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Interaction between Light Quality and Light Quantity in the Photoregulation of Anthocyanin Production

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ABSTRACT

The interaction between phytochrome photoequilibrium (ϕ) and photon flux in the photoregulation of anthocyanin production under prolonged irradiation was studied in seedlings of *Brassica oleracea* L. and *Lycopersicon esculentum* Mill. In cabbage, anthocyanin production increases with decreasing ϕ, reaching a maximum at the lowest value (ϕ = 0.13) used in this study; in tomato, the extent of the response is higher at intermediate values, reaching a maximum at ϕ = 0.46. In cabbage, the response increases with increasing photon flux at all ϕ values; however, the response to changes in photon flux is minimal at ϕ = 0.85, and, at ϕ = 0.13, minimal at photon fluxes higher than 5 micromolar per square meter per second. In tomato, the response increases with increasing photon flux at ϕ = 0.46, 0.65, and 0.85, the response to changes in photon fluxes being minimal at ϕ = 0.85; at ϕ = 0.13 and 0.29 the response first increases (significantly at ϕ = 0.29 and minimally at ϕ = 0.13) and then decreases with increasing photon fluxes, the transition occurring at about 1 micromolar per square meter per second at ϕ = 0.13, and at 5 micromolar per square meter per second at ϕ = 0.29. The patterns of light quality-quantity interaction in the photoregulation of anthocyanin production are significantly different in cabbage and tomato and are also significantly different than those observed for other photomorphogenic responses to prolonged irradiations.

The production of high levels of anthocyanin in young seedlings requires prolonged exposures to visible and near-visible radiation at relatively high photon fluxes[1]; the extent of the response is a function of light quality and quantity (9, 10). Anthocyanin production and other plant photomorphogenic responses to prolonged irradiation in the R[2] to FR (600–760 nm) spectral region are mediated by phytochrome (14, 17). Phytochrome, a plant photosensory pigment, is reversibly interconverted by light between two forms, Pr and Pfr; Pfr is generally considered as the physiologically active form (17). According to current hypotheses (5), the state of phytochrome under prolonged irradiation is a state of dynamic equilibrium, which is a function of wavelength and photon flux. This state can be defined, in first approximation, by the values of ϕ and H: ϕ, the Pfr/Ptot ratio at photoequilibrium, is a function of light quality; H, the rate of cycling between Pr and Pfr at photoequilibrium, is a function of light quality and photon flux. Reasonably enough, in current hypotheses (5, 14), the wavelength and photon flux dependence of the state of phytochrome at photoequilibrium is considered the basic factor responsible for the wavelength and photon flux dependence of phytochrome-mediated responses to prolonged irradiations. However, the variability in the wavelength and photon flux dependence of phytochrome-mediated responses to prolonged irradiations (14) has not allowed the development of a unified model for the relationships between phytochrome states and extent of the expression of the response.

Previous studies in my laboratory (10, 16) have shown that the R/FR effectiveness ratio for anthocyanin production under continuous irradiation can be significantly affected by the duration and the photon flux of the light treatments. However, specific studies on the relationships between the state of phytochrome, as defined by ϕ and H, and anthocyanin production, under continuous light treatments, at values of ϕ different than those established by R and FR, had not been carried out. The purpose of this study was to determine the extent of the differences in the pattern of interaction between light quality and quantity on phytochrome-mediated anthocyanin production in the seedlings of two species, *Brassica oleracea* L. and *Lycopersicon esculentum* Mill., which show large differences in the sensitivity of the response to R and FR (10, 16, 20). Anthocyanin production in both species shows a marked response to BL, which is mediated by cryptochrome (21). The simultaneous excitation of cryptochrome and phytochrome complicates the interpretation of results in terms of the state of phytochrome. To avoid this problem, the light sources used in this study contained no detectable radiation at wavelengths shorter than 550 nm, and thus, on the basis of present understanding of the characteristics of the two photoreceptors (18), they should produce only a minimal, if any, excitation of cryptochrome.

