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UV-B Inhibition of Phytochrome-Mediated Anthocyanin Formation in *Sinapis alba* L. Cotyledons[1]

ACTION SPECTRUM AND THE ROLE OF PHOTOREACTIVATION

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ABSTRACT

An action spectrum was measured for ultraviolet (UV) radiation-induced damage to (inhibition of) phytochrome-induced anthocyanin formation in cotyledons of 40-hour-old *Sinapis alba* L. seedlings. The action spectrum showed maximum effectiveness in the 260 to 280 nanometer waveband with little effect above 295 nanometers. The damaging effect of UV could be photorepaired by subsequent exposure to sunlight or to long wavelength (360 nanometers) UV radiation. Because this form of damage is subject to photorepair (photoreactivation), it is probably due to the formation of pyrimidine dimers, and the results suggest that it would not be ecologically relevant even if there was an increase in solar UV due to a decrease in stratospheric ozone levels of about 30%. If a dark period of more than 1 hour is interspersed between the phytochrome induction and the UV irradiation, the inhibition of the phytochrome induction gradually decreases with increasing dark period.

The claims made in the 1970s that various man-made pollutants (in particular, nitrogen oxides and chlorofluoromethanes) could cause a significant reduction in the natural atmospheric ozone equilibrium (10, 15) have led to an upsurge of interest, both in the possible effect such a reduction would have on the amounts of UV radiation reaching the earth's surface and the consequences of such changes for living systems.

A survey by the U.S. National Academy of Sciences (16) has suggested an eventual decrease of 16% in the stratospheric ozone level, should production of chlorofluoromethanes remain at present rates, and more than 30% should these rates increase at about 7% annually. Whereas ozone absorption in the UV-C[2] range is so strong that no changes in terrestrial levels would be expected, in the UV-B waveband biologically significant increases may well be possible (6).

It has long been known that UV radiation can cause damage to living organisms, and the action spectra for such effects usually show a peak at 260 to 280 nm (see 10). Most of the work to date has, however, been obtained using microorganisms and animal tissues as the experimental objects. Although the data from plants has tended to show similar action spectra (see 4, 5), the situation is complicated by the existence of parallel 'positive' or 'non-damaging' UV-B effects (see 23). These effects are often similar in response type to other photomorphogenic responses to UV-A and visible radiation, but do not appear to be mediated directly by phytochrome or the blue light receptor(s). It has been suggested that they may well have a protective function for the plant against damaging effects from shorter wavelength UV-B radiation (6, 23). The positive nature of UV-B-induced flavonoid and anthocyanin formation is fairly clear, but in the case of UV-B-induced growth reduction it is not always easy to determine whether this is a protective effect (reducing cell division where danger of exposure of DNA to damaging UV is present) or is simply a result of damage.

This paper seeks to investigate the damaging effect of UV-B radiation on a previously induced positive photomorphogenic effect, namely, phytochrome-induced anthocyanin formation in cotyledons of *Sinapis alba* L. It is thought that the effect studied here can be considered to be a true damaging effect as it negates a previously induced positive response which is not found in the dark-grown seedling. The effect is characterized by an action spectrum, which is compared to that for other UV-B effects, and its possible mechanism and significance in an environment under increased UV-B radiation is discussed.

MATERIALS AND METHODS

Sinapis alba L. seeds (harvest 1977) were obtained from the Asgrow Co., Freiburg-Ebnet, West Germany, and selected and sown as described by Mohr (12). They were then allowed to germinate and grow in darkness at 25°C for 40 h. The cotyledons were excised, separated from each other, and placed on two layers of filter paper (Schleicher and Schüll 2043 bmgl) and irrigated with 1.3 ml distilled H_2O. The cotyledons were arranged so that the underside was uppermost. In each case, one cotyledon from a single plant was used as the experimental material and the other as the control. The cotyledons were excised under a dim green safe light (13). The cotyledons were then subjected to the relevant light treatment and returned to darkness at 25°C for 24 h before the anthocyanin was extracted.

Anthocyanin extraction and measurement followed the method of Lange *et al.* (11). Ten cotyledons per probe were extracted with 1 ml *n*-propanol/HCl/H_2O (18/1/81). Anthocyanin was measured as extinction at 546 nm using an Eppendorf photometer (Eppendorf Gerätebau, Hamburg, West Germany).

