittent irradiation as predicted by the model for a "high-energy" reaction proposed by SALE, VINCE and PRUE (1964) on the basis of results obtained with blue light in the control of stem elongation in *Pisum* and *Tropaeolum*. The low anthocyanin yield obtained with intermittent far-red shots alone suggests that the second light step which finally results in anthocyanin synthesis requires a higher concentration of P_{fr} (a terminal exposure to red) or the maintenance of a low concentration over a long period (exposure to continuous far-red). It is also clear that several exposures to red increase anthocyanin yield when compared with one; the slope of the response to an increasing number of red shots appears to increase slightly as the total pre-treatment dose of far-red light is increased, and this point is being investigated further.

Previously (GRILL and VINCE, 1965) the effect of a long exposure to far-red was shown to be markedly reduced if plants were first exposed to continuous or intermittent red light; results obtained at low temperatures and in nitrogen suggested that the pre-treatment with red acted by destroying phytochrome. From the results presented in Fig. 3 it can be seen that the enhancement effect of a 6-hour exposure to far-red given before transfer to continuous red was almost completely prevented if preceded by four hours of red light; it is, therefore, concluded that any additional effect of far-red compared with red is lost if phytochrome is first destroyed as a result of a pre-irradiation treatment with red.

3. *The Effect of Blue*

In the previous paper it was shown that, in contrast to the results obtained with far-red, the substitution of red for the first six hours of a long irradiation with blue light did not decrease the yield of anthocyanin and it was concluded that the "high-energy" reaction in blue light probably operated through a photoreceptor other than phytochrome. The results of Fig. 3 support this conclusion: a pre-treatment with six hours of blue or far-red both increased the subsequent effect of continuous red but the enhancement by blue light was only slightly decreased if preceded by red.

The interrelationships between blue and red, or far-red, effects were examined further by again reducing the total duration of light. A 4-hour pre-treatment with continuous or intermittent red, far-red or blue was given before transfer to a main light treatment consisting of six hours of continuous blue; plants were then placed in the dark immediately, or after exposure to 10 minutes red light. All treatments followed a previous exposure to high temperature. The results (Fig. 4) show that the effect of six hours of blue was increased by the terminal red exposure; the

absolute increase (0.150) was similar to that after far-red, but the percentage increase was less.

Considering only those treatments receiving a terminal red exposure (Fig. 4, hatched bars), a pre-irradiation sequence with continuous or intermittent red did not change the yield from six hours blue; however, pre-treatment with either intermittent far-red or blue resulted in a small

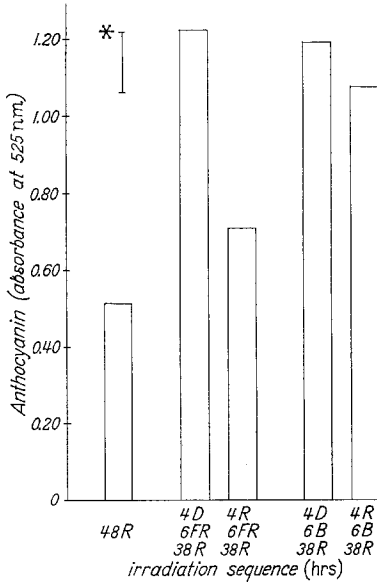


Fig. 3

Fig. 3. The effect of a pre-treatment with red light on anthocyanin synthesis during a subsequent irradiation sequence with far-red/red or blue/red. Seedlings were 60 hours old and grown in 5×10^{-3} M. phenylalanine. D = dark; R = red; B = blue; FR = far-red. * Significant difference at $P = 0.05$

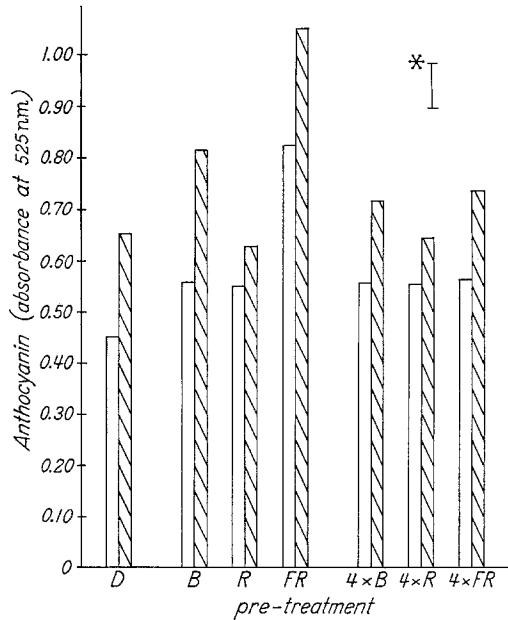


Fig. 4

Fig. 4. The effect of irradiation pre-treatments given before exposure to continuous blue light. All seedlings received high temperature before beginning the irradiation sequences. During the four hour pre-treatment plants received continuous blue (B), far-red (FR) or red (R), or four 5-minute shots of blue (4x B), far-red (4x FR), or red (4x R) given once every hour. The plants without pre-treatment (D) remained for a further 4 hours in darkness at 35°C before transfer to blue light. After the main light treatment of 6 hours continuous blue, plants were placed in darkness immediately (open bars) or after 10 minutes of red light (hatched bars). Seedlings were 58 hours old and grown in 5×10^{-3} M. phenylalanine. * Significant difference at $P = 0.05$

increase in yield. A continuous pre-irradiation with blue increased yield slightly, but significantly, more than intermittent blue or far-red; continuous far-red resulted in a further large increase in yield.