MATERIALS AND METHODS

Plant Material

Seeds of cabbage (*Brassica oleracea* L., cv Red Acre, Burpee No. 5291, lot 8793-78) and tomato (*Lycopersicon esculentum* Mill., cv Beefsteak, Burpee No. 6127, lot 44411-79) were sown in Petri dishes on filter paper moistened with a solution

[1] Photon flux: quantity of photons per unit area per unit time intercepted by a flat surface, as measured with a radiation detector having an angle of acceptance equal or close to 180° and with a cosine correction applied to radiation arriving at angles which are not normal to the receiver surface (see ref. 7).

[2] Abbreviations: R, red light: FR, far red light; $\phi = k_1/k$, Pfr/Ptot at photoequilibrium; Ptot, (Pr + Pfr) total phytochrome; H, rate of cycling between Pr and Pfr at photoequilibrium; BL, blue light; k_1, rate constant for Pr to Pfr photoconversion; k_2, rate constant for Pfr to Pr photoconversion; k, k_1, + R_2, rate constant for phytochrome photoconversion; RF, simultaneous irradiation with R + FR.

of streptomycin (200 μg/mL; 8 mL per dish) and were incubated in darkness until the beginning of the light treatments, which started 4 d after sowing. Streptomycin inhibits Chl synthesis and enhances anthocyanin production (13) and was used to reduce the differences in the screening effects of Chl on the state of phytochrome during the course of the light treatments. The seedlings grew in the presence of streptomycin from sowing to the end of the light treatments. Temperature during the 4 d dark incubation was 20°C for cabbage and 25°C for tomato; temperature during the light treatments was 22 to 23°C.

Extraction and Assay of Anthocyanin

Lots of 30 (cabbage) and 55 (tomato) seedlings were extracted with 18 ml of 1% HCl (w/v) in methanol for 2 d at 3 to 5°C with occasional shaking. The absorbance of the extracts, clarified by filtration, was measured at 530 nm (peak of absorption of anthocyanin) and 657 nm (peak of absorption of degradation products of Chl in acidic methanol). The formula, $A_{530} - 0.25A_{657}$, was used to compensate for the contribution of degradation products of Chl to the absorption at 530 nm. The absorbance values reported in the figures were corrected by subtracting the absorbance values of the dark controls (cabbage, 0.045; tomato, 0.015) and represent the means of 16 replicate samples (each sample consisting of 30 cabbage or 55 tomato seedlings) for each treatment, from two independent experiments. The standard errors were about 3 to 5% of the mean values.

Light Sources

The R and FR light sources were mounted on a steel frame, housed in a temperature-controlled room. The R source, placed at the top of the frame, consisted of 8 Sylvania special phosphor (F36T12/236/HO) fluorescent lamps wrapped with Roscolux red filter No. 27. The FR source consisted of 16 (in two banks of 8 each, mounted on the sides of the frame) Sylvania special phosphor (F36T12/232/HO) fluorescent lamps wrapped with Roscolux filters, blue No. 83 and red No. 27. The spectral photon flux distribution of the two light sources was reported previously (11). There was no detectable photon flux at wavelengths shorter than 550 nm. The maximum photon fluxes delivered at seedling level (the light measuring instrument was an ISCO Spectroradiometer model SR) were: R, 24 μmol m^{-2} s^{-1}; FR, 51 μmol m^{-2} s^{-1}. Photon fluxes were reduced as required by using Cornelia black polypropylene fabric of various mesh sizes (E.C. Geiger, Inc.) with flat transmission curves (maximum deviation 3%) between 350 and 800 nm. The two sources were used alone or in combination to produce five values of ϕ: 0.13, 0.29, 0.46, 0.65, and 0.85. The light treatments for anthocyanin production started with 5 min at the maximum photon flux available for the selected value of ϕ, to ensure rapid establishment of photoequilibrium, and then continued at the specified photon flux.

