15 Assessment of DNA Damage as a Tool to Measure UV-B Tolerance in Soybean Lines Differing in Foliar Flavonoid Composition

Joseph H. Sullivan[1], Linda C. Pope, Betsy M. Sutherland[2], Paula V. Bennett[2], James E. Blum[3], Ann E. Stapleton[4], and Dennis C. Gitz III[5]

[1] Department of Plant Science and Landscape Architecture, 2122 Plant Sciences Building
University of Maryland, College Park, MD 20742, USA
E-mail: jsull@umd.edu

[2] Brookhaven National Laboratory, P.O. Box 5000, Upton, NY 11973-5000, USA
E-mail: bms@bnl.gov

[3] Department of Mathematics and Statistics
University of North Carolina at Wilmington, Wilmington, NC 28403, USA
E-mail: blumj@uncw.edu

[4] Department of Biology and Marine Biology
University of North Carolina at Wilmington, Wilmington, NC 28403, USA
E-mail: stapletona@uncw.edu

[5] USDA-ARS, 3810 4th Street, Lubbock, Texas 79415,USA

Abstract Damage to DNA, in the form of cyclobutane pyrimidine dimers (CPD), may occur in soybean (*Glycine max* (L.) Merr) plants when they are exposed to increasing levels of ultraviolet-B (UV-B) radiation. Flavonoids and other phenolics accumulate in the epidermal layer of leaves and may provide protection for sensitive tissues including DNA molecules. We evaluated the steady state levels of accumulated damage and the protection afforded by flavonoids in two soybean isolines: Clark producing high levels of flavonoids, and Clark-magenta producing extremely low flavonoid levels. Both cultivars were grown in the field under ambient and supplemental UV-B radiation. Leaf tissue was harvested in a diurnal sequence, and the samples were analyzed. Two methods of analysis were used in order to develop a common reference point between the two. In one method, DNA was isolated and treated with UV endonuclease, and the DNA fragments were separated using unidirectional pulsed field electrophoresis and quantified through electronic imaging. In the alternate method, a western blotting procedure, immobilized DNA was reacted with monoclonal antibodies specific to CPD DNA damage. Results were similar in both techniques and show lesion frequency to be low in both isolines. However significant differences

were found between cultivars, UV treatments, time of day collected, and levels of PAR. The average level of dimers per megabase for the isoline Clark was ~4 (with or without supplemental UV), and for Clark-magenta, ~4 for samples with no supplemental UV and ~6 for those exposed to supplemental UV radiation. Diurnally, dimer levels were frequently higher in the Clark-magenta isoline, especially when exposed to supplemental UV-B. Both isolines appear to be either well-protected from DNA damage, or repair is efficient enough to minimize biologically significant accumulation of DNA damage. This suggests that protection mechanisms, other than flavonoids alone, contribute to maintenance of DNA integrity in soybean.

Keywords Glycine max, soybean, DNA damage, pyrimidine dimers, UV-B radiation, stratospheric ozone depletion

15.1 Introduction

Continued stratospheric ozone depletion and the resultant increase in ultraviolet-B radiation (UV-B) raises a concern for a potential decrease in crop yields and impacts on agricultural and natural ecosystems. Although the implementation of regulations that minimize inputs of chlorofluorocarbons into the stratosphere is resulting in recovery of the ozone layer, there is still uncertainly about the stability of future ozone levels (WMO, 2003). For example, the link between global warming and ozone depletion is not fully understood and warrants further investigation.

Warming trends in the Pacific Ocean affect the strength of the vortex at the South Pole allowing the photochemical reactions that deplete ozone to persist longer in the spring (Kerr, 1995). As CO_2 levels increase and the troposphere warms, the associated cooling of the stratosphere leads to increased transport of water vapor to the stratosphere (Kirk-Davidoff et al., 1999). This water vapor is the basis for the formation of the polar stratospheric clouds (PSCs) that provide a surface on which ozone destruction takes place. Persistence of PSCs into the Antarctic spring due to stratospheric cooling may lead to enhanced rates of catalytic ozone destruction. This may be further exaggerated by atmospheric denitrification that reduces the effectiveness of nitric acid to combine with free chlorine and prevent ozone destruction (Salawich et al., 2002). Thirty-five percent of the ozone lost in the Arctic winter of 1994 – 1995 was attributable to stratospheric denitrification (Waibel et al., 1999). Concerns such as these suggest that the possible implications of continued ozone depletion and concomitant increases in solar UV-B radiation (between 290 nm and 315 nm) need further evaluation.

