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PHOTOCONTROL OF ANTHOCYANIN FORMATION IN TURNIP SEEDLINGS

I. DEMONSTRATION OF PHYTOCHROME ACTION

By

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Wit 3 Figures in the Text

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Summary

The participation of the red/far-red reversible reaction of phytochrome in the control of anthocyanin formation in turnip seedlings has been demonstrated. A brief exposure to red light following a preliminary irradiation period in blue, increased anthocyanin content compared with blue alone; this effect was reversed by a subsequent short exposure to far-red. The sensitivity to red light was largely restricted to 24 hours old seedlings when grown in water at 25° C. Sensitivity was restored in older seedlings when they were grown in phenylalanine and kept in high temperature (35° C) for several hours before light was given; under these conditions, the phytochrome effect was greater in 48 hours old than in 24 hours old seedlings. In the youngest seedlings the largest increase occurred when red followed a preliminary blue exposure of at least 12 hours; in older seedlings the maximum response to red was almost attained after only 4 hours of blue light. Hypocotyl elongation was shown to be hardly affected by the reversible reaction of phytochrome. Possible reasons for these changes in sensitivity to phytochrome are discussed.

1. Introduction

Although the role of light in anthocyanin synthesis has been studied in many plants, it is still not certain how many photoreceptors are involved. This question has been discussed in several recent reviews (MOHR, 1964, VINCE, 1964, DOWNS, 1964). The participation of both the red/far-red reversible reaction of phytochrome and of a prolonged light reaction has been confirmed for seedlings of *Sinapis alba* (MOHR, 1957), *Fagopyrum* (MOHR and VAN NES, 1963), *Sorghum* (DOWNS and SIEGELMAN, 1963), and for apple skin (DOWNS, 1964). In turnip seedlings, however, an attempt to demonstrate phytochrome control of anthocyanin formation gave negative results and only a prolonged light reaction could be shown (SIEGELMAN and HENDRICKS, 1957). Observations (GRILL and VINCE, 1964) that seedling age, as well as other conditions, markedly affected anthocyanin content in turnip seedlings suggested that it would be worthwhile re-examining the question of whether phytochrome is really without effect on anthocyanin synthesis in this plant.

2. Materials and Methods

These were similar to those used previously (GRILL and VINCE, 1964). Seedlings of *Brassica rapa* "Sutton's Red Globe" were grown on units consisting of three microscope slides wrapped in filter paper (Whatman No. 1); 25 seeds were sown on each unit and two units were placed in a transparent plastic box ($14 \times 8 \times 6$ cm) containing 20 ml de-ionized water or 5×10^{-3} M phenylalanine. Seeds were germinated and grown in the dark at 25° C for 24, 48 or 72 hours before illuminating, and measurements of anthocyanin content were made 48 hours after the beginning of the light treatments. Removal of the seed coats was carried out over a green safe light, as described previously. The primary irradiation treatments were given with blue light; afterwards the seedlings were either transferred directly to darkness or were given 10 min red, or 10 min red followed by 10 min far-red, before transfer to the dark. During the illumination treatments the boxes were placed in lighted cabinets. These were 1) blue: blue fluorescent lamps with Strand No. 32 medium-blue cinemoid filter giving a wave-band from 380—520 nm (SALE and VINCE 1959), 2) red: magnesium arsenate fluorescent lamps with Strand No. 6 (primary red) and No. 1 (yellow) cinemoid filters giving a wave-band from 600—700 nm with a maximum at 656 nm (VINCE, BLAKE and SPENCER, 1964) and 3) far-red: 1 kW tungsten-filament tubular lamp with a 2 mm thick glass filter (Schott & Gen. R.G. 9) and a 7 cm thick flowing water screen; emission begins at 695 nm and rises to a maximum at 840 nm. The energy levels were 6.16, 3.58 and 3.51 Kergs cm^{-2} sec^{-1} in the blue, red and far-red cabinets respectively, giving approximately equal incident quanta.

The temperature was maintained at 25° C throughout or, in the high temperature treatments, was raised to 35° C overnight before the light treatment began, giving about nine hours at high temperature.