When treatments without a terminal exposure to red are considered (Fig. 4, open bars), pre-irradiation with intermittent or continuous blue or red, and intermittent far-red gave approximately equal yields. Pre-treatment with continuous far-red again resulted in an appreciably greater yield.

A further comparison was made between the effects of continuous or intermittent exposures to blue with, and without, a terminal red treatment. When followed by red, the yields in blue (Table 2) were similar to those obtained with the comparable far-red pre-treatments (Fig. 2); without a terminal exposure to red all treatments with blue gave higher anthocyanin yields than the equivalent far-red pre-treatments. Intermittent blue resulted in the formation of an appreciable amount of

Table 2. *The Effect of a Single 5-Minute Exposure to Red Light Given After Various Pre-Treatments with Blue*

All seedlings were given high temperature before beginning the irradiation sequence. They were 60 hours old and grown in $5 \times 10^{-3} M$ phenylalanine

Light treatment		Antho- cyanin (absorbance at 525 nm)	Effect of red light
During first four hours	Immediately before transfer to darkness		
—	none	0.126	+0.041
	5 min red	0.167	
$8 \times (5 \text{ min})$ shots of blue	none	0.201	+0.027
	5 min red	0.228	
$16 \times (5 \text{ min})$ shots of blue	none	0.240	+0.044
	5 min red	0.284	
Continuous blue	none	0.317	+0.090
	5 min red	0.407	

Significant difference at $P = 0.05; 0.040$.

anthocyanin and here it does not appear to be possible to separate the first from the second light step. It is, therefore, presumed that the P_{fr} concentration in blue light is sufficiently high to permit the formation of some anthocyanin even with one 5-minute exposure every 30 minutes.

IV. Discussion

1. *The Effect of Far-Red and Red*

Two lines of evidence have been presented in this paper. Results from experiments in which exposure to far-red has preceded exposure to red have shown a synergistic increase in anthocyanin yield compared with the yield from the two wave-lengths given separately. Furthermore, the effect of a single short exposure to red light, which alone causes almost no anthocyanin synthesis, is markedly increased by a prior irradiation with a few hours of continuous or intermittent far-red light, and this is completely reversible by a subsequent short exposure to far-red. The results with intermittent far-red are particularly interesting as, when

given alone, this yields little anthocyanin. It is interesting to compare here the results of KONISHI and GALSTON (1963), who found that brief far-red flashes were more effective than brief red flashes in causing the synthesis of some unidentified phenolic compounds; it is therefore possible that a phenolic precursor of anthocyanin is produced as a result of the first light step in turnip.

These results taken alone can be interpreted to mean that two photochemical reactions are involved in the formation of anthocyanin in turnip. One occurs in continuous or intermittent far-red light and appears to lead to the production of a precursor, perhaps a phenolic compound; the second is the photochemical formation of P_{fr} phytochrome, which results in the synthesis of anthocyanin; an adequate concentration of P_{fr} is maintained with intermittent red and in continuous far-red, but not with intermittent far-red. A different approach also led to the conclusion that two light steps are necessary for anthocyanin synthesis in turnip (GRILL and VINCE, 1964).

We may now turn to the second line of evidence, which shows that a prior irradiation with red light almost completely prevents the promoting effect of far-red. It was shown (Fig. 3) that, if four hours of red preceded the exposure to six hours far-red + 38 hours red, the final yield was similar to that obtained in continuous red. Similarly it was found previously (GRILL and VINCE, 1965) that a pre-treatment with red for a period of several hours markedly depressed the amount of anthocyanin formed in continuous far-red; brief exposures to red and far-red showed that this depended on the formation of P_{fr} . Furthermore, as the depression was largely prevented if plants were maintained at low temperature or in nitrogen during the pre-treatment period, it was suggested that it resulted from the destruction of phytochrome. The photoreceptor in both red and far-red was, therefore, assumed to be phytochrome, the yield in each wave-band depending on the relative rates of formation and destruction of P_{fr} .

The situation appears to be more complex than this, however, if two inter-dependent photochemical reactions are involved, the first (far-red promoted) yielding products for the second (red-promoted) reaction. There seems little doubt that the latter is the red/far-red reversible reaction of phytochrome and depends on the production of P_{fr} in red or continuous far-red. The far-red promoted reaction also seems to require phytochrome, as the far-red enhancement is almost entirely prevented by a pre-treatment with red light, presumed to act by destroying phytochrome. Thus there appear to be two phytochrome dependent steps involved in anthocyanin synthesis in turnip, the first occurring better in far-red light because of the rapid rate of phytochrome destruction in red.