Although it represents only a fraction of the total solar electromagnetic spectrum, UV-B may exert substantial photobiological effects when absorbed by important macromolecules such as proteins and nucleic acids (Giese, 1964). The highly energetic photons of UV-B radiation may reduce photosynthesis, alter

stomatal development and functioning, damage proteins and membranes, and induce DNA lesions if absorbed in sufficient quantity (e.g., Caldwell et al., 2003; Sullivan, 2005). However, sensitivity to UV-B varies greatly among plant species. Some plants are quite tolerant to high fluences of UV-B (Sullivan et al., 1992; Ziska et al., 1992), while others are sensitive to present ambient fluences (Bogenrieder and Klein, 1982; Krizek et al., 1998). This variation in response to UV-B makes it difficult to generalize about UV-B effects, but overall meta-analysis of UV studies through the end of the Century suggested that major reductions in growth or biomass were rare in realistic field studies (Searles et al., 2001). This infers the importance of UV-B throughout the evolution of land plants that has led to well-developed UV protection mechanisms in many plant species (Beggs et al., 1986).

One of the protective responses that have generally been considered adaptive is the accumulation of epidermal UV-screening compounds that serve as UV filters. The accumulation of phenolics, and flavonoids in particular, has been frequently reported in response to UV-B radiation (e.g., Robberecht and Caldwell, 1978; Caldwell et al., 1983; Sullivan and Teramura, 1989; Tevini et al., 1991). In fact, Searles et al. (2001) found through a meta-analysis of 103 published articles that the most common response of plants to UV-B is an increase in UV-screening compounds. Flavonoids and other phenolics, especially hydroxycinnamic acids (Sheahan, 1996), absorb strongly in the UV-B range. The accumulation of these compounds in the epidermis has been shown to reduce UV-B radiation transmittance and hypothesized to protect sensitive targets (Robberecht and Caldwell, 1978; Robberecht and Caldwell, 1983; Beggs et al. 1986; and many others). The accumulation of these compounds is dependent upon a number of factors, including both visible and UV fluence (Mohr and Drumm-Herrel, 1983; Wellman, 1983) and many other environmental factors (McClure, 1986). The mechanistic basis of light or UV-induced accumulation lies at the gene level as several key enzymes in the flavonoid biosynthetic pathway, such as, phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), are induced by UV radiation (Chappell and Hahlbrock, 1984; Beerhues et al., 1988; Liu and McClure, 1995; Schnitzler et al., 1997). There is little doubt that phenolics accumulate in leaf tissue in response to UV-B radiation, and that they are important in conferring photoprotection to “target” molecules that may be damaged by UV-B radiation.

One of the best methods utilized to date to evaluate the screening effectiveness of phenolics has been to actually measure UV-B penetration inside a leaf and compare this to expected values based on soluble phenolic levels. The research by Day et al. (1994), and other related studies (see Vogelmann, 1994, and references therein), have shown that the penetration of UV-B through the epidermis is quite variable and may be related to plant life form and growth habit. However, a rather poor correlation ($r^2 = 0.21$) was found between epidermal transmittance of UV-B at 300 nm and absorbance of soluble phenolics (Day, 1993). Also, Day et al. (1994) measured epidermal transmittance of both UV-B at 300 nm and total

UV-B weighted with one of several weighting functions. They found that the expected extinction rates based on absorbance values across a wide range of species within a life-form group followed the theoretical expectation in only 1 of 42 species. In other words, "Beer-Lambert" type extinction was rarely observed, based on concentrations of soluble UV-absorbing compounds alone. The presence of bound phenolics in the epidermis that are not extracted, but alter epidermal transmittance, could contribute to this relationship.

