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Quantitation of Pyrimidine Dimers in DNA from UVB-irradiated Alfalfa (*Medicago sativa* L.) seedlings

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Abstract

Depletion of stratospheric ozone will increase the solar ultraviolet radiation in the range from 290-320 nm (UVB) that reaches the surface of the earth, placing an increased UV burden on exposed organisms. One consequence of increased UVB may be decreased productivity of crop plants. A principal lesion caused by UV in DNA is the cyclobutyl pyrimidine dimer. We have adapted a method for measuring these dimers in nanogram quantities of non-radioactive DNA for use in UV-irradiated plants. We find that biologically relevant doses of broad band UVB radiation induce easily detectable frequencies of pyrimidine dimers in the DNA of irradiated alfalfa sprout leaves and that the dose response for dimer formation is linear up to doses of at least 690 J/m^2. We also find easily measurable frequencies of dimers in the leaves of seedlings grown in glass filtered sunlight but not exposed to additional UVB, suggesting that significant numbers of dimers are formed in plants exposed to normal sunlight.

Introduction

Depletion of stratospheric ozone, resulting from the release of chlorine-containing molecules, is predicted to cause an increase in the solar ultraviolet radiation in the range 290-320 nm reaching the biosphere (Caldwell *et al.*, 1989). Since man is dependent upon crop plants for food and fibre, understanding the consequences of increased UVB on agricultural production is of importance.

Most of the research on the potential impact of an increase in UVB radiation on plants has centered on changes in growth and physiology. Studies of plants grown in artificial white light supplemented with UVB have shown that an increase in UVB radiation induces a variety of responses. These include a reduction in net photosynthesis (Murali and Teramura 1986, 1987; Tevini *et al.*, 1988), an increase in stomatal closure (Teramura *et al.*, 1983; Negash, 1987), and a reduction in photosystem II activity (Iwanzik *et al.*, 1983; Tevini and Pfister 1985; Bornman *et al.*, 1984; Renger *et al.*, 1989). The effects of UVB on plant growth and morphology include a reduction in leaf size (Biggs and Kossuth, 1978), reduction in leaf weight (Biggs *et al.*, 1981), bronzing, glazing and chlorosis (Vu *et al.*, 1982), stunting (Teramura, 1980), reduction in dry matter (Teramura, 1980), seed yield (Teramura *et al.*, 1988) and seedling height (Sullivan *et al.*, 1988).

These experiments indicate the range of physiological and morphological effects produced by supplemental UVB. Many variables, including the variety of a particular species and the level of photosynthetically active radiation in different growth regimes, play a role in the effects

observed. Studies such as those described above do not, however, elucidate the mechanisms by which UV produces these deleterious effects.

At the molecular level, UV can damage DNA, RNA, proteins and many other cellular constituents. DNA is found to be the most sensitive target molecule in most systems that have been studied in detail. Unlike proteins and RNA, which can be replaced by new synthesis, damaged DNA must be repaired to restore functionality and repaired correctly to avoid mutation. UV radiation causes the formation of several types of lesions or photoproducts in DNA, the most numerous of which are cyclobutyl pyrimidine dimers. Reports on the direct effects of UVB on plant DNA have focused on water plants (*Wolffia microscopia*; Degani *et al.*, 1980), carrot protoplasts (*Daucus carota*; Eastwood and McLennan, 1985), bean seedlings (*Phaseolus vulgaris* L.; Langer and Wellman, 1990) and *Arabidopsis thaliana* (Pang and Hays, 1991). Most of these reports center on the repair of pyridimine dimers. The methods used in these studies, requiring radiolabeling of the DNA of an intact higher plant or plant cell, tend to be difficult and laborious.

This report describes an adaption of a non-radioactive electrophoretic method, developed originally for measurement of dimers in DNA from UV-irradiated human skin (Sutherland *et al.*, 1980; Freeman *et al.*, 1986; Sutherland *et al.*, 1987a, 1987b; 1990), for the quantitation of pyrimidine dimers in the DNA of seedlings of alfalfa (*Medicago sativa*) irradiated *in situ* by broad band UVB.

