Problems of Reporting Spectral Quality and Interpreting Phytochrome-mediated Responses

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Plant growth depends on radiation as a source of energy for photosynthesis, the process that transforms radiation energy into chemical energy. In addition to photosynthesis, plant growth and development depends on radiation through its effects on photomorphogenesis, i.e., processes that control growth and differentiation by photon flux in specific regions of the spectrum, independent of photosynthesis. Photon flux required for photosynthesis is much greater than that required for photomorphogenesis and is mainly absorbed by chlorophyllous pigments. In photomorphogenesis, photon flux in specific regions of the spectrum is perceived by biological photoreceptors present in small quantities. Upon absorption of photons in specific wavelengths of the spectrum, these photoreceptors induce signals that can lead to a specific plant response.

There are at least three photoreceptor systems present in higher plants. Two light-absorbing photoreceptors [ultraviolet blue (UV-B) and blue (B)] detect wavelengths only in the 370- to 500-nm range of the spectrum. Phytochrome, the widely studied photoreceptor system in higher plants, is capable of detecting wavelengths from 300 to 800 nm with maximum sensitivity in red (R) (600–700 nm) and far-red (FR) (700–800 nm) wavelengths of the spectrum. Phytochrome has at least two reversible light-absorbing forms; the red-absorbing phytochrome (P_r) absorbs R light maximally and upon absorption is transformed into the far-red-absorbing phytochrome (P_{fr}) which absorbs FR light maximally and is transformed into the P_r form. Of the two forms, the P_{fr} form is assumed to be the active form that controls signal transduction and plant response.

Interest in photomorphogenic responses has increased in recent years due to the increased use of electric light sources with a variety of spectral qualities. Photon ratios between the R and FR region of the spectrum (R : FR ratio) and in vitro estimates of phytochrome photoequilibrium [ø = amount of phytochrome in the P_{fr} form relative to total phytochrome ($P_{fr} : P_{tot}$)], based on photochemical properties of purified phytochrome and spectral photon flux distribution of light source, have been commonly used in discussing the relationship between light environment and phytochrome-mediated photomorphogenic responses. Although these parameters have been used to describe phytochrome-mediated responses, there are numerous problems in using them for comparing plant responses. The objective of this paper is to review the problems and difficulties of using photon ratios and in vitro estimates of ø in interpreting results in phytochrome-mediated photomorphogenic responses.

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R : FR ratio

Basic research on phytochrome has shown that phytochrome maximally absorbs photons in the R and FR regions of the spectrum and, therefore, the ratio of photon flux in the R region to that of the FR region (or vice versa) is often used to express light environment quantitatively (Smith, 1982).

[1]

A survey of literature indicates that a wide range of wavelengths centered around peak absorption of P_r [between 665–670 nm for phytochrome isolated from etiolated oat tissue and 650–655 nm for phytochrome from green oat (*Avena sativa* L.) tissue] and P_{fr} (between 730–735 nm for phytochrome from etiolated oat tissue and 725–730 nm for phytochrome from green oat tissue) has been used for R : FR ratio calculations. Some researchers have used narrow band widths (i.e., 10 nm) centered around peak absorbance [i.e., R = 655–665 nm and FR = 725–735 nm (Smith, 1982); R = 640–650 nm or 645–655 nm and FR = 725–735 nm (Kasperbauer et al., 1963)]; while others have used broad band widths (100 nm) [i.e., R = 600–700 nm and FR = 700–800 nm (Mortensen and Stromme, 1987)] for R : FR ratio calculations. The selections of band widths for R : FR ratio determination rely mainly on the inclination of the researcher.

Problems in correlating plant responses with R : FR ratio

To illustrate the difficulty of selecting band widths for the R : FR ratio and using the ratio for correlating plant response with the spectral quality, consider the following example. In previous papers, we have shown that light transmitted through $CuSO_4$ filters (4% to 40% $CuSO_4 \cdot 5H_2O$) reduced plant height of chrysanthemum [*Dendrathema ×grandiflorum* (Ramat.) Kitamura] compared to control (water) filters, and we hypothesized that the response under $CuSO_4$ filters may be mediated through the phytochrome system (Rajapakse and Kelly, 1992; Rajapakse et al., 1992, 1993). Initially, we attempted to explain the plant response under $CuSO_4$ filters using R : FR ratios calculated using broad (100 nm) and narrow (10 nm) band widths.

