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Isoflavonoid Formation as an Indicator of UV Stress in Bean (*Phaseolus vulgaris* L.) Leaves[1]

THE SIGNIFICANCE OF PHOTOREPAIR IN ASSESSING POTENTIAL DAMAGE BY INCREASED SOLAR UV-B RADIATION

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ABSTRACT

Induction of the isoflavonoid pigment, coumestrol (3,9-dihydroxy-6H-benzofuro-[3,2-c] [1] benzopyran-6-one), in primary leaves of beans (*Phaseolus vulgaris* L. var Saxa) by ultraviolet (UV) radiation was used as a quantifiable marker for UV damage to a plant system. Coumestrol was induced only by wavelengths below 300 nanometers and its formation could be reversed by treatment with white, but not red light after the UV irradiation period. Formation of coumestrol by UV could also be prevented over a period of 14 hours by simultaneous irradiation with blue light provided that the blue fluence rate was high enough. The results suggest that coumestrol formation is mediated via UV-induced pyrimidine dimer formation in the plant DNA and the photorepair properties of blue light are discussed with respect to possible increases in solar UV due to stratospheric ozone depletion.

Studies of the effects on plants of increased UV radiation caused by a reduction in stratospheric ozone levels (see, *e.g.* 1, 21) have so far paid little attention to the role of possible photorepair systems in determining the plant response. It has long been known that one of the most damaging effects of UV radiation is the production of cyclobutane-type pyrimidine dimers in DNA (16) and that these products can lead to cell transformation and tumor production in eukaryotic cells (15). Furthermore, it is known that pyrimidine dimers of the cyclobutane type can be removed (monomerized) through a visible light-dependent enzymic reaction whereby the photoreactivating enzyme or photolyase binds to the dimer site in a dark reaction and then, using light energy (maximal effectiveness, about 350–450 nm), splits the dimer, thereby restoring the DNA to its original state (28).

Photorepair of UV damage in plants has been known since the early 1950's (2) and evidence has been presented for a photoreactivating enzyme with maximal photon effectiveness at around 420 nm (25, 26). Recently, Wellmann *et al.* (30) presented evidence for photoreactivation of UV-B inhibition of phytochrome-mediated anthocyanin formation in *Sinapis alba* as part of an approach to be able to quantify UV damage effects in plants.

Another quantifiable effect is UV-induced isoflavonoid formation in members of the Leguminosae. Isoflavonoid compounds, which often show phytoalexin properties, are frequently produced in plants of this family in response both to infection and to various so-called biotic and abiotic elicitors (10). One such abiotic elicitor is UV radiation (7, 12). Work by Hadwiger and coworkers (11–14, 20) has shown that the abiotic elicitors are mainly agents which cause changes in cellular DNA and Bridge and Klarman (7) showed that subsequent irradiation with blue light prevented formation of hydroxy-phaseollin (= (−) Glyceollin I) induced by UV radiation.

It seems likely that stimulation of isoflavonoid production by UV radiation is mediated via DNA damage in the form of pyrimidine dimers. For this reason we have used UV-induced isoflavonoid production as a marker to quantify DNA damage in the plant. The paper describes UV induction of the isoflavonoid coumestrol (3,9-dihydroxy-6H-benzofuro-[3,2-c] [1] benzopyran-6-one) in *Phaseolus vulgaris* leaves and its 'photorepair' by visible light given both after UV irradiation and simultaneously.

MATERIALS AND METHODS

Seeds of *Phasaeolus vulgaris* L. var Saxa were obtained from G. Vath, Freiburg, West Germany and sown in earth in small (6 × 6 × 5 cm) plastic pots. The plants were grown for 2 weeks in a phytochamber at 25°C under a 12-h light:12-h dark photoperiod (white light source: Osram HQIL 3.5 kW mercury lamps filtered through 3 mm KG1 heat-absorbing glass from Schott and Genossen, Mainz, West Germany and 6-mm-thick Thermopane glass. The fluence rate in the 400 to 750 nm waveband was about 400 $\mu mol^{-2}\ s^{-1}$).