Materials and methods

Plant material

Seeds of alfalfa (*Medicago sativa*) were germinated on filter paper (Whatman #3) in petri dishes at 20°C under glass. After 6-7 days the cotyledons were fully expanded and the seedlings were used in irradiation experiments.

Irradiation of alfalfa seedlings

Seedlings in the open petri dishes were exposed to radiation from a 20W Westinghouse FS20 fluorescent sunlamp, with its principal emission from 275 to 350 nm. The fluence was 1.15 W/m^2 as determined by a Jagger meter (Jagger, 1961) calibrated using a Molectron radiometer (model PR200) with a quartz filter. Duplicate dishes were exposed to 0, 69, 138, 345 or 690 J/m^2. All subsequent manipulations were carried out in dim yellow ($\lambda > 500$ nm) light to prevent photoreactivation.

DNA extraction

DNA was extracted by a method designed to minimize photoreactivation and/or excision repair and for production of high molecular weight DNA, which will be described in detail elsewhere. Briefly, 12 cotyledons were macerated in Buffer A [0.5 M ethylene-diaminetetraacetate (EDTA), 10 mM Tris-HCl pH 8.0, 10% sarcosyl, 1 mg/ml proteinase K], embedded in agarose plugs, and incubated in Buffer A for 48 hrs at 60°C. After treatment with phenylmethylsulfonylfluoride (PMSF), the plugs were stored in 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

Treatment of alfalfa DNA with UV-endonuclease from Micrococcus luteus

UV-endonuclease from *M. luteus* was partially purified as described by Carrier and Setlow (1970). Agarose plugs were equilibrated with a buffer containing 30 mM Tris-HCl pH 8.0, 40 mM NaCl, 1 mM EDTA. A portion of each plug was digested for 30 min at 37°C with sufficient activity of the UV-endonuclease to yield complete cleavage at all the dimer sites. A companion slice from each plug was incubated in buffer under otherwise identical conditions. The reactions were stopped and the DNA denatured by the addition of a stop mix comprising 0.5 M NaOH, 50% v/v glycerol and 0.25% w/v bromocresol green.

Alkaline agarose electrophoresis

The DNA's were dispersed according to their single strand molecular length by electrophoresis in alkaline agarose (Freeman *et al.*, 1986). 0.4% (w/v) agarose gels in 1 mM EDTA, 50 mM NaCl were prepared and equilibrated with 2 mM EDTA, 30 mM NaOH. Agarose plugs from the untreated and treated samples were placed in adjacent wells and samples from the complete irradiation series (i.e., 0 - 690 J/m^2) were electrophoresed on the same gel, along with molecular length standards [T4, 170 kb; lambda, 48.5 kb; T7, 40 kb and a Bgl I digest of T7 (22.5 kb, 13.5 kb, 4 kb)]. Unidirectional pulsed field gel electrophoresis was carried out for 18 hrs with 0.3 s pulses (15 V/cm) and 10 s interpulse period (Sutherland *et al.*, 1987a). The plugs were then removed and electrophoresis continued for 7 hr. The gel was then neutralized (2x30 min in 100 mM Tris-HCl, pH 8.0), stained with ethidium bromide (1 μg/ml) for 30 min and destained in H_2O overnight at 4°C.