Based on narrow-band R : FR ratios (Table 1), one would expect the plant height of the control and under the 40% $CuSO_4$ filter to be similar. Plant height under 40% $CuSO_4$, however, was reduced similarly as for plants grown under a 6% $CuSO_4$ filter (Table 2), which had a very high narrow-band R : FR ratio (Table 1). This result suggests that, based on wavelength selection, R : FR ratio of a light source can vary considerably and the use of the R : FR ratio to explain differences in plant response could lead to wrong conclusions when evaluating light sources with little FR light or spectral filters that remove FR light (Fig. 1).

Phytochrome-related parameters

Most plant physiologists and photobiologists recognize that the R : FR ratio of a light source does not accurately explain phytochrome-mediated plant responses and that estimates of in vivo phytochrome parameters, such as ø, and phytochrome cycling rate should be used when correlating phytochrome-mediated responses with the light environment. Hayward (1984) argued that the R : FR ratio can be directly transformed into ø because established ø mainly depends on the energy in the R and FR wavelengths. However, this relationship holds true only when light sources with R and FR wavelengths are involved. The ø estimated only from the R : FR ratio has little value in explaining plant responses under broad-spectrum light sources, because phytochrome is capable of absorbing wavelengths from 300 to 800 nm.

When spectroradiometric data for 300- to 800-nm wave bands are available, ø can be estimated over the complete spectrum by inte-

Table 1. Variation in R : FR ratio, as influenced by band width, of light transmitted through $CuSO_4$ filters (adapted from Rajapakse et al., 1992).

$CuSO_4$ concn	R : FR[z]		
(%)	NB1	NB2	BB
0	1.1	1.2	1.1
4	2.8	3.4	3.3
6	4.0	5.0	5.2
10	4.7	6.3	6.7
15	3.5	5.6	6.0
20	1.7	2.7	3.0
30	1.3	1.7	2.8
40	1.1	1.3	2.0

[z]NB1 = narrow-band R : FR ratio using R = 655–665 nm and FR = 725–735 nm (Smith, 1982). NB2 = narrow-band R : FR ratio using R = 640–650 nm and FR = 725–735 nm (Kasperbauer et al., 1963). BB = broad-band R : FR ratio using R = 600–700 nm and FR = 700–800 nm (Mortensen and Stromme, 1987).

Table 2. Influence of control and $CuSO_4$ filters on height of 'Bright Golden Anne' chrysanthemum plants.

$CuSO_4$ concn (%)	Shade (%)	Plant ht (cm)
0 (Control 1; C1)	37	34.8
0 (Control 2; C2)	58	32.3
6	37	24.4
40	58	21.6
Contrasts[z]		
6 $CuSO_4$ vs. C1		***
40 $CuSO_4$ vs. C2		***
C1 vs. C2		*
6 $CuSO_4$ vs. 40 $CuSO_4$		**

[z]Single degree of freedom contrasts.
*,**,***Significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

grating photon fluxes with extinction coefficients of P_r and P_{fr} and the quantum efficiencies of P_r to P_{fr} and P_{fr} to P_r phototransformations. Methods for estimating ø from photochemical properties of purified phytochrome and spectral photon flux from the 300- to 800-nm range have been described by several researchers (Butler et al., 1964; Gardner and Graceffo, 1982; Kelly and Lagarias, 1985; Sager et al., 1988). In estimating ø, these researchers have used the photochemical properties of purified phytochrome obtained from various sources.

Gardner and Graceffo (1982) estimated ø as the sum of the product of photon flux (I) of the radiation sources and the in vivo relative quantum efficiencies (ϕ) of purified phytochrome phototransformation derived by Pratt and Briggs (1966), as described in Eq. [2]. Relative quantum efficiencies for P_r ($\phi_{R\lambda}$) and P_{fr} ($\phi_{FR\lambda}$) photoconversions at a given wavelength are estimated as the product of extinction coefficient (ε) and quantum yield of photoconversion (Φ) [$\phi_{R\lambda} = \varepsilon_{R\lambda} \cdot \Phi_R$; $\phi_{FR\lambda} = \varepsilon_{FR\lambda} \cdot \Phi_{FR}$].