Light Sources. The standard red and blue sources used in these experiments were as described by Schäfer (18): red λ_{max}, 660 nm; half bandwidth, 18 nm; energy fluence rate, 0.67 or 6.7 w m^{-2}; blue λ_{max}, 434 nm; half bandwidth, 43 nm; energy fluence rate, 7 w m^{-2}. Short wavelength (UV-B) UV radiation was obtained either from Philips TL 40W/12 fluorescent tubes (λ_{max}, 310 nm; half bandwidth, 40 nm; energy fluence rate, 1.5 w m^{-2})

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[2] Abbreviations: UV-A, ultraviolet radiation (320–390 nm); UV-B, ultraviolet radiation (280–320 nm); UV-C, ultraviolet radiation (200–280 nm); PAL, phenylalanine ammonia-lyase.

or by using Leitz Wetzlar projectors fitted with Osram XBO 450W xenon arcs and quartz optics (14) in conjunction with Schott UV-M-IL interference filters (half bandwidth about 10 nm) (Schott and Gen., Mainz, West Germany). Long wavelength (UV-A) UV radiation was obtained from Osram 40W/73 fluorescent tubes (λ_{max}, 350 nm; half bandwidth, 40 nm; energy fluence rate, 7.8 w m^{-2}).

Fluence rate measurements were made using a thermopile connected to a AL1 galvanometer (both from Kipp and Zonen, Bergisch Gladbach, West Germany). The thermopile was calibrated against a Gamma Scientific (San Diego) model 2900 digital photometer with 700-31 VTM scanning monochromator, 2020-10C photomultiplier system (tube type PM6), and flexible fiberoptic tube fitted with a miniature cosine corrected receptor head (type 700-8B).

RESULTS

In preliminary experiments, the kinetics of anthocyanin formation induced by red pulses in intact and excised *Sinapis* cotyledons were measured and compared. No significant differences were found between the two cotyledon types or between them and the previously published results of Lange *et al.* (11). Following these workers, anthocyanin was extracted 24 h after irrradiation.

Figure 1 shows the effect of the length of UV irradiation on anthocyanin formation. Anthocyanin formation was first induced in the cotyledons by a 5-min red light pulse and this was followed by UV radiation pulses of various lengths from the Philips TL 40W/12 source. The amounts of anthocyanin (*A* at 546 nm) produced were then measured after 24 h in darkness. It can be seen that even 1 min UV has a clear effect. The decrease in anthocyanin is linear up to about 2 min, at which point the effect becomes more or less saturated.

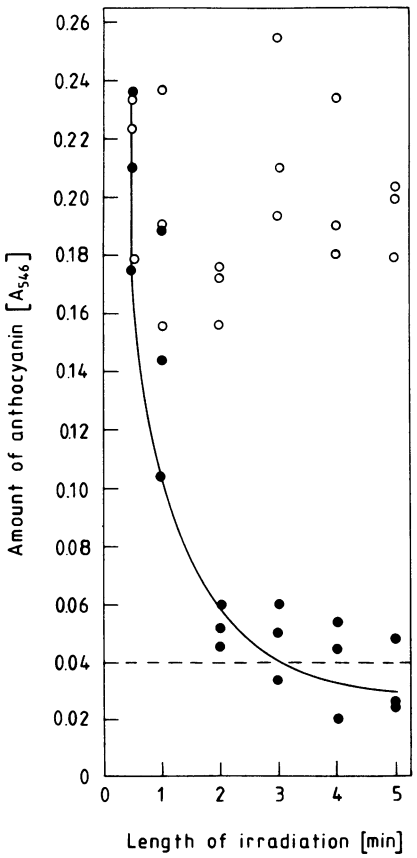

FIG. 1. The effect of the length of irradiation with broadband UV-B (TL 40W/12) radiation on red light-induced anthocyanin formation. The irradiation program was 5 min red + varying times of UV-B. Control values were for 5 min red alone and relate to the other half of the cotyledon pair. Anthocyanin measurement (expressed here as A_{546}) was carried out after a further 24 h in darkness. (●), with UV-B; (○), control; (– – –), level of dark (*i.e.* not induced by red light) anthocyanin.

Figure 2 shows a series of similar curves using monochromatic UV radiation isolated with interference filters. In this case, the effect is plotted as a percentage of the control values and the fluence in J m^{-2}. The figure enables a comparison of the effectiveness of the various wavelengths to be made. The wavelengths 260, 277, and 291 nm can be seen to be very effective (damaging to anthocyanin formation) and saturate at approximately 500 J m^{-2}. Radiation of 298 nm is somewhat less effective, whereas wavelengths above 305 nm have little effect even at high fluences (*i.e.* after lengthy irradiation periods). A test of reciprocity is included in the curves as explained in the figure legend.