Image analysis and construction of dose-response curves

An image of the distribution of ethidium fluorescence in the destained gels was recorded using an improved version of an electronic imaging system described previously (Sutherland *et al.*, 1987b). The migration position of the molecular length standards was used to determine the dispersion function of the electrophoresis system (i.e. the relationship between molecular length and migration position) and the number average molecular length (L_n) of each DNA sample was calculated by the method described by Freeman *et al.*, (1986). Since under our conditions the UV-endonuclease cleaves quantitatively, the number of endonuclease sensitive sites (ESS) and hence the number of single strand breaks will be equivalent to the number of pyrimidine dimers. The number of UV-endonuclease sites per 10^6 bases (ESS/Mb), *i.e.* the dimer frequency, p, for each sample was calculated using the equation

$$p = \frac{1}{L_n(+endo)} - \frac{1}{L_n(-endo)}, \qquad Eqn.\ 1$$

where $L_n(+endo)$ and $L_n(-endo)$ are the number average molecular lengths of the DNA treated and not treated with UV-endonuclease, respectively. Quadruplicate estimates of dimer frequencies at each exposure were calculated for the two separate irradiation series. Mean dimer frequency (ESS/Mb) was plotted as a function of UV exposure in J/m^2.

Results

A typical gel showing the distribution of DNA after alkaline electrophoresis is shown in Fig. 1. The first and last lanes show the molecular length standards as described in materials and methods. Lanes 2, 4, 6, 8, and 10 show the single strand size distribution of DNA from plants irradiated with 0, 69, 138, 345, and 690 J/m^2 respectively and not treated with UV endonuclease. Samples in lanes 3, 5, 7, 9, and 11 represent the corresponding samples after treatment with UV-endonuclease. Some of the DNA treated with UV-endonuclease migrates further through the gel in a dose dependent manner and hence has progressively lower number-average lengths. Fig. 2 shows the integrated lane profiles from lanes 4 and 5 of the gel. The profile of lane 5, (138 J/m^2, + UV-endonuclease) demonstrates a shift in the distribution of DNA to shorter lengths compared to lane 4, which received the same dose of UV but was not treated with the endonuclease.

The dimer frequency per unit exposure is found from the slope of the linear dose response curve shown in Figure 3. Table 1 gives the slope calculated for each gel, together with the corresponding correlation coefficients. The slopes from the different gels range from 2.9×10^{-2} to 3.8×10^{-2} $ESS \cdot Mb^{-1} \cdot J^{-1} \cdot m^2$, with a mean of 3.2×10^{-2} and standard deviation of 3.43×10^{-3}.

Discussion

We have developed an electrophoretic method that does not require radioactivity for the quantitation of pyrimidine dimers in plant DNA. This, in conjunction with the use of agarose plugs, which eliminate the need for organic DNA extraction and precipitation, allows the facile manipulation of many samples in parallel.

The broad band UVB source used in this study is one commonly used in tanning salons. Freeman *et al.* (1986) examined the efficacy of a similar source in producing pyrimidine dimers in the skin of several volunteers of different skin types. They found that an exposure of 1 J/m^2 produced from 2×10^{-2} to 11.2×10^{-2} dimers per Mb in human skin depending on skin type. The values obtained from human skin are similar to those reported here for plant DNA (3.2×10^{-2}), however a smaller variation is seen. A possible explanation for the smaller scatter seen here is that each DNA sample is comprised of portions of several individual plants. Another possibility is that the population of plants is more homogenous than the range of human volunteers studied by Freeman *et al.*

Fig. 3 shows that DNA from alfalfa seedlings raised under glass filtered sunlight contained about 10 ESS/Mb even without sunlamp exposure. Subsequent experiments with seedlings grown in a growth chamber showed much higher 'zero dose' levels. However these values were reduced to near zero by imposing between the growth chamber lamps and the plants a plastic filter that absorbs strongly any radiation below 400 nm.

The frequency of dimers produced per unit exposure is strongly dependent upon the spectral distribution of radiation in the source used. Accurate prediction of the level of dimers induced by solar radiation requires knowing both the spectral distribution of the radiation, which is a function of time, location and ozone levels and the wavelength dependent cross section (or 'action spectrum') for dimer formation. The latter can be determined for a given biological system using the methods reported here but irradiating with monochromatic, rather than broad band, radiation.