Spectrophotometric Measurements of Phytochrome *in Vivo*

A custom-built, computer-controlled dual-wavelength spectrophotometer (19) was used for the assay of phytochrome *in vivo*. The measuring wavelengths were 730 and 800 nm. The actinic light source of the spectrophotometer consisted of a 500 W CBA projection lamp and 660 and 740 nm interference filters, half-bandwidth about 15 nm. The seedling sample (25 seedlings for cabbage and 40 for tomato) was packed into a cylindrical aluminum cuvette (i.d. 1 cm, transparent Plexiglas bottom) to a depth of 3 mm and kept cold during the measurement cycle using ice in the cuvette holder. The values of ϕ, k, k_1, and k_2 were determined as described in detail previously (11). The H values cannot be measured directly and were calculated from the formula $H = (1 - \phi)k_1 = \phi k_2$ (2). Values of ϕ and k under R and FR were determined first, and used to calculate the R/FR photon flux ratios required in simultaneous R + FR irradiations to produce ϕ values of 0.30, 0.45, and 0.65; the average, measured values were 0.29, 0.46, and 0.65. The experiments for each combination of ϕ and photon flux proceeded as follows: the light source, R, FR, or combination, was set as required; ϕ and k were measured; subsequently, the seedlings were exposed to the 24 h treatment used for anthocyanin production. Rates of photoconversion at photon fluxes higher than 5 to 10 μmol m^{-2} s^{-1}, depending on the ϕ value, were too fast for accurate determination and were extrapolated from the data obtained at lower photon fluxes; ϕ values were measured at all photon fluxes used. Measured values of ϕ and k *in vivo* were preferred over projected values (calculated from the spectral photon flux distribution of the light sources used and photochemical parameters of purified phytochrome) because projected values may vary significantly depending upon the set of photochemical parameters of phytochrome used in the calculations (12) and are also significantly different than those measured *in vivo* (11). Even though measured values were used, there are some limitations that must be mentioned. The values of ϕ and H used in these study were obtained from measurements in 4 d old, dark-grown seedlings. It is not known if these values remain the same throughout the course of the 24 h light treatments used for anthocyanin production. As a consequence of phytochrome destruction in light, the phytochrome content is soon reduced to levels that are too low for accurate measurements of photoconversion rates, especially in tomato, whose phytochrome content (0.007–0.009 $\Delta\Delta A$ per 40 seedlings) is much lower than in cabbage (0.019–0.025 $\Delta\Delta A$ per 25 seedlings). A few measurements of ϕ (2–4 per treatment), taken about 4 h after the beginning of the light treatments, showed no significant differences from the ϕ values given above.

RESULTS AND DISCUSSION

Anthocyanin production was expressed as a function of photon flux (Fig. 1), H (Fig. 2), and ϕ (Fig. 3) to emphasize, respectively, the dependence on these three variables and to facilitate the comparison with data from other studies. Expressing the response as a function of ϕ and H (Figs. 2 and 3), instead of ϕ and photon flux (Fig. 1), is preferable for the

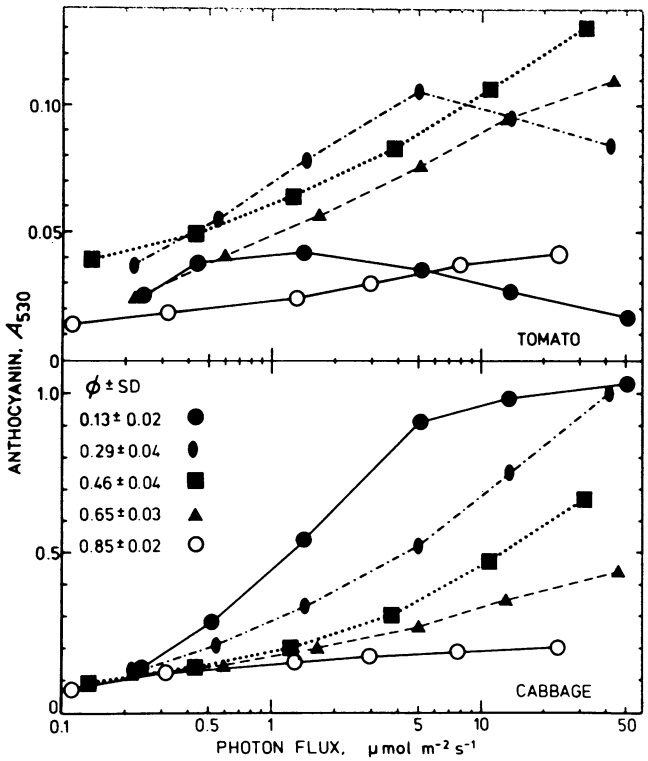

Figure 1. Photon flux dependence of anthocyanin production in cabbage and tomato seedlings at various values of ϕ. Anthocyanin was extracted after 24 h continuous irradiation.