Figure 3 shows an action spectrum for decrease in anthocyanin formation. The spectrum was constructed from the data of Figure 2 plotted logarithmically with respect to fluence (curves not shown) and for 50% anthocyanin reduction. The action spectrum demonstrates clearly that wavelengths below 300 nm alone are harmful and presumably disrupt the chain leading from phytochrome phototransformation to anthocyanin formation.

Figure 4 and Table I show results designed to investigate the mechanism of the UV effect and its possible role in the environment. Figure 4 shows the effect of a dark period interspersed between the inductive red light pulse and the damaging UV pulse (for 5 min Philips TL40W/12 source). The results are shown as per cent of control plotted against length of the dark period between the pulses. It is apparent that, after a lag phase of about 1 h, the effectiveness of the UV radiation decreases steadily as the dark period increases.

Table I shows the results of experiments to determine the possible role of photoreactivation in the UV-induced damage. It shows that the effect of a damaging pulse of UV radiation can be at least partially reversed by immediate subsequent exposure to 1 h of sunlight. UV-A radiation was also very effective in reversing the damaging effect, blue light and red light being completely ineffective. Three possible explanations for this reversal of the UV effect can be considered, namely, photosynthesis, phytochrome, and photoreactivation. The fact that red light is ineffective argues strongly against an involvement of photosynthesis and phytochrome. Thus, the most likely explanation is that the results represent a photoreactivation effect.

The greater efficiency of sunlight relative to UV-A, in reversing the damage can probably be explained by two factors. First, the published action spectra for photoreactivation in plants show a peak of action at about 410 nm (17). Thus, the UV-A source does not have an optimal emission spectrum for photoreactivation, whereas the sunlight emission spectrum also includes the higher wavelengths. Second, the absolute fluence rate emitted by the sun in this waveband is much higher. The UV-A source emits about 3.5 w m^{-2} in the relevant 350 to 400 nm waveband, while summer afternoon sunlight at temperate latitudes, as used in these experiments, produces, in this waveband, about 20 to 40 w m^{-2}, depending on cloud cover (J. E. Hughes, personal communication).

DISCUSSION

The results presented here appear to show a definite damaging effect of UV radiation in a plant system. The action spectrum shows that the peak of effectiveness is at about 280 nm or lower and that 290 nm radiation is already significantly less effective. It thus differs from the various 'positive' effects of UV-B on anthocyanin formation, where effectiveness is found at higher wavelengths (see 23 for details). It is, on the other hand, very

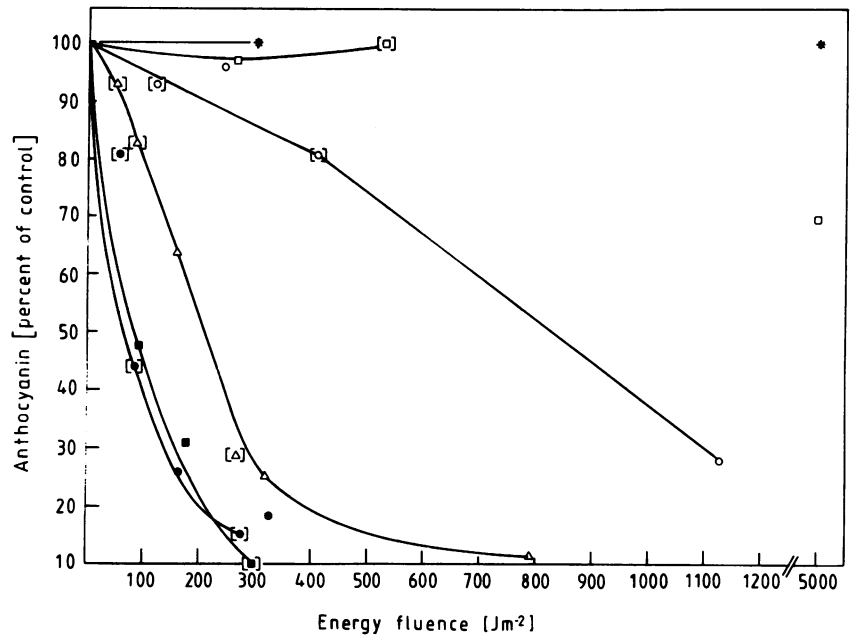

FIG. 2. The effect of monochromatic UV-B radiation on red light-induced anthocyanin formation. The program was as in Figure 1, except that in order to demonstrate reciprocity, UV fluence was varied either by varying the time of irradiation (symbols with brackets). or by varying the fluence rate (symbols with brackets). The results are expressed as percentage of the control cotyledons (5 min red alone). (●), 260 nm; (■), 277 nm; (△), 291 nm; (○), 298 nm; (□), 305 nm; (*), 317 nm.