For the experiments with monochromatic UV-irradiation: in each case one leaf of a primary leaf pair was irradiated and the other used as the unirradiated control. This leaf was covered with black paper during the period of UV-irradiation. After irradiation, the paper was removed from the control leaf and the plant transferred to red light until harvesting and extraction of isoflavonoids. Red light was used, as it was desired that no photoreactivation should take place. Red light was chosen rather than darkness as this waveband does not affect the photoreactivating enzyme (26, 28) and preliminary experiments had shown that the synthesis of coumestrol was higher in red light than in darkness. This is possibly due to a requirement for photosynthetic energy. The control values for unirradiated material which were subtracted from a particular measurement were always those from the corresponding unirradiated leaf from the same plant.

For the experiments with simultaneous UV and blue irradiation: the two leaves from the same plant were used. One leaf received UV alone and the other was covered with black paper during this irradiation. This irradiated leaf was then extracted

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and the other (previously covered) leaf irradiated with UV plus blue light. This leaf served as control for its pair irradiated with UV alone. Preliminary experiments had shown that there was no difference in the results resulting from whether the leaf first irradiated had received UV alone or UV plus blue.

Light Sources. Red light was obtained from Philips TL 40W/15 fluorescent tubes and filtered through 3 mm Plexiglas PG 501 (Röhm GmbH., Darmstadt, West Germany; λ_{max} was 660 nm, halfbandwidth 18 nm, and fluence rate 6.7 W m^{-2}). White light was as for the phytochamber. UV-B was obtained from a Philips TL 40W/12 fluorescent tube (λ_{max}, 310 nm; halfbandwidth, 40 nm) or monochromatic UV-B by using light from a Leitz Wetzlar projector fitted with an Osram XBO 450W xenon arc and quartz optics, filtered through Schott and Genossen UV-M-IL interference filters (halfbandwidth about 11 nm). Blue light was obtained from Philips TL 40W/18 fluorescent tubes (λ_{max} about 420 nm). Fluence rates were measured as described previously (3, 30). Cosine correction of the thermopile measurements was carried out using a factor derived from a cosine-corrected luxmeter (Pocketlux, Lichtmesstechnik, Berlin).

Extraction. Isoflavonoids were extracted as follows: 6 cm^2 of leaf material per determination were cut up into small pieces, added to 1 ml of 50% ethanol and heated to 85°C in a water bath. After 15 min at 85°C, 200 μl of the extract were centrifuged for 2 min at 5000*g* and applied (in 5-cm-long bands) to silica gel TLC plates (Merck, Darmstadt, West Germany, Kieselgel 60_{F254}, 20 × 20 cm, 0.25 mm thickness) with added fluorescence indicator. The plates had previously been washed three times with analytical grade diethyl ether. The chromatograms were developed with diethyl ether. Coumestrol was identified on the plates by its strong violet fluorescence under 350 nm UV light (approximately R_F value 0.8). The band corresponding to coumestrol was scraped off and eluted with 1 ml 50% ethanol. Further chomatography of the eluant on silica gel with methanol or chloroform:methanol (50:2) revealed no further bands. The amounts of coumestrol were determined by *A* at 334 nm using an Eppendorf Photometer 1101M (Eppendorf Gerätebau GmbH., Hamburg, West Germany). In the Figures, each point represents the mean from at least eight independent determinations.

Larger quantities of coumestrol for identification purposes were obtained by irradiating leaves for 24 h with the Philips TL 40W/12 source and then after a further 24 h in darkness, extracting the leaves with ethanol. The leaf material was powdered in a mortar with liquid N_2 and the ethanol added. The extract was evaporated to dryness at 35°C in a rotary evaporator and washed several times with petroleum ether (boiling point 50–70°C) to remove Chl, carotenoids, and other lipophilic substances. The washed residue was redissolved in distilled H_2O and applied to a cellulose column (Avicel, Merck, 35 × 2.5 cm). Using distilled H_2O as eluant, coumestrol remains at the origin together with a methoxy-coumestrol which was not further investigated. Other phenolics are eluted by this step. The coumestrol-containing (violet-fluorescing) portion of the column was cut out, freeze dried, and extracted with 50 ml of acetone for 48 h in a Soxhlet apparatus. The acetone extract was reduced in volume in a rotary evaporator and applied to washed silica gel plates as described above. This chromatographic step separated the coumestrol from the other coumestan. The coumestrol-containing band was eluted with diethyl ether, evaporated to dryness, and redissolved immediately in ethanol. The coumestrol was then characterized by UV spectroscopy and MS.