The ability of plants to withstand an increased UVB burden depends on both protection and repair. As most crop plants are sessile organisms, they are exposed continually to the environment and have evolved extensive protective mechanisms to deal with adverse environmental conditions. With respect to UV, one defensive strategy is the biosynthesis of UV-absorbing compounds such as flavonoids and anthocyanins. Another defensive mechanism is the repair of UV-damaged DNA by the removal of UV-induced photoproducts. Both photoreactivation and excision repair have been reported in plant systems (Degani *et al.*, 1980; Eastwood and McLennan, 1985; Langer and Wellman, 1990; Pang and Hays, 1991).

The methods described in this paper allow the facile detection and quantitation of pyrimidine dimers in plant DNA. As repair, both by photoreactivation and excision, will reduce the number of pyridimine dimers and hence the number of UV-endonuclease sites in the DNA, the removal--i.e. the repair of pyridimine dimers--in plant systems can also be quantified.

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Figure Legends

Figure 1. Photograph of the monitor of the imaging system showing the ethidium fluorescence of a typical gel. Lanes 1 and 12 show the distribution of the markers, (170, 48.5, 40, 22.5, 13.5, 4 Kb), lanes 2, 4, 6, 8, 10, show DNA samples extracted from plants having UVB exposures of 0, 69, 138, 345, and 690 J/m^2 respectively and not treated with UV endonuclease. Lanes 3, 5, 7, 9, and 11 show the same samples treated with UV endonuclease.

Figure 2. Lane profiles of lanes 4 (solid line) and 5 (dashed line) of the gel shown in Figure 1. Both samples were subjected to a UV exposure of 69 J/m^2. The DNA in lane 4 was not treated with UV-endonuclease, while the DNA in lane 5 was treated with UV-endonuclease prior to electrophoresis.

Figure 3. Dose response curve calculated from the pooled mean of all the UV exposures (i.e. gels 1-4 from both irradiation series). Error bars represent the standard error of the mean.