Anthocyanin content was measured as follows. Hypocotyls or cotyledons from 25 seedlings were extracted in 5 ml 1% aqueous hydrochloric acid for 48 hours at 5° C; 0.2 ml HCl (s.g. 1.16) was then added to the extract including the plant material and the tubes placed in boiling water for 20 minutes. The extract was made up to 5 ml with water and centrifuged at $3{,}100 \times G$ for 20 minutes. Absorbance of the clear supernatant was measured at 525 nm using a 1 cm path. Each datum is the mean for three or four experiments. Although hypocotyls and cotyledons were extracted separately, only values for total anthocyanin content are given as the treatments with red and far-red, phenylalanine and high temperature were not found to affect differentially the amounts of anthocyanin formed in hypocotyls and cotyledons.

3. Experimental Results

If phytochrome participates in a photoresponse, the effect of red light will be prevented or reduced by a subsequent brief exposure to far-red light. In turnip, when 3 days old seedlings were given a primary irradiation with 4 hours of white light to induce some anthocyanin formation, subsequent brief irradiations with red or far-red did not affect the amount of pigment formed and, consequently, the absence of phytochrome control was assumed (SIEGELMAN and HENDRICKS, 1957). As both seedling age and duration of irradiation are important factors determining the yield of anthocyanin in turnip, experiments with younger seedlings as well as with increased durations of a primary blue irradiation period were carried out. The results given in Fig. 1

show clearly that, for seedlings grown in water (W) at 25° C throughout, there was no reversible phytochrome effect of any magnitude, except in the youngest seedlings. In these the main effect of the second irradiation was to increase anthocyanin content when red followed blue compared with blue given alone; when far-red immediately followed

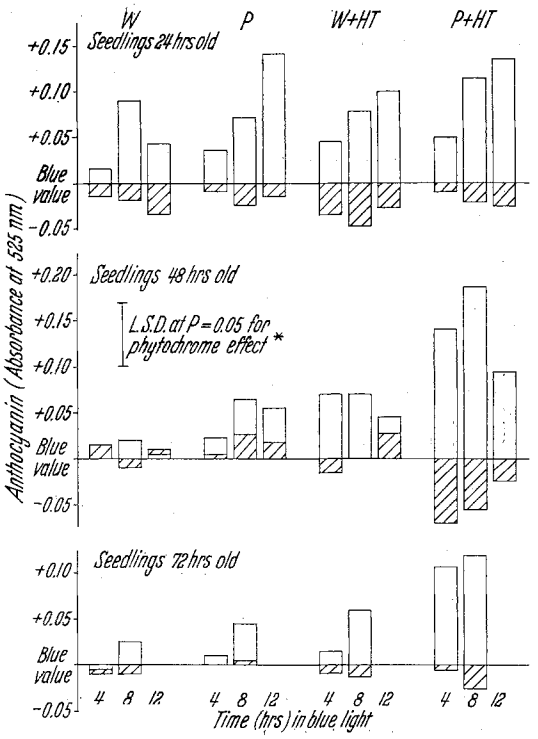

Fig. 1. Increase in the amount of anthocyanin formed by 10 minutes red light (open bars) and its reversal by 10 minutes of far-red given subsequently (shaded bars): the red and red/far-red treatments followed various durations in blue light and the results are given as increases (plus values) and decreases (minus values) compared with the appropriate blue control. Seedlings of three ages were grown in water (W) or phenylalanine (P), and were either kept at 25° C throughout or pretreated with high temperature (HT)

red the amount of anthocyanin formed was reduced only slightly compared with blue. The effect of phytochrome was only significantly large after at least 8 hours of blue light, and was still considerable after 16 hours (data from another experiment not given in Fig. 1). For seedlings older than 24 hours at the beginning of the blue light treatment the effects of the post-irradiation treatments were negligible; the lack

* The analysis was carried out with the differences between red and red/far-red values in each treatment; from the data presented the phytochrome effect for any one treatment can be calculated by subtracting the red/far-red value (positive or negative) from the red value.

of effect of brief exposures to red or far-red following a primary 4 hour irradiation of 3 days old seedlings, as found by SIEGELMAN and HENDRICKS (1957) was thus confirmed.