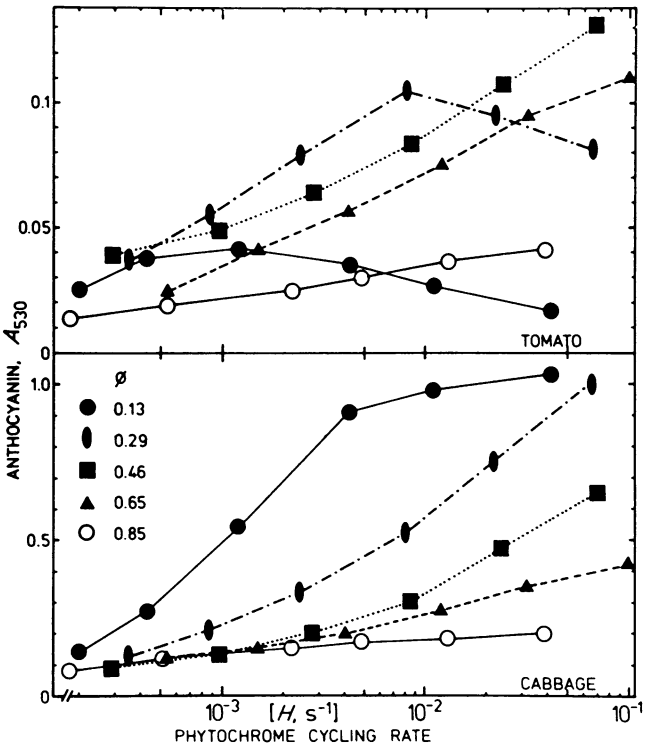

Figure 2. Dependence of anthocyanin production in cabbage and tomato seedlings on phytochrome cycling rates at various values of ϕ. Anthocyanin was extracted after 24 h continuous irradiation.

analysis of the relationships between phytochrome state and extent of phytochrome-mediated responses (2, 6), because, in Figure 1, points at the same abscissa (same photon flux) on the different ϕ curves differ not only in ϕ, but also in H. The relationships between photon flux, k, and H for the values of ϕ used in this study are given in Table I. Both k and H increase with increasing photon flux; the fact that the maximum values of H and k are not at the same value of ϕ is a consequence of the relationships between cycling rates, ϕ, and rates of photoconversion, as defined in the equation for the rate of cycling, $H = (1 - \phi)k_1 = \phi k_2$ (data on the relationships between ϕ, k, and H at wavelengths between 310 and 790 nm can be found in Tables 3 to 6 in ref. 12). In terms of the general aspects of the effects of light quality and quantity on the expression of the response, the two presentations convey essentially the same information.

The first noticeable difference between the two species is the extent of the response: maximum anthocyanin production in tomato is only about 20% of that in cabbage under the same treatment and only about 13% of maximum production in cabbage (Figs. 1 and 2). The large difference in phytochrome content between tomato and cabbage (see "Materials and Methods") might be one of the factors responsible for the difference in the extent of the response. The results of preliminary studies with phytochrome-poor tomato mutants (1) and phytochrome-enriched transgenic tomato seedlings (3) suggest a positive correlation between the phytochrome content and the extent of anthocyanin production and other phytochrome-mediated responses. But other factors, in addition to differences in phytochrome content, might be responsible for the difference in anthocyanin production between cabbage and tomato. For example, the *hp* tomato mutant, whose phytochrome content is about the same as that of the wild type, produces about three times as much anthocyanin as the wild type, possibly as a consequence of increased responsiveness toward Pfr (1).

The second noticeable difference between the two species is in the dependence of the response on ϕ. In cabbage, anthocyanin production increases with decreasing ϕ (Fig. 3), as expected: in this species, the peak of action for anthocyanin production under prolonged irradiation is in the FR waveband (9, 10). In tomato, anthocyanin production is higher at intermediate ϕ values (Fig. 3).