Even when the concentration or screening ability of soluble and bound phenolics are considered, these parameters do not always correlate well with UV tolerance (e.g., Barnes et al., 1987; Dillenburg et al., 1994; Sullivan et al., 1996), and simple correlations may not exist between the apparent concentrations of soluble UV-absorbing compounds and UV-sensitivity. Barnes et al. (1987) suggested that some species adapted to high ambient UV-B fluxes were inherently tolerant to UV-B radiation, but we do not yet have a complete understanding of all tolerance mechanisms.

In addition to their role as sunscreens, phenolics are involved in numerous aspects of plant growth and development, such as serving as antioxidants (Sheahan, 1996), involvement with auxin-mediated responses (Stafford, 1991), cell wall extension (Dale, 1988; Liu et al., 1995), and lignification (Raven, 1977). Phenolic concentrations and composition also have important ecological implications for decomposition rates and nutrient cycling, as well as for plant-insect and plant-pathogen interactions. Therefore, the physiological and ecological roles of phenolics in plants, and the role that solar UV radiation plays in regulating the composition and concentration of them, needs further evaluation. For example, the specific dose response of accumulation of protective compounds, the action spectrum for this response, and the contribution of environmental factors other than UV-B to this response, have not been clearly resolved in field studies.

Soybean has been intensively studied for UV-B responses for the past quarter century and its production of primary flavonols in response to UV-B radiation has frequently been reported (Murali and Teramura, 1986; Sinclair et al., 1990; Mazza et al., 2000). Therefore, since its chemistry and response to UV-B are rather well-known, soybean is a good candidate for a model system to evaluate the protective role of phenolics, flavonoids in particular, in response to UV radiation. This chapter assesses the protective role of flavonoids in protecting soybean DNA from damage due to exposure to high levels of UV-B radiation in the field. DNA damage, primarily cyclobutane pyrimidine dimers (CPDs) caused by UV-B exposure in plants, has been studied in a variety of plant systems, including soybean (Sutherland et al., 1996; Mazza et al., 2000; Bennett et al., 2001; and others). In addition, it is known that DNA damage may accumulate in some species. However, it is not clear whether the absence of flavonoids as screening compounds would alter the diurnal progression of CPD formation.

In this study, we utilized soybean lines that differ in flavonoid quantity and composition in order to evaluate the protective role of flavonoids against UV-B

damage. These cultivars included: (1) the Clark cultivar, a moderately tolerant cultivar that produces primarily the flavonols quercetin and kaempferol for putative UV-screening; and (2) an isoline of Clark, Clark-magenta (Buzzell et al., 1977) that produces essentially no flavonols, but accumulates cinnamic acids in response to UV radiation. Two of three goals of this research were to determine if: (1) CPDs accumulate over the course of the day; and (2) the absence of flavonol glycosides lead to the formation of more photoproducts in the DNA.

A third goal was to compare two methods of CPD analysis. One method previously utilized to evaluate CPDs uses a gel electrophoresis and imaging system to quantify dimers, while many researchers employ an alternate method using a western blotting procedure, in which immobilized DNA is reacted with monoclonal antibodies specific to CPD DNA damage (Mori et al., 1991; Stapleton et al., 1993; Stapleton and Walbot, 1994; Mazza et al., 2000). As previous research has rarely used both methods of dimer quantification, a comparison of these two methods will lend perspective and increase the interpretive power of past and future studies that may utilize one method or another.

15.2 Materials and Methods

15.2.1 Plant Growth and UV Irradiation

Soybean seeds (Glycine max L., cultivars Clark and Clark-magenta) were planted in plots (4.5 m×2.5 m) at the USDA Agricultural Research Center (ARS), South Farm, Beltsville, MD, with a spacing between rows of 0.4 m, and a density of ~35 seeds m^{-1}. One row (4.5 m) of Clark seed was planted next to one row of Clark-magenta seed, and bordered by additional rows of soybeans to simulate field-crowding conditions and to create even sunlight exposure and shading. After germination, plants were thinned to ~25 plants m^{-1} (see (Teramura et al., 1990) for additional information regarding the field sites). In order to reduce water or nutrient stress that can mask the response to UV-B radiation, the plants were watered daily when natural precipitation did not occur, and the plots were fertilized before planting according to recommendations from the Maryland Soil Testing Laboratory.