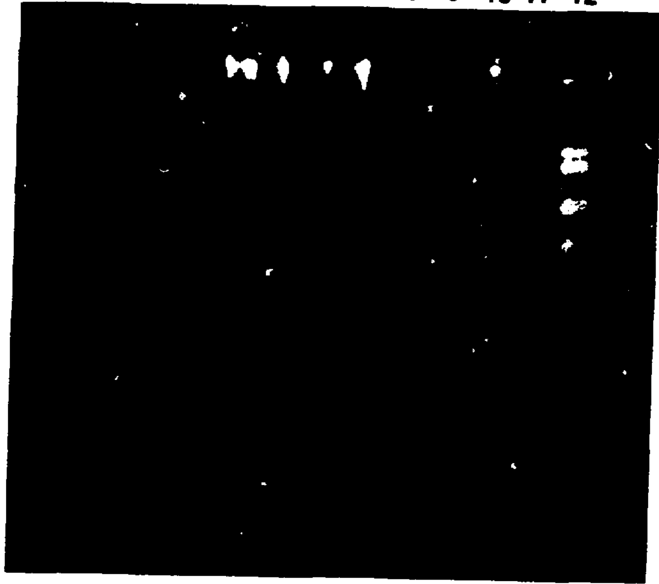
IRRADIATION SERIES 1

GEL	SLOPE (x 10^7)	CORRELATION COEFFICIENT
1	3.4	0.97
2	3.1	0.89
3	3.0	0.91
4	3.8	0.91

IRRADIATION SERIES 2

GEL	SLOPE (x 10^7)	CORRELATION COEFFICIENT
5	3.5	0.91
6	3.1	0.87
7	2.9	1.00
8	3.6	0.93

Table 1. The slope of the dose response curves and the correlation coefficient calculated from the observed dimer frequencies.