[2]

Kelly and Lagarias (1985) and Sager et al. (1988) estimated ø as the sum of the product of I and photochemical cross sections (σ) of purified phytochrome phototransformation, as described by Eq. [3]. Photochemical cross sections for P_r (σ_R) and Pfr (σ_{FR}) photoconversions at a given wavelength are estimated as $2.3 \cdot \varepsilon_{R\lambda} \cdot \Phi_R$ and $2.3 \cdot \varepsilon_{FR\lambda} \cdot \Phi_{FR}$, respectively.

[3]

Problems in correlating plant response with ø estimates

To illustrate a problem one may face when using ø to explain plant response, let us consider the spectral filter example again. We estimated ø under 0 to 40% $CuSO_4$ filters as described by Gardner and Graceffo (1982) (G&G), Kelly and Lagarias (1985) (K&L), and Sager et al. (1988) (SSEC), and attempted to correlate plant response under spectral filters with these estimates. In general, the estimated ø of light transmitted through $CuSO_4$ filters (Table 3) increased as concentration of $CuSO_4$ increased from 0 to ≈10% and decreased thereafter. This pattern could be attributed to the greater removal of FR than R wavelengths by 0 to 10% $CuSO_4$ filters (Fig. 1). Most FR wavelengths of transmitted light were removed when concentration was increased to 10%. Further increase in $CuSO_4$ concentration (from 10% to 40%) reduced R wavelengths of transmitted light, thus resulting in a reduction of estimated ø as $CuSO_4$ concentration increased. The estimated ø values were not significantly different from each other using the methods described by SSEC and K&L, but were higher than the values estimated using the G&G method (≈11% difference in 40% $CuSO_4$ to ≈30% difference in control). This difference in estimated ø values between the SSEC (or K&L) and G&G method may be attributable to the lower B light sensitivity of purified phytochrome and the differences in photochemical properties of purified phytochrome used by G&G as described later in this paper. The $CuSO_4$ filters did not significantly alter photon flux in B wavelengths of transmitted light but removed a significant portion of R and FR wavelengths as concentration increased. The phytochrome parameters used by G&G had less sensitivity to B light than those used by K&L or SSEC, and maximum ø at 660 nm is ≈10% less with the G&G method compared to that estimated by the K&L or SSEC method (Gardner and Graceffo, 1982; Kelly and Lagarias, 1985; Sager et al., 1988).

Clearly, when estimated by the K&L or SSEC methods, ø values for light transmitted through 40% $CuSO_4$ and the control were similar (only about a 1% to 3% difference) (Table 3). However, when estimated using the G&G method, 40% $CuSO_4$ had higher ø values than the control (≈15% difference). Based on ø estimates from SSEC or K&L methods, one would expect plant height under the control and 40% $CuSO_4$ filter to be similar. However, as with the 6% $CuSO_4$ filter, which had a higher ø than control, plant height under 40% $CuSO_4$ filters was reduced by ≈30% compared to the corresponding control filter. From this example, it is clear that ø estimates may vary considerably with the estimation method and that correlation of phytochrome-mediated plant response with phytochrome-related parameters also should be done carefully to avoid wrong conclusions.

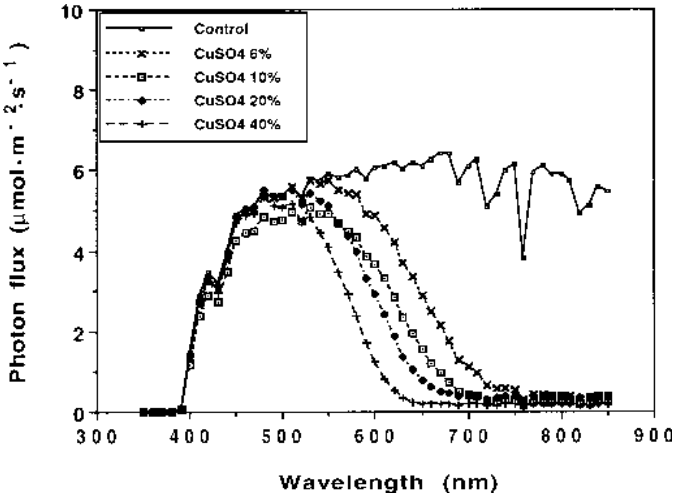

Fig. 1. Photon flux distribution under polycarbonate panels filled with water (control), or 6%, 10%, 20%, or 40% $CuSO_4$ solutions.