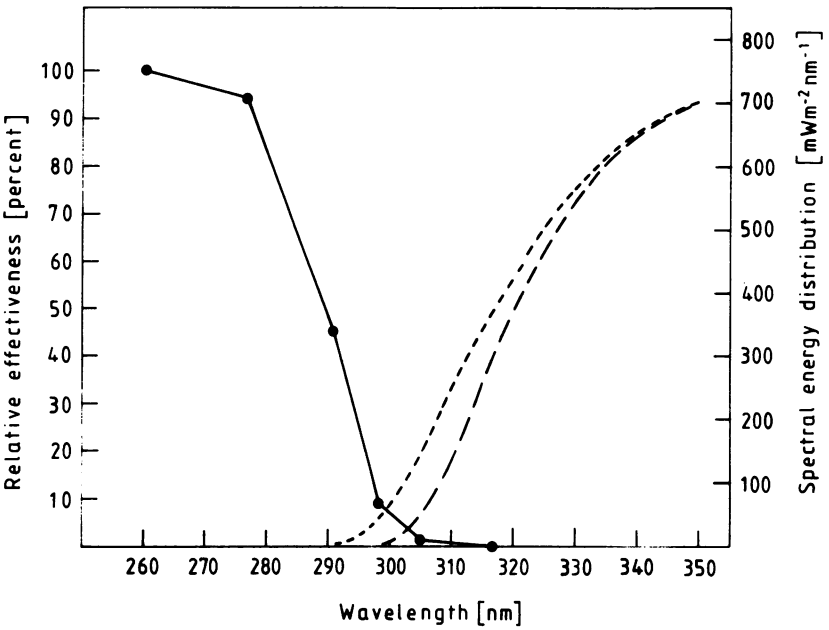

FIG. 3. Action spectrum for UV-B-induced inhibition of red light-induced anthocyanin formation constructed from the data of Figure 2 (see text) for 50% anthocyanin reduction. The effectiveness is normalized to 260 nm = 100%. Also shown are the expected changes in solar UV following a decrease of 60% in the ozone layer as taken from references 6 and 7. (— —), at present day ozone levels; (- - - -), assuming a 60% reduction.

similar to the many action spectra for plant responses which are believed to be due to absorption of radiation by either proteins or nucleic acids (see 4) and also to the results of Arthur (1), where it was found that 254 nm radiation from a mercury vapor arc prevented a later response to inductive radiation (290–313 nm UV-B—a positive UV-B effect) in anthocyanin formation in apple skin.

Figure 3 shows the action spectrum for damage and also the expected changes in terrestrial UV following a large decrease (of 60%) in the ozone level, as calculated by Caldwell (6) from the model of Green *et al.* (7). It can be seen that only the waveband between 290 and 300 nm is relevant for a discussion of the role of increased solar UV on the environment, as above 300 nm little or no damage is found and below 290 nm no UV reaches the earth's surface. Calculation from our fluence response curves and the values given by Caldwell (6) show that for 60% ozone depletion, approximately 6 h 298 nm solar radiation would be necessary to achieve 50% of the maximum inhibition caused by approximately 1 min of 260 nm radiation if, as has not been shown, reciprocity is valid for such long term irradiations. For comparison, the figure would be about 100 h for present day solar radiation. In drawing conclusions with respect to the effect of such a decrease in the ozone layer for life on earth, it should be noted that the model calculation was for a clear sunny day and an ozone depletion far greater than the most pessimistic estimates. Furthermore, the action spectrum takes no account of photoreactivation by longer wavelengths, which are always present in sunlight and which will always be simultaneously absorbed by the plant. Table I shows that photoreactivation by sunlight is very effective and can almost completely reverse the damage of up to 10 min UV. This supports the view that such damage would not occur in the environment after the level of ozone reduction that has been predicted.