Ultraviolet-B radiation was provided in a squarewave system by Q-Panel (Cleveland, OH) with UVB-313 sunlamps (12 lamps per plot, 30 cm apart) suspended over and perpendicular to the rows of soybean plants (Teramura et al., 1990). Lamps suspended over the supplemental UV-B plants were wrapped with either cellulose diacetate (CA) which transmits UV-B radiation down to 290 nm, or with polyester filters that block almost all UV-B below 316 nm. Therefore, plants received similar levels of PAR and UV-A radiation, but differed in

exposure to UV-B radiation. Spectral irradiance beneath the lamps was measured with an Optronic Laboratories Inc. (Orlando, FL) Model 754 spectroradiometer. The height of the lamps above the plants was maintained at approximately 75 cm.

All filters were presolarized for eight hours, and changed weekly for CA and every two weeks for polyester to compensate for filter degradation. Plants in the field received either ambient UV-B (polyester) or ambient UV-B plus a maximum of 5 kJ m^{-2} d^{-1} UV-B_{BE} (Biologically Effective UV-B when weighted with the action spectrum of Caldwell (1971)). This represented the maximum supplemental level of UV-B radiation that would be received at College Park, MD, during a cloudless day at the summer solstice, with a 25% reduction in ozone, according to the model derived by Green et al. (1980), with later derivations by Björn and Murphy (1985).

15.2.2 Field Sampling

Samples were collected on two days, July 9 and July 22 (2000) from the newest, fully-expanded leaves. Average daily UV-B radiation values and climatological data were obtained from the USDA UV-B Monitoring and Research Program website (http://uvb.nrel.colostate.edu/UVB/index.jsf) for the Beltsville, MD station, located approximately 300 m from the study site. Overall average daily radiation was 68.9 kW/m^2 at 300 nm on July 9th and 46.7 kW/m^2 at 300 nm on July 22nd. This led to ambient daily weighted (Caldwell, 1971) UV-B levels of 4.2 and 3.1 kJ m^{-2} on the two days respectively, and 9.2 and 8.1 kJ m^{-2} respectively of UVB_{BE} when ambient and supplemental levels were combined. Relative humidity ranged from 27% to 94.7% on July 9 and between 56% and 100% on July 22. Daily average temperature was 81.7°F (27.6°C) on July 9 and 80.7°F (27.0°C) on July 22. Plants were watered daily.

Sampling in the field began at dawn, (4:00 Solar time) on July 9th and continued at two hour intervals until after dark (22:00 Solar time). At each time point, at least six samples were collected from each cultivar, UV treatment, and replicate block. A second set of samples was collected using approximately the same method on July 22, 1999. The weather on July 22nd was overcast with light rain. In all cases, samples (two leaf disks, 1 cm in diameter) were taken from the newest, fully expanded trifoliate in the upper canopy, immediately wrapped in a labeled foil packet, dropped into liquid nitrogen (LN_2), and stored over dry ice (CO_2, −78.5°C). In order to avoid variation from plant to plant and leaf to leaf, the same trifoliate was sampled five times over the course of the day. However, to reduce the chance that wounding affected dimer levels, at least one leaf was sampled for the first time in each sampling period, and a rotation of leaves sampled was implanted such that each measurement period (after the fifth each day) included leaves that had been previously sampled from one to five times.