UV spectrum: λ_{max} (in ethanol) 244, 264 (shoulder), 304, 344, 360(sh) nm.

MS: M^+ = 268 (base peak), other main peaks: m/z 240, 212, 211, 184, 183, 155, 149, 134, 128, 127, 120. (see 5, 6, 24 for comparative reference values).

RESULTS

Figure 1 shows an accumulation kinetic for UV-induced coumestrol accumulation in *Phaseolus vulgaris* primary leaves. The leaves were irradiated for 4 h with monochromatic UV radiation (277 nm, 0.44 W m^{-2}) and then placed in red light until extraction. No coumestrol is detectable for approximately 4 h. There then follows a rapid accumulation up to 20 h. After 20 h a slow decrease in extractable pigment occurs. For further experiments coumestrol was thus extracted after a 24-h period in red light following the appropriate UV treatment.

Fluence-response relationships for 277 nm are shown in Figure 2 where the total UV fluence was varied both by different irradiation times and by placing wire gauze between the radiation source and the irradiated leaves. Within the range tested the fluence-response relationship was linear and the law of reciprocity held. From the fluence-response curve shown in Figure 2 and response values for irradiation with other wavelengths at known fluence rates and times, an action spectrum for UV-induced coumestrol formation could be constructed and this is shown in Figure 3. Maximal effectiveness, within the wavelength range tested, was found at 260 nm and little or no effect was found for wavelengths above 300 nm.

Further experiments investigated the possible effect of a photoreactivation treatment. The term 'photoreactivation' is used here as it is the usual term for such effects. It is, of course, somewhat inappropriate for a situation where the response diminishes on treatment. It should be borne in mind that it is probably the DNA which is being photoreactivated (photorepaired) rather than the isoflavonoid as such. Preliminary experiments showed that in leaves treated with 4-h monochromatic UV at 277 nm and then transferred to either red or white (phytochamber) light for 24 h, those leaves receiving white light after UV produced only 10% of the extractable coumestrol found in plants left under red light.

This effect was studied further in the results shown in Figure 4. Leaves were irradiated using the broadband fluorescent tube UV-B source at a fluence rate of 1.5, 1.9, or 2.8 W m^{-2} and then transferred to red light for 24 h before extraction. Owing to the low fluence rate of the broadband source below 290 nm, it was necessary to irradiate the plants for a longer time in order to obtain a large response. Plants were therefore irradiated for 14 h rather than the 4 h used for the experiments with monochromatic UV. The plants were either irradiated with UV alone or with addition of varying fluence rates of blue light. The results show the difference in coumestrol content between leaves irradiated with UV alone and those irradiated with UV + different fluence rates of blue. The difference is designated as per cent photoreactivation, where the coumestrol content of leaves irradiated with UV alone is set as 0% and that of leaves receiving no UV irradiation as 100%. Approximately 10 W m^{-2} of simultaneously applied blue light were required to negate fully the effects of 2.8 W m^{-2} UV. When the UV fluence was lowered, considerably less blue light was required for full photoreactivation (Fig. 4).

DISCUSSION

UV-radiation can induce the formation of isoflavonoids (in this case coumestrol) in leaves of *Phaseolus vulgaris* L., as has previously been shown for other members of the Leguminosae (see, *e.g.* 7, 12). The action spectrum demonstrates that short wavelengths are responsible for the response with maximal effectiveness below 280 nm. This is very similar to the situation for UV-induced stimulation of the stilbene resveratrol in leaves of grape (*Vitis vinifera*) and peanut (*Arachis hypogaea*) callus cultures (8, 18). These spectra show that below 260 nm the effectiveness declines. This is probably due to optical screening reducing the amount of UV reaching the receptor molecules. In