Limitations for in vitro ø estimates

Although estimated ø has been the preferred quantitative parameter to explain phytochrome-mediated plant responses, uncertainties may arise in correlating plant response with the light environment in some instances. The variation in ø estimates and the lack of correlation between estimated ø and plant response could be due, at least partly, to several constraints, as discussed in the following sections.

Photochemical properties of purified phytochrome. Since its discovery in the 1950s, phytochrome has been isolated, and the photochemical properties of purified phytochrome have been derived in several laboratories.

Table 3. Variation in estimated phytochrome photoequilibrium (ø) of light transmitted through $CuSO_4$ filters using different photochemical properties of phytochrome (adapted from Rajapakse et al., 1992).

$CuSO_4$ concn	ø[z]		
(%)	SSEC	K&L	G&G
0	0.72	0.71	0.55
4	0.80	0.79	0.65
6	0.81	0.80	0.67
10	0.80	0.80	0.70
15	0.79	0.78	0.70
20	0.76	0.75	0.67
30	0.73	0.72	0.65
40	0.70	0.70	0.63

[z]SSEC, K&L, and G&G represents ø values estimated by Sager et al. (1988), Kelly and Lagarias (1985), and Gardner and Graceffo (1982), respectively.

Considerable variation in photochemical properties of purified phytochrome can be found in comparing phytochrome preparations from different laboratories. This variation is due to differences in purity of phytochrome preparations or the use of different methods for estimating photochemical parameters. Vierstra and Quail (1983) reported that properties for 124-kDa phytochrome, which is assumed to be highly purified, is different from those of partially degraded 118-kDa, 114-kDa, or 60-kDa phytochrome and, therefore, recommended the use of photochemical values obtained for 124-kDa phytochrome. However, several laboratories have determined the photochemical properties of 124-kDa phytochrome and, depending on the methods used for estimating these parameters, considerable variation in photochemical properties of 124-kDa phytochrome has been reported in the literature, making it difficult to select which set of properties for 124-kDa phytochrome to use. To illustrate the variability in photochemical properties of 124-kDa phytochrome, Kelly and Lagarias (1985) and Lagarias et al. (1987) showed that quantum yield of P_r to P_{fr} phototransformation (Φ_R) varied from 0.152 to 0.254, quantum yield of P_{fr} to P_r phototransformation (Φ_{FR}) varied from 0.069 to 0.165, and $\Phi_R : \Phi_{FR}$ ratio varied from 1.54 to 2.20 depending on the method used to estimate [approach-to-equilibrium method (Kelly and Lagarias, 1985) or initial-rate analysis (Butler, 1972)] these parameters. Depending on the source (purity) of phytochrome preparation, values ranging from 0.11 to 0.27 for Φ_R, 0.10 to 0.18 for Φ_{FR}, and 0.98 to 1.76 for the $\Phi_R : \Phi_{FR}$ ratio have been reported for 118-kDa, 114-kDa, and 60-kDa phytochrome, respectively (Butler, 1972; Pratt, 1975; Vierstra and Quail, 1983). The reported extinction coefficients (ε) for P_r and P_{fr} from different laboratories also vary considerably; for example, values ranging from 68.3 to 125.9 mmol·cm^{-1} for ε_{R660} and 49.0 to 85.5 mmol·cm^{-1} for ε_{FR730} have been reported (Kelly and Lagarias, 1985).