In cabbage, anthocyanin production increases with increasing photon flux and H (Figs. 1 and 2). The response to changes in photon flux and H is minimal at $\phi = 0.85$ and increases with decreasing ϕ. At $\phi = 0.13$, the response to changes in photon flux is pronounced between 0.25 and 5 μmol m^{-2} s^{-1} and minimal between 5 and 50 μmol $m^{-2}s^{-1}$. At the highest photon flux and H used in this study, there is no significant differences in anthocyanin production at $\phi = 0.13$ and 0.29.

In tomato, anthocyanin production increases with increasing photon flux and H at $\phi = 0.46$, 0.65 and 0.85 (Figs. 1 and 2). The response to changes in photon flux and H is minimal at $\phi = 0.85$, as observed in cabbage. At $\phi = 0.13$ and 0.29, anthocyanin production first increases, minimally at $\phi = 0.13$, and significantly at $\phi = 0.29$, and then decreases with increasing photon flux, the transition occurring at about 5 μmol m^{-2}

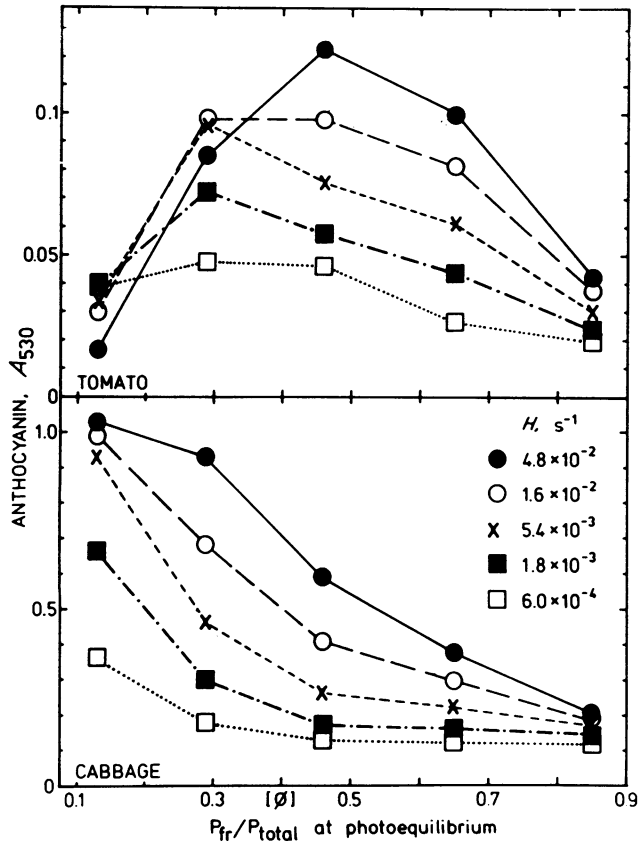

Figure 3. Effect of phytochrome photoequilibrium on anthocyanin production in cabbage and tomato seedlings at various cycling rates. Anthocyanin was extracted after 24 h continuous irradiation.

Table I. *Relationships between Photon Flux and Phytochrome Cycling Rate (H) at Different Values of ϕ*

Values of H calculated according to the equation $H = (1 - \phi)k_1 = \phi k_2$, using values of ϕ and rates of phytochrome photoconversion measured *in vivo* (see "Materials and Methods").

	Photon flux (μmol m^{-2} s^{-1})						
	2		4		8		
ϕ	k	H	k	H	k	H	H/k
	$10^3 \times s^{-1}$						
0.13	14.1	1.59	28.2	3.18	56.3	6.37	0.113
0.29	15.6	3.22	31.3	6.44	62.6	12.88	0.206
0.46	17.9	4.45	35.8	8.90	71.7	17.81	0.248
0.65	21.5	4.89	43.0	9.77	85.9	19.55	0.228
0.85	27.3	3.48	54.6	6.96	109.1	13.91	0.128

s^{-1} at $\phi = 0.29$, and at about 1 μmol m^{-2} s^{-1} at $\phi = 0.13$. A decrease of anthocyanin production in tomato with increasing irradiances of continuous FR ($\phi < 0.05$) had been reported before (20). The rate of phytochrome degradation under continuous irradiation is a function of ϕ and photon flux (4); an increase in the rate of degradation results in the decrease of Ptot and, consequently, of the Pfr level. The decrease of the Pfr level might be critical at low ϕ, especially in a system with a low phytochrome content such as tomato, and might be responsible for the observed decrease of anthocyanin production with increasing photon fluxes. Experiments are being designed to test this hypothesis.