It is known that there are two kinds of phytochrome; of the total phytochrome present in etiolated tissue a large fraction is photolabile, while a small fraction remains active even in continuous light. It is possible that the pigment mediating the "far-red promoted" reaction is the destructible type of phytochrome, while the final red-promoted reaction depends on the non-destructible type.

At present this suggestion cannot be supported by evidence of any chemical difference between the two kinds of phytochrome and it must be emphasised that the depressing effect of a pre-treatment with red light has not been conclusively proved to be due to phytochrome destruction. Other interpretations are possible. For example, red may act to cause the dissipation of a stored precursor by forming a substance which cannot react to yield anthocyanin in the absence of the product of the far-red reaction. If this were the case, then the pigment mediating the response to far-red is not necessarily phytochrome.

2. *The Effect of Blue Light*

In blue light the situation is equally complex. Some P_{fr} must be present because of the absorption band of P_r in blue, although the effect of a six-hour irradiation was considerably increased by a short terminal exposure to red (Fig. 4). This cannot, however, be the only reaction occurring because a few hours of blue, given before transfer to continuous high intensity red light, results in a large increase in yield. Pre-irradiation with red does not markedly reduce anthocyanin formation in blue light nor does it prevent the enhancement effect when blue is followed by prolonged red; furthermore, pre-treatment with blue light markedly decreases the yield in far-red (GRILL and VINCE, 1965) and presumably, therefore, causes phytochrome destruction. These points seem strong evidence in favour of the existence of another photoreceptor, in addition to phytochrome, mediating the reactions that occur in blue light. The marked synergism that occurs when blue light precedes exposure to red suggests that, in blue light also, substrates for phytochrome action may be produced.

The interrelationship between the reactions occurring in blue and far-red is still obscure. Results from experiments not reported here show a synergistic increase in yield when blue follows far-red. Here (as in the experiment shown in Fig. 4) blue light probably has two actions: firstly the presence of P_{fr} during the irradiation with blue and for some time afterwards results in anthocyanin formation utilising substrates accumulated during the first irradiation with far-red and, secondly, the blue "high-energy" reaction will lead to anthocyanin formation (possibly also by the production of substrates for phytochrome action). The observed synergism between far-red and blue may thus depend entirely on P_{fr}

formation in blue light; how closely the "high-energy" reaction in blue is linked with the reactions proceeding in far-red is not known.

Zusammenfassung

Die Substitution der ersten 6 Std einer Hellrot-Dauerbestrahlung durch Dunkelrot führte zu einem starken Anstieg im Anthocyangehalt, der höher war als die Summe aus Dunkelrot und Hellrot, wenn beide Bestrahlungen getrennt gegeben wurden. Folgte auf intermittierende Dunkelrot-Bestrahlung eine einmalige Dosis Hellrot, bildete sich eine beträchtliche Menge Anthocyan, obwohl jede Bestrahlung für sich kaum wirksam war. 4 Std Dauerdunkelrot induzierten bereits meßbare Anthocyanbildung, die durch kurze Hellrot-Bestrahlung weiter gesteigert werden konnte; der Effekt dieser terminalen Dosis Hellrot konnte durch nachfolgende kurze Dunkelrot-Bestrahlung wieder rückgängig gemacht werden. Daraus wird geschlossen, daß wenigstens zwei photochemische Reaktionen bei Bestrahlung mit Hellrot und Dunkelrot ablaufen, wobei die erste Substrat(e) für die zweite produziert.

Wurde vor einer Dunkelrot-Hellrot-Sequenz mit Hellrot bestrahlt, ging die fördernde Wirkung von Dunkelrot fast vollständig verloren und der Anthocyangehalt entsprach annähernd dem in Hellrot. Der Effekt der Hellrot-Vorbestrahlung wird auf die Destruktion von Phytochrom zurückgeführt und es wird vermutet, daß Phytochrom das einzige Pigment ist, das bei der Anthocyan-synthese in Hellrot und Dunkelrot beteiligt ist. Eine mögliche Interpretation wäre, daß die „Hochenergiereaktion“ in Dunkelrot und die Hellrot-Dunkelrot reversible Niederenergiereaktion durch verschiedene Formen von Phytochrom vermittelt werden.

Die Substitution der ersten 6 Std einer Dauerbelichtung mit Hellrot durch Blau ergab ebenfalls eine synergistische Zunahme im Anthocyangehalt. Der fördernde Effekt von Blaulicht konnte jedoch durch Vorbestrahlung mit Hellrot nicht verhindert werden. Daraus wird geschlossen, daß Phytochrom nicht das einzige Pigment sein kann, das die Reaktionen in Blaulicht vermittelt. Der Synergismus zwischen Blau und Hellrot läßt vermuten, daß die Hochenergiereaktion in Blau zur Produktion von Substrat führt, mit dem Phytochrom reagieren kann.

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