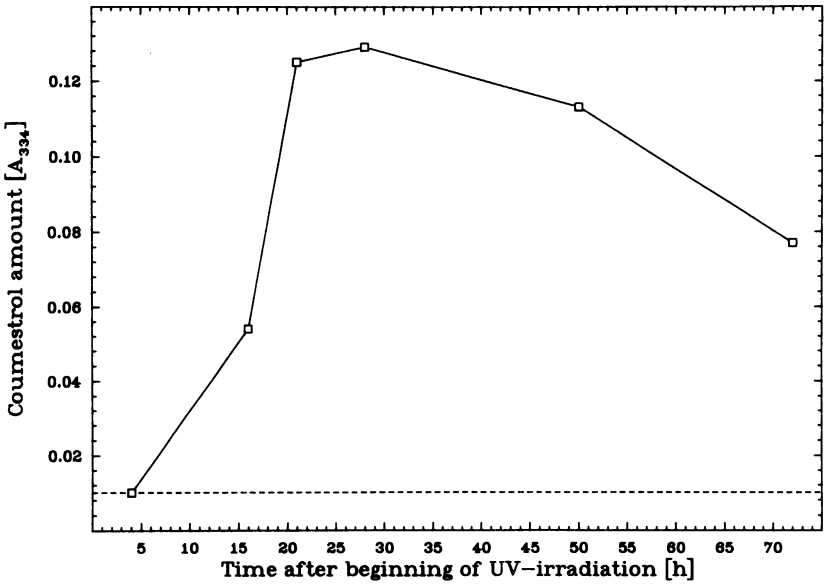

FIG. 1. Accumulation kinetic for UV-induced coumestrol in *Phaseolus* primary leaves. Leaves were irradiated for 4 h with 277 nm UV (fluence rate, 0.44 W m^{-2}) and then placed under red light (fluence rate, 6.7 W m^{-2}) until extraction. The dotted line at $A_{334} = 0.01$ represents the absorbance of an ethanolic extract of silica gel alone. Standard errors of the mean were less than 5%.

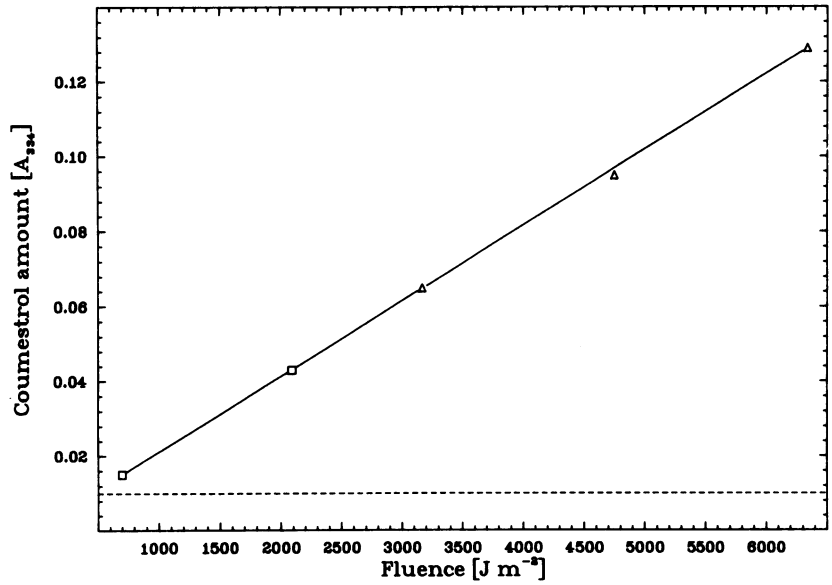

FIG. 2. Fluence-response curve for UV-induced coumestrol formation. Leaves were irradiated with 277 nm UV and the fluence varied by varying the time of irradiation (△) or the fluence rate (□). (---), at A_{334} = 0.01, represents the absorbance of an ethanolic extract of silica gel alone. Standard errors of the mean were less than 5%.

our experiments, measurements below 260 nm were not carried out as these wavelengths are not relevant for the natural environment. In all cases, wavelengths above 300 nm are not effective.

The work of Hadwiger *et al.* (11–14, 20) and the photoreactivation experiment of Bridge and Klarman (7) both strongly suggest that many elicitors of isoflavonoid (including UV radiation) act via direct interaction with plant DNA. This is also supported by the action spectra which peak at shorter wavelengths than those for UV-induced flavonoid or anthocyanin synthesis (4, 29). These latter results have been interpreted as a nondamaging protective response. The action spectra for isoflavonoid and stilbene formation are also very similar to that published by Wellmann *et al.* (30) for inhibition of phytochrome-mediated anthocyanin formation in *Sinapis alba* cotyledons. This inhibition could also be reversed by subsequent irradiation with sunlight or UV-A but not by red light.