The estimates of ø can vary considerably depending on which set of purified phytochrome properties is used in the calculations. The considerable variation of ø values in Table 3, calculated as described by the SSEC, K&L, and G&G methods, can be explained, in part, by the variation of phytochrome properties used in their calculations. As there are many phytochrome data sets available, the selection of one set of photochemical properties of phytochrome to be used in ø estimations can be a difficult choice. Mancenelli (1986) proposed that the use of photochemical properties determined on phytochrome isolated using recent techniques may provide better ø estimates because of high purity and less degradation of the isolated sample. A critical review of the methods used for determining photochemical properties may provide some basis for selecting a set of photochemical properties.

"Etiolated" or "green" phytochrome. The amount of total phytochrome in light-grown plants is low compared to that in dark-grown plants due to decreased synthesis and degradation of P_{fr} when plants are exposed to light. Tokuhisa and Quail (1989) estimated that phytochrome in green tissue is only about 0.002% to 0.003% of the total crude protein extract. Therefore, isolating phytochrome from green tissue is difficult and almost all of the photochemical properties of phytochrome in the literature have been derived on phytochrome isolated from dark-grown, etiolated seedlings. However, recent research has demonstrated that the phytochrome pool is heterogeneous and that the most abundant type of phytochrome present in green tissue is different from that isolated from etiolated tissues (Shimazaki and Pratt, 1985; Tokuhisa et al., 1985).

More recently, researchers, using monoclonal antibodies, showed that green oat leaves contain at least three types of phytochrome (Pratt et al., 1991; Wang et al., 1991). Both showed that only one of the three phytochromes is abundant in etiolated tissues, indicating that there are at least two types of phytochromes (123 kDa and 125 kDa) present in green tissue that are different from the phytochrome in etiolated tissue (124 kDa). They also showed that the two phytochromes in green tissue are different from each other. The photochemical properties and physiological roles of the phytochromes in green tissue may be different from those isolated from etiolated tissue. McCormac et al. (1992), using photomorphogenic mutants, showed evidence for differential roles of light-stable ("green") and light-labile ("etiolated") phytochrome in photomorphogenic response. Currently, our understanding of different phytochrome pools (i.e., etiolated vs. green) is poor, and to our knowledge, there are no published reports on photochemical properties of green phytochrome. Our lack of understanding the physiological role of green phytochrome and the use of photochemical properties of etiolated phytochrome to estimate ø of light-grown plants can raise serious problems in explaining photomorphogenic responses in light-grown plants, such as in the spectral filter experiments.

Dark reversion or destruction. The actual in vivo ø is established by the balance between photochemical and nonphotochemical (dark) conversions of phytochrome. A certain amount of P_{fr} is lost due to dark reversion and destruction, independent of photochemical conversions, thus resulting in lower in vivo P_{fr} content than that estimated in vitro. Both dark reversion and destruction of P_{fr} lead to overestimating in vivo ø, especially if the dark destruction of phytochrome is much greater than that of photochemical conversion.

Source of phytochrome. Most of the photochemical properties in the literature have been obtained from phytochrome isolated from etiolated oat or rye (*Secale cereale* L.) seedlings. Lagarias et al.'s (1987) phytochrome analysis indicated that photochemical properties differed for phytochrome isolated from oat and rye seedlings (i.e., Φ_R and Φ_{FR} for oat phytochrome was 0.152 and 0.063 and those for rye phytochrome was 0.173 and 0.076, respectively; the $\Phi_R : \Phi_{FR}$ ratio was 2.52 and 2.34 for oat and rye phytochrome, respectively). If phytochrome properties vary from one species to another, using photochemical parameters derived from one species may result in variation in ø estimates in other species.

Critical P_{fr} level. A complication in interpreting plant responses based on ø may also arise due to a lack of understanding of the critical amount of P_{fr} required to produce a response. Since ø is a ratio, it does not indicate the absolute amount of P_{fr} required to initiate a response. If a tissue has accumulated a sufficient amount of P_{fr}, the signal transduction may take place regardless of the estimated ø. In such a case, one may find it difficult to explain plant response with the ø estimates, as in the spectral filter examples shown in this paper.