The decline in responsiveness to red and far-red with increasing age may be caused by lack of substrates or may be due to some more basic change in phytochrome physiology or both. Phenylalanine was observed previously to increase anthocyanin synthesis in turnip seedlings and also to shift the maximum anthocyanin content in blue light from

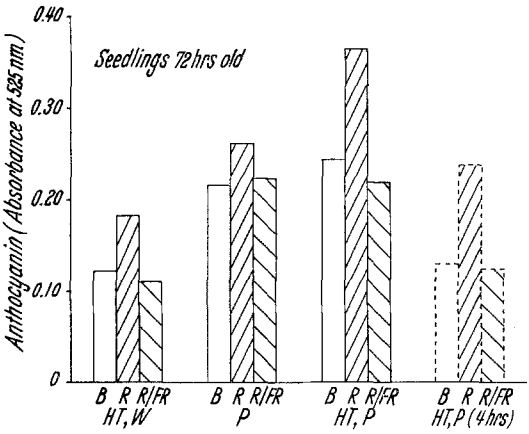

Fig. 2. Red/far-red reversibility effects in 72 hours old seedlings showing that the magnitude of the red/far-red effect is not necessarily related to the amount of anthocyanin formed in response to blue light. Seedlings were grown in water (W) or phenylalanine (P) and were either kept at 25° C throughout or pre-treated with high temperature (HT) before irradiation with 8 hours (or 4 hours) of blue light (B) followed by 10 minutes red (R) or 10 minutes red then 10 minutes far-red (R/FR)

24 hours old to 48 hours old seedlings (GRILL and VINCE, 1964, Fig. 1); therefore, red/far-red reversibility was tested for the three seedling ages in the presence of phenylalanine. Although the total anthocyanin content was increased, phenylalanine did not much affect the responsiveness to red and far-red (Figs. 1 and 2), except in the 24 hours old seedlings following exposure to 12 hours of blue light, where the red promotion was considerably increased.

Exposure to high temperature has been reported by TOOLE (1959) to increase red sensitivity in *Lepidium* seeds, which require light to promote germination. The temperature was, therefore, raised from 25° C to 35° C overnight and reversibility tested as before. The exposure to high temperature increased the response to red and far-red slightly in all treatments. However, when both phenylalanine and high temperature were given, the effects for the 48 and 72 hours old seedlings were synergistic and large increases in the responsiveness to red and far-red were found (Fig. 1); these increases were not dependent on the amount

of anthocyanin formed during the blue irradiation (Fig. 2). No synergistic effect was observed in the 24 hours old seedlings.

An analysis of the data of Fig. 1 for the 24 and 48 hours old seedlings showed a significant interaction between age and duration of blue light ($P < .001$); in the younger seedlings the magnitude of the phytochrome effect increased with increasing duration of blue light from 4 to 12 hours but in the older seedlings the maximum phytochrome effect was almost attained after only 4 hours of blue and the response decreased after 8 hours.

Discussion

The results afford physiological evidence for the participation of the red/far-red reversible reaction of phytochrome in the control of anthocyanin synthesis in turnip seedlings. The effect, however, seems rather small compared with that of the prolonged light reaction. It is interesting that the effect of phytochrome on hypocotyl elongation was negligible, and was hardly affected by the various treatments (Fig. 3).

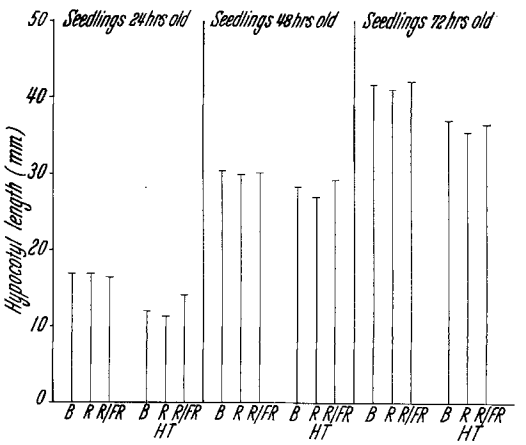

Fig. 3. Effects of phytochrome on hypocotyl elongation. Seedlings of three different ages were grown at 25° C throughout or pre-treated with high temperature (HT) before irradiation with 4 hours of blue light (B) followed by 10 minutes red (R) or 10 minutes of red then 10 minutes far-red (R/FR). Seedlings were grown in phenylalanine