The experiments described in this paper (Fig. 4) involving simultaneous irradiation with short wavelength blue light (λ_{max} about 420 nm) strongly suggest that a genuine photoreactivation is taking place. This, in turn, supports the conclusion that UV-induced coumestrol synthesis in *Phaseolus* occurs via direct damage to DNA in the form of photoreparable cyclobutane-type pyrimidine dimer formation, since it is known that the photoreactivating enzyme confines its activity to repair of such lesions (16, 28). It seems, therefore, reasonable to use coumestrol synthesis as a marker for UV damage to DNA in the plant.

The action spectrum shows that wavelengths in sunlight at the earth's surface do not elicit coumestrol formation. On the other hand, calculations of the possible effects of stratospheric ozone reduction on solar UV reaching the earth's surface predict a definite increase at wavelengths around 290 nm (9). According to our action spectrum, these wavelengths, although suboptimal, could induce coumestrol, very probably via dimer production in the plant DNA.

When considering the relevance of this type of effect for the natural situation under reduced ozone levels, one must take into account the presence of those wavelengths which are effective in reversing (preventing) coumestrol formation. Such wavelengths are always present in natural sunlight at fluence rates far higher than those for UV-B might ever be expected to reach. Indeed, it

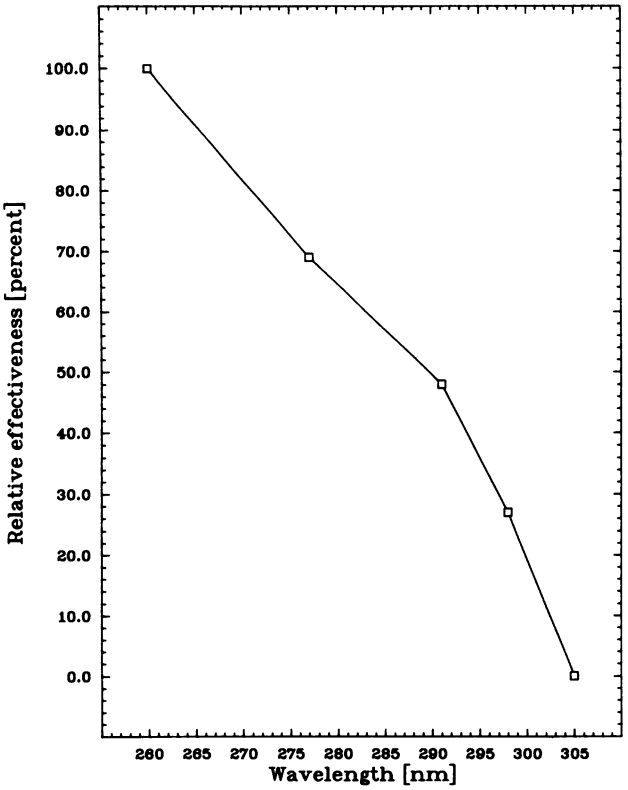

FIG. 3. Action spectrum for UV-induced coumestrol formation. Constructed from the data of Figure 2 and values for the other wavelengths at fixed fluences.

must be supposed that for photoreversal processes, the amount of effective light available will never become limiting, although the capacity of the biochemical process(es) involved may well do so. Figure 4 shows that when *Phaseolus* plants are irradiated with 2.8 W m^{-2} broadband UV-B (of which fluence rate, about 0.3 W m^{-2} are below 290 nm) coumestrol formation can be fully negated by simultaneous irradiation with about 10 W m^{-2} of blue light (or less if the UV fluence rate is still lower). In temperate latitudes (50 ° N), calculations of Gerstl *et al.* (9) for 20% ozone depletion show a fluence rate at 290 nm of about 1.5 $\times 10^{-5}$ W m^{-2} nm^{-1} for minimum solar zenith angle in summer with a cloudless sky. For the same latitude and conditions, the waveband 350 to 450 nm has a fluence rate of about 100 W m^{-2} (calculated from the data of Hughes *et al.* [17]). Thus, not only is light not rate limiting, but the capacity of the photoreversal system appears to be sufficient to reverse the effect caused by UV of far higher fluence rates than might be expected after extreme ozone reduction. It seems most likely that coumestrol formation is mediated via the production of pyrimidine dimers in the DNA and that the photoreversal of this effect by blue/UV-A light represents a photoreactivation (photorepair) of these dimers. On this assumption, one can similarly conclude that the photoreactivation capacity is sufficient to deal with the increased dimer formation which might result from an increase in solar UV-B.