Because of these limiting factors, in vivo ø may be different from in vitro estimates, and the correlation with plant response may be difficult. The use of ø estimates or R : FR ratio for explaining photomorphogenic response is also based on the assumption that phytochrome is the major photoreceptor involved in the expression of a response. However, the involvement of other photoreceptors, such as B light receptors, may complicate the explanation of plant response, especially when comparing light sources with little R and FR or spectral filters that remove R and FR.

Blue light responses. Blue light reduces stem elongation, and FR light in combination with B light reverses B-light-induced responses (Kadman-Zahavi and Ephrat, 1976). R light, in the absence of B, may not be effective in reducing chrysanthemum stem elongation, indicating that B light, acting on the phytochrome system or through a B light receptor, may be eliciting the reduction in plant height under spectral filters (McMahon et al., 1990). Recent work by Britz and Sager (1990) with soybean [*Glycine max* (L.) Merr.] and sorghum [*Sorghum bicolor* (L.) Moench] has shown that light sources high in R (ø = 0.70), but deficient in B wavelengths, produce taller plants, similar to high FR irradiance. Warpeha and Kaufman (1989) reported that B light inhibited epicotyl elongation of pea (*Pisum sativum* L.) seedlings under R light (to saturate phytochrome conversion), indicating that excitation of a B light receptor was involved in the process. Adamse et al. (1988), working with a tomato (*Lycopersicon esculentum* Mill.) mutant, reported that B light inhibited hypo-

Table 4. Variation in B : R and B : FR ratios of light transmitted through $CuSO_4$ filters (adapted from Rajapakse et al., 1992).

$CuSO_4$ concn (%)	B : R[z]	B : FR[y]
0	0.7	0.9
4	1.2	4.1
6	1.4	7.7
10	2.1	14.0
15	3.2	19.1
20	3.8	14.0
30	7.7	18.6
40	11.4	20.9

[z]B : R ratio using B = 400–500 nm and R = 600–700 nm.

[y]B : FR ratio using B = 400–500 nm and FR = 700–800 nm.

cotyl elongation of both the normal and mutant tomatoes lacking in labile (etiolated) phytochrome, suggesting that B light can produce responses similar to R light independent of phytochrome involvement.

Although we focused on ø and R : FR ratio in explaining plant response under $CuSO_4$ filters, the separate involvement of B light in regulating plant response under these filters cannot be neglected. The $CuSO_4$ filters did not significantly alter photon flux in B wavelengths (400–500 nm) (Fig. 1). Because of the unchanged B wavelengths and the continued reduction of R and FR wavelengths, B : R and B : FR ratios of transmitted light increased as the $CuSO_4$ concentration increased from 0 to 40% (Table 4). The morphological differences observed under 6% and 40% $CuSO_4$ filters could be better explained by the involvement of B light in the regulatory process. It seems possible that the amount of B wavelengths relative to R or FR wavelengths in light may be more important in regulating plant response under the $CuSO_4$ filters than the absolute amount of B wavelengths received by the plant. However, this type of measurement is also derived, as mentioned earlier, by determining band width for measurements.

Conclusion

Interpreting plant response under various light environments is complicated by the lack of a reliable quantitative light quality parameter. The R : FR ratio of a light source varies considerably with the wavelengths used in the calculations and could lead to wrong conclusions, especially when evaluating light sources with little or no R and FR light or evaluating spectral filters that remove R and FR light. Currently, ø estimates provide a useful measure to compare plant response under various light sources; however, the correlation of plant response with estimated ø and the comparison of experimental results from other laboratories should be done carefully to avoid wrong conclusions. Estimated ø values also vary considerably depending on the selection of photochemical properties of purified phytochrome in the calculations. In addition, our lack of knowledge of the physiological roles and photochemical properties of various phytochrome pools may result in significant errors in in vitro ø estimates.

A coordinated effort of interested researchers may help in developing a standard quantitative parameter to express spectral quality and in the discussion of photomorphogenic responses. Due to a current lack of correlation between predicted ø (or photon ratios) and plant responses, the presentation of complete spectral data, as shown in Fig. 1, is probably most useful in explaining plant response to light environment. In addition to complete spectral data, R : FR, B : R, and B : FR ratios and estimated ø may be useful until more information is available.

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