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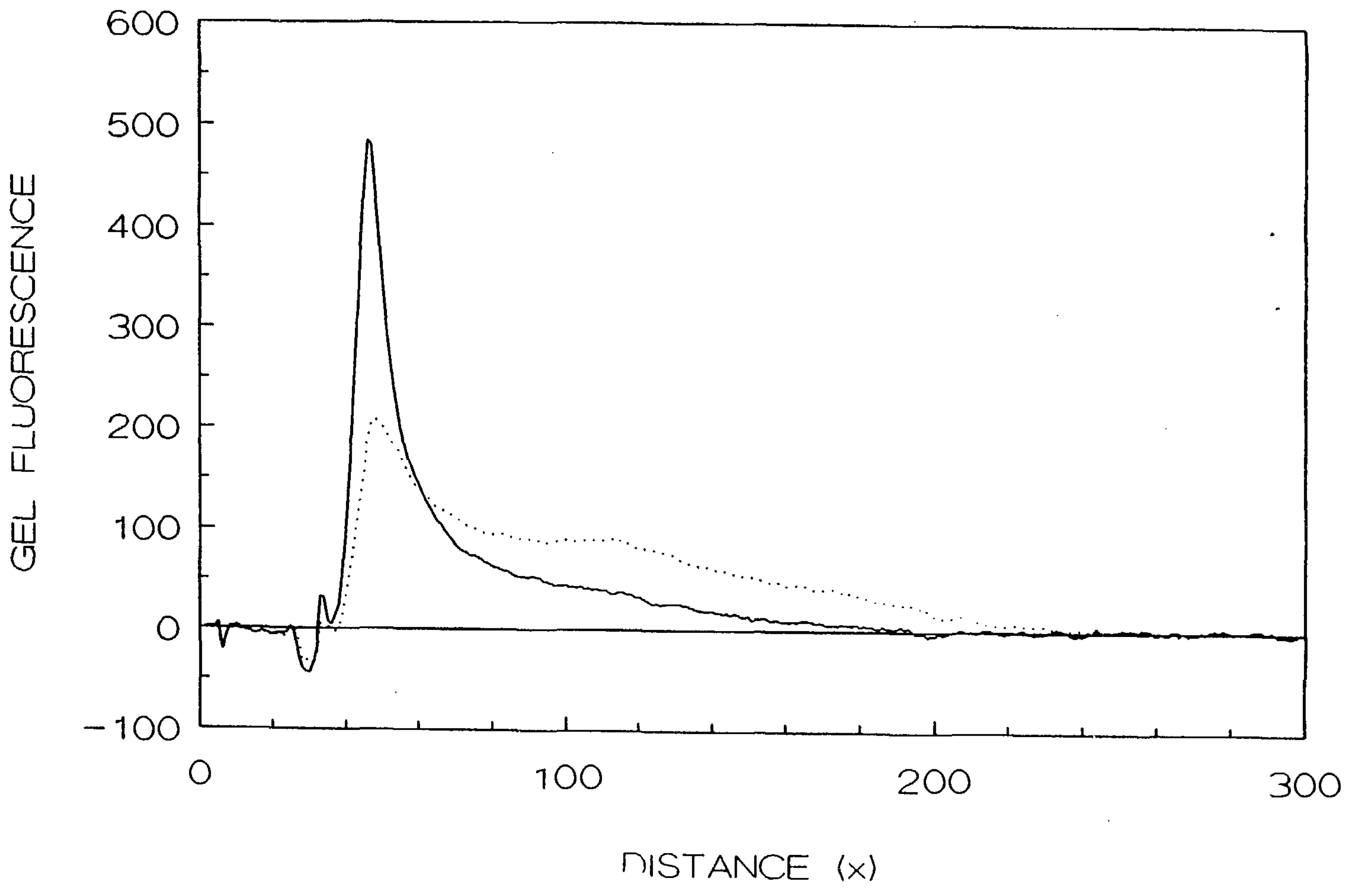
GEL FLUORESCENCE
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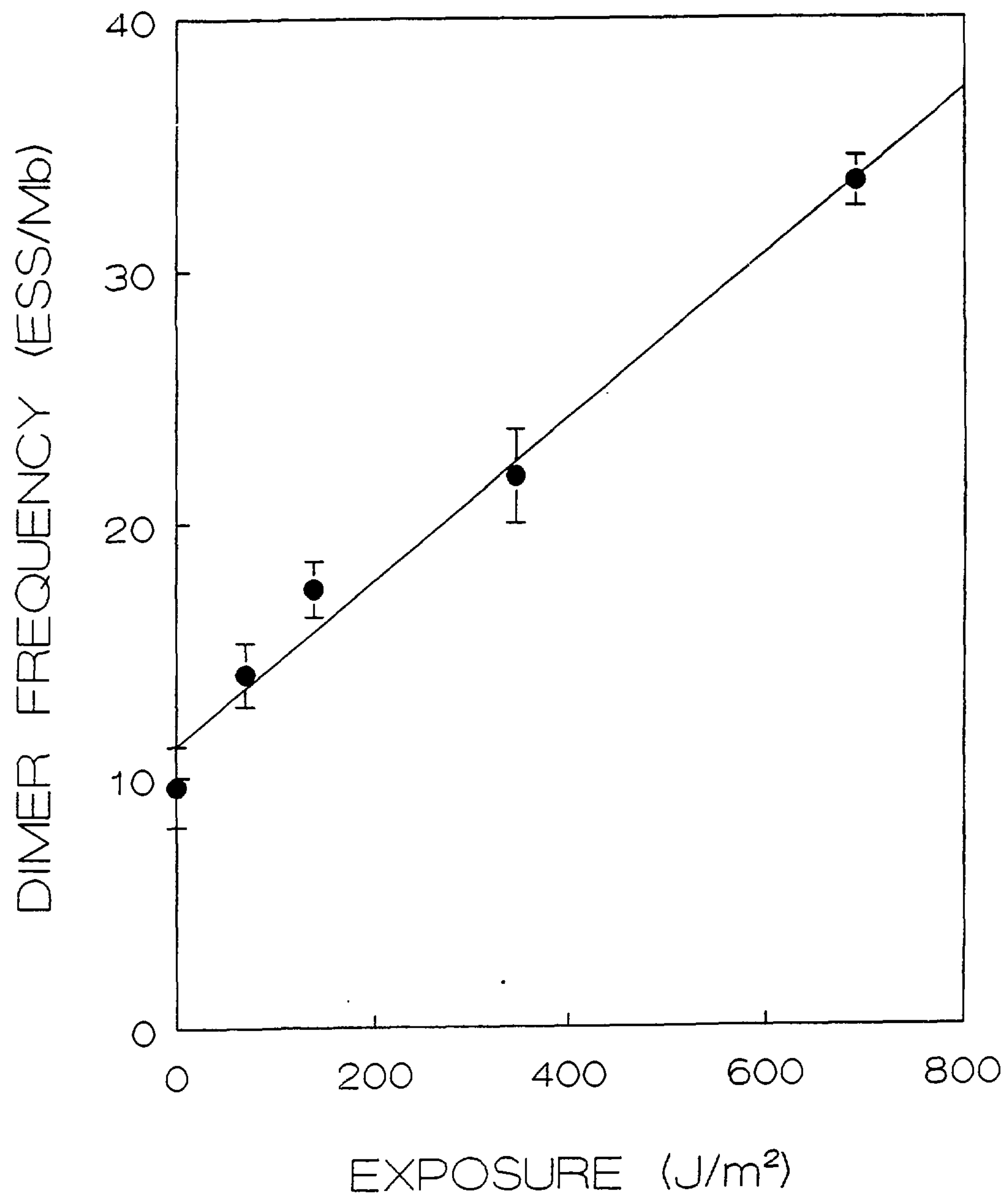
500
400
300
200
100
0
-100
0
100
200
300
DISTANCE (x)



DIMER FREQUENCY (ESS/Mb)
40
30
20
10
0
0
200
400
600
800
EXPOSURE (J/m²)