The results also provide useful information with respect to the mechanism of the damage. The exact photoreceptor for such damage is unknown, but it has been suggested, on the basis of action spectra, that proteins and nucleic acids are likely candidates. Photoreactivation is, however, a specific process whereby

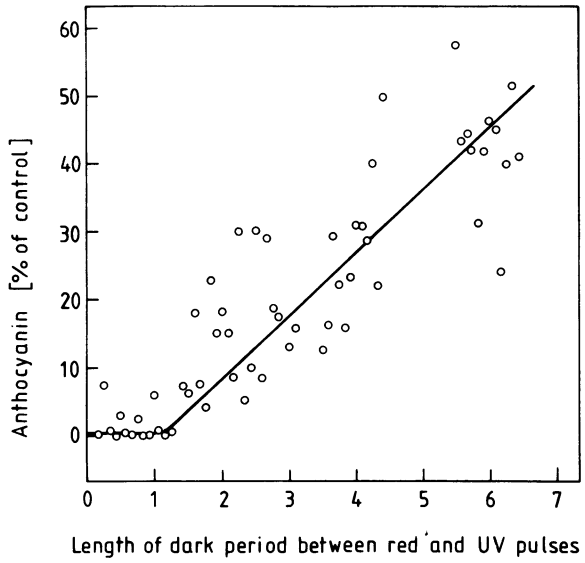

FIG. 4. The effect of a dark period between the inductive red light pulse and the UV-B pulse. The UV-B treatment was 5 min broadband (TL 40W/12) radiation. In this figure, the level of dark anthocyanin has been subtracted from the values.

Table I. *Effect of Light of Various Qualities Given after the Red and UV-B (TL 40W/12) Pulses on Anthocyanin Formation*

Sources (except sunlight) were standard sources; in the case of red light, the 6.7 w m^{-2} source was used. In all cases, anthocyanin was measured 24 h after the UV-B treatment.

Irradiation Program	Anthocyanin
	A_{546}
5 min red + 10 min UV-B + 1 h blue	0.055, 0.015, 0.045
5 min red + 10 min UV-B + 1 h red	0.03, 0.01, 0.03
5 min red + 10 min UV-B + 1 h UV-A	0.16, 0.195, 0.195
5 min red + 10 min UV-B + 1 h sunlight	0.15, 0.28, 0.32
5 min red + 10 min UV-B + dark	0.01, 0.02, 0.025

UV damage to DNA is repaired in a light-dependent, enzyme-mediated process. UV radiation leads commonly to the production of pyrimidine dimers in the DNA, which are split by the photoreactivating enzyme(s) (see 19, 20). Both photoreversible harmful effects of UV radiation (2, 3, 8, 22,) and the presence of a photoreactivating enzyme (17) have been demonstrated in plants. According to Setlow (20), the photoreactivation process is limited to repairing (monomerization) of pyrimidine dimers. It is therefore reasonable to assume that also in the case described here, the damage is caused by such lesions in the DNA, thus indicating that DNA itself is the photoreceptor.

Hadwiger and Schwochau (8) showed that in *Pisum sativum*, PAL, a key enzyme in phenylpropanoid biosynthesis, is stimulated by UV-B radiation, whereas in our experiments, it is Pfr (red light) which stimulates anthocyanin production and UV-B which inhibits. It is known from the literature (23) that photostimulation of PAL occurs through various photoreceptors, the visible light receptors phytochrome and the blue light receptor, the UV-B receptor for positive responses, and as a photostress product probably mediated via damage to DNA. Anthocyanin in *Sinapis* is not a stress product and it is reasonable to assume that our experiments do not concern stress-stimulated PAL. Whether our UV-B treatment also stimulates such a PAL, not concerned with anthocyanin synthesis, was not tested.

Both Table I and Figure 3 also provide interesting insights into the mode of action of the red light (phytochrome) effect. As stated above, the fact that the anthocyanin inhibition can be photoreactivated shows that the inhibition occurs via the formation of pyrimidine dimers in the plant DNA. It is, as yet, not possible to determine which DNA is involved. It is possible either that those genes responsible for anthocyanin, which are activated by phytochrome (Pfr) are rendered inactive by the UV-B radiation and then reactivated via photoreactivation, or that the UV-B radiation has its effect on other genes whereby some necessary substance for Pfr-mediated anthocyanin formation is no longer available until the photoreactivation process allows the supply to be resumed. On the other hand, Figure 4 shows that when a dark period is interspersed between the red and UV treatments and this period exceeds about 1 h, the damage done to the phytochrome effect begins to decrease. This implies that within about 1 h the phytochrome signal must have passed the point at which UV radiation acts on the system. Were this point of UV action the anthocyanin genes themselves, this would imply that the phytochrome signal requires about 1 h, on phototransformation, to reach and act upon these genes. Should other nonspecific genes be involved, it would still imply that the maximum time for the phytochrome signal to reach its primary target would be of the order of 1 h. It is not, as yet, possible to interpret the slope of the gradual loss of phytochrome system vulnerability to UV-B radiation.

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