In extrapolating to the natural environment, one should, however, be careful not to neglect the possible role of other simultaneously occurring stress factors. Our laboratory plants are grown under carefully controlled, fairly optimal conditions. In the natural environment, additional stresses may combine to make the plant more susceptible to UV stress. Preliminary experiments have shown that older plants and plants subjected to water or mineral stress produce considerably more coumestrol in response to the same UV fluence used with younger, 'healthier' plants. Such interactions are the object of further study.

With respect to the question of how DNA lesions should lead to specific activation of the genes necessary for coumestrol synthesis, the answer remains unclear. The possibility exists that the control mechanism is similar to that found in the SOS response of *E. coli*, where DNA damage or the blocking of DNA replication leads to activation of various genes through cleavage of a common repressor by a DNA damage-activated protease (19). Similar responses to DNA damage have been described for mammalian cell culture systems (22, 23, 27). As in these mammalian and bacterial systems, plants too produce not only low mol wt phytoalexins in response to UV (and other elicitors and infections) but also a whole series of so-called resistance proteins of, as yet, unknown function (20). In the mammalian cell cultures the response (to UV) is considerably stronger in cell lines from xeroderma pigmentosum patients, where DNA repair systems are known to be deficient. Thus, where DNA damage is quickly and efficiently repaired, a smaller stress response occurs. It would,

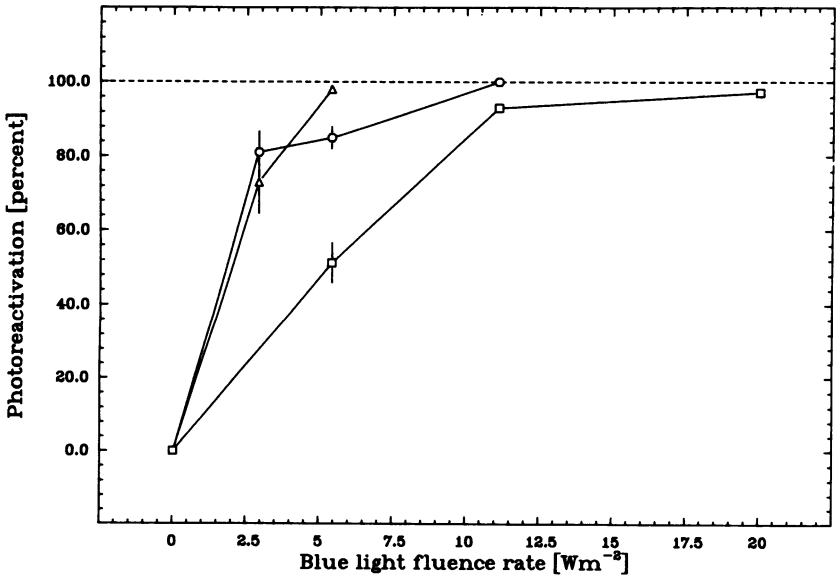

FIG. 4. Photoreactivation of UV-induced coumestrol formation by simultaneously applied blue light. Leaves were irradiated for 14 h with the broadband UV-B (Philips TL 40W/12) source, at various fluence rates (△, 1.5 W m^{-2}; ○, 1.9 W m^{-2}; □, 2.8 W m^{-2}) and simultaneously with blue light at varied fluence rate. After irradiation the plants were transferred to red light for 24 h before extraction. Results are expressed as per cent photoreactivation (0% = coumestrol content of leaves irradiated with 14 h of UV alone: 100% = coumestrol content of leaves receiving no UV radiation). Error bars represent standard errors of the mean.

therefore, not be unexpected if immediate activation of an efficient photorepair system should lead to lowered coumestrol levels or, indeed, to complete cessation of its synthesis.

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