The results obtained in this study support the argument on the importance of using appropriate operational criteria (18) to determine photoreceptor involvement in photomorphogenesis. Under irradiation from the light sources with no detectable BL used in this study, anthocyanin production in tomato reaches a maximum at $\phi = 0.46$ (Fig. 3), a value close to that (0.40–0.45) established by BL (11), the most effective spectral region for anthocyanin production in tomato (15). These observations, combined together, may suggest that the high efficiency of BL for anthocyanin production in tomato may be linked to maintaining an optimal value of ϕ rather than to the involvement of a specific blue-light photoreceptor (cryptochrome). However, experiments based on the principle of equivalent light action show that BL is significantly more effective than RF (a mixture of R and FR with no detectable BL) for anthocyanin production in tomato under conditions in which BL and RF maintain the same state of phytochrome ($\phi_{BL} = \phi_{RF}$; $k_{BL} = k_{RF}$; $H_{BL} = H_{RF}$; 21). Since BL excites both cryptochrome and phytochrome and RF excites phytochrome, but not cryptochrome, the difference in anthocyanin production between BL and RF treatments maintaining the same state of phytochrome can reasonably be attributed to the involvement of cryptochrome in the mediation of the action of blue light (21).

The extent of the response to changes in photon flux is significantly affected by ϕ, as shown by the differences in the slopes of the curves for different ϕ values (Figs. 1 and 2). Conversely, the relative efficiency of light of different spectral quality, establishing different values of ϕ, is significantly affected by the photon flux of the light treatment (Fig. 3). A valid argument can be made for the necessity of using a wide range of experimental conditions in studies of the basic characteristics of photomorphogenic responses to prolonged irradiations. The ϕ values used in this study are within the range of those that can be obtained using monochromatic radiation of wavelengths between 670 and 710 nm ($\phi_{670} = 0.83$–0.87; $\phi_{710} = 0.08$–0.14; 12). The results presented here suggest that, for example in studies of the wavelength dependence of the response, the wavelength of the peak of action, the width of the band at the peak of action, and the relative effectiveness of different wavelengths may vary significantly, depending on the photon flux.

The patterns of interaction between light quality and quantity on anthocyanin production are significantly different in cabbage and tomato and are also significantly different from those reported in studies of other responses to prolonged irradiation, for example, light-dependent inhibition of hypocotyl elongation. In etiolated mustard seedlings (8), the extent of the response (% inhibition of hypocotyl elongation) is a function of photon flux only at ϕ values between about 0.05 and 0.80; a response to changes in ϕ was observed only at $\phi < 0.05$. In light-grown cucumber (6) and mustard (8) seedlings, the extent of the response (% inhibition of hypocotyl elongation) increases with increasing ϕ. Anthocyanin production increases with decreasing ϕ in cabbage and peaks at intermediate ϕ in tomato (Fig. 3).

The variability in the characteristics (extent, spectral sensitivity, photon flux and duration dependence) of phytochrome-mediated responses to prolonged irradiations, often called high irradiance responses, is well documented in the literature (9, 10, 14), and is confirmed by the results reported here. The causes of the variability cannot be explained satisfactorily on the basis of the results available at present. This variability, some limitations in the methods that can be used to determine its causes, and the limitations in the interpretation of the results of photomorphogenesis research, imposed by the unknown nature of the mechanism of action of phytochrome at the molecular level, the unknown nature of the signal transduction chain, and the difficulties in the estimation of the state of phytochrome *in vivo*, are some of the reasons why these responses are still the least understood among those mediated by phytochrome.

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