The phytochrome effect clearly decreases as the duration of growth in darkness before irradiation is increased. The responsiveness can be completely restored (and indeed increased in 48 hours old seedlings) by a combination of phenylalanine feeding and high temperature pretreatment. The first question arising is what causes the loss in sensitivity to red and far-red and why it is restored by these particular treatments. One possibility is that the phytochrome content decreases during prolonged growth in darkness. De Lint and Spruit (1963) observed a decrease in the phytochrome content of etiolated maize mesocotyls over a period of three days; this decrease also occurred at low temperature. Furuya and Hillman (1964) on the other hand could detect no significant decrease in the phytochrome content of etiolated pea seedlings over a 10 day period. It seems unlikely, however, that a decreased

phytochrome content explains the loss of response with age, unless the combined treatments have the effect of maintaining phytochrome content in the dark, or perhaps of retarding phytochrome destruction in the light. Continuous irradiation has been found to cause a loss of phytochrome as a result of conversion to the less stable P_{FR} form (BUTLER et al., 1963). The former seems less likely as, in the 72 hours old seedlings, the high temperature treatment was given and was effective at a time when the response would already have been rather low without it. As the high temperature treatment was found slightly to inhibit growth of the hypocotyls (Fig. 3) additional materials may be made available for anthocyanin synthesis. However, the high temperature may cause some change in the conditions in the tissue that indirectly favours a phytochrome response.

Table. *Cotyledons fresh weight (mg) after 48 hours of continuous irradiation with blue or far-red, with or without phenylalanine*

Seedling age (hr.)	blue		far-red	
	water	phenylal. 5×10^{-3}M	water	phenylal. 5×10^{-3}M
24	83.9	92.0	116.0	90.5
48	85.3	118.8	116.6	151.3
72	84.6	119.9	99.9	143.8

The effect of phenylalanine is presumably mainly as a precursor of the flavonoid B-ring, and both the reduction in total anthocyanin synthesis with increasing duration of growth in darkness and the increased phenylalanine effect (GRILL and VINCE, 1964) suggest that anthocyanin precursors are utilised during growth of the seedlings in the dark. Phenylalanine may, however, be used in other ways than in synthesis of phenolic compounds, as for instance in protein synthesis (MCCALLA and NEISH, 1959), the higher fresh weight value for cotyledons after feeding (Table) indicate that here its effect is not only to give rise to a pool of hydroxycinnamic acids.

The second problem is why the 24 hours old seedlings should be increasingly responsive to phytochrome as the duration of blue light increases from 4 to 12 hours while older seedlings show almost the maximum response after only 4 hours. It is possible that phytochrome is being synthesised during the first 48 hours of growth as has been found in maize (BUTLER and LANE, 1965) even though its effectiveness is reduced; certainly the biggest phytochrome effect occurs in the 48 hour old seedlings when they are given a high temperature pretreatment and are not limited by B-ring precursors. The subsequent decline in responsiveness in older seedlings and with longer durations of blue light may result from a variety of causes, e.g. phytochrome destruction in light or with increasing age, decrease in substrates other than phenylalanine or change in tissue sensitivity. It is probably perti-

nent that the synthetic ability of the seedlings in response to prolonged irradiations also declines with age and cannot be restored by feeding with phenylalanine (GRILL and VINCE, 1964).

Zusammenfassung

Die Beteiligung des reversiblen Hellrot-Dunkelrot-Pigmentsystems Phytochrom wurde bei der Kontrolle der Anthocyansynthese in Keimlingen von *Brassica rapa* nachgewiesen. Durch kurze Hellrotbestrahlung wird der Anthocyangehalt gegenüber dem der vorausgehenden Bestrahlung mit Blaulicht erhöht. Dieser Effekt wird durch eine nachfolgende kurze Bestrahlung mit Dunkelrot wieder mehr oder weniger aufgehoben. Die Empfindlichkeit gegen Rotlicht ist weitgehend auf 24 Std alte, in Wasser gewachsene Keimlinge beschränkt. Die Empfindlichkeit konnte in älteren Keimlingen wieder hergestellt werden, wenn diese in Phenylalanin aufgezogen und mehrere Stunden vor der Bestrahlung bei hoher Temperatur (35° C) gehalten wurden. Unter diesen Bedingungen war der Phytochrom-Effekt in 48 Std alten Keimlingen größer als in 24 Std alten Keimlingen. In den jüngsten Keimpflanzen wurde die größte Zunahme durch Hellrot erzielt, wenn wenigstens 12 Std mit Blaulicht vorbestrahlt worden war. In älteren Keimlingen wurde die maximale Hellrotwirkung schon nach etwa vierstündiger Vorbelichtung erreicht. Das Hypokotylwachstum war durch das Phytochromsystem kaum beeinflußbar. Mögliche Gründe für die Veränderungen in der Phytochrom-Empfindlichkeit werden diskutiert.

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