15.2.3 Analysis of Phenolics

Leaf samples were collected on plants at predetermined times following the commencement of irradiation in order to verify their chemical composition and to assess their response to UV-B radiation. Leaf disks (1.13 cm^2) were made on the first true leaf of the plants for studies on emerging plants and on the most recently mature leaf on mature plants. These were placed on ice and taken to the laboratory where they were placed in 20 ml high density polyethylene (HDPE) scintillation vials covered with 10 ml of slightly acidified aqueous methanol (MeOH:H_2O:AcOOH, 50:50:1, v:v:v), tightly capped, and held in the dark at room temperature with gentle agitation (50 rpm) on a G10 Gyrotory Shaker (New Brunswick Scientific, Edison, NJ, USA) for 48 hrs. − 72 hrs. During extraction, vials were sealed with polyethylene lined caps; since it was found that foil lined caps would occasionally corrode and contaminate the extracts leading to the non-uniform formation of metal-flavonol complexes and bathochromic shifting of the absorbance spectra. The extract absorbance was determined from 260 nm − 760 nm at 1 nm intervals with a Shimadzu UV-1601 dual beam spectrophotometer (Shimadzu Scientific Corp., Columbia, MD, USA), and data were expressed as absorbance units per unit leaf area (A cm^{-2}). Since UV level did not lead to qualitative differences in spectra within a species or cultivar, absorbance values at 300 nm were used for comparative purposes between the treatment and controls.

In order to verify presence or absence of flavonoids in the Clark and Clark-magenta isoline of soybean, High Performance Liquid Chromatography (HPLC) separation and determination of flavonoids were conducted. Leaf disks were milled under liquid nitrogen in microcentrifuge tubes, hydrolyzed in 1 N H_2SO_4 in 1 ml 50% aqueous methanol at 80° for 30 minutes under nitrogen, brought to 5 ml, and filtered. Forty μL of the filtrate was directly injected onto a 250 × 4.6 mm column packed with 7 μ spherical C8 stationary phase with gradient elution by 20% solvent B (aqueous 1% orthophosphoric acid) in solvent A (acetonitrile in 1% orthophosphoric acid) increased to 60% B over 20 min at a flow rate of 1.5 ml min^{-1}. Detection at 370 nm was accomplished using a Waters 490E programmable multiwavelength detector. Naringenin (5μL) in 50% aqueous methanol (1 mg ml^{-1}) was coinjected at the beginning of each run as a standard.

15.2.4 Determination of DNA Lesions—Gel Electrophoresis Method

1. Leaf tissue processing: Unless otherwise noted, all procedures were performed under dim yellow light (General Electric, fluorescent; >~500 nm) to reduce the possibility of undesired photoreactivation of the samples during processing. Leaf tissue was processed according to the methods outlined by several researchers (Quaite et al., 1992; Quaite et al., 1994; Sutherland et al., 1996; Kang et al., 1998;

Bennett et al., 2001). Leaf disks were chopped and ground to a fine, light green powder in liquid nitrogen (LN_2), dropped into Lysis Buffer #1 (10 mM Tris-HCl, pH 8.0, 0.83 M EDTA, 13% mannitol, 2% sarcosyl, and 1 mg/ml Proteinase K (Boehringer Mannheim, Indianapolis, IN), 40℃ – 45℃) and vacuum-infused. Agarose Sea Plaque (2-1/2% w/v, FMC, Rockland, ME) in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, heated to 70℃ – 75℃) was added and the mixture pipetted into chilled molds (GeNunc Module, Nalge Nunc, catalog no. 2-32549). The solidified samples were covered with Lysis buffer #2 (10 mM Tris-HCl pH 8.0, 0.83 M EDTA, 2% sarcosyl, and 1 mg/ml Proteinase K), wrapped with aluminum foil, and incubated 24 hours at 45℃. Lysis buffer #2 was replaced with Lysis buffer #3 (10 mM Tris-HCl pH 7.8 – 8.0, 0.1 M EDTA, 20 mM NaCl, 1% sarcosyl, and 1 mg/ml Proteinase K), rewrapped in foil, and incubated for 24 to 48 hours at 45℃. Lysis buffer #3 was refreshed, and the plugs incubated for an additional 24 to 48 hours at 45℃ (total of 72-hour minimum in Lysis buffer #3).

2. Plug processing: Plugs were soaked in TE (pH 7.6) in the dark and over ice. Plugs were subsequently soaked in TE and phenylmethyl sulfonyl fluoride (PMSF) (2.2 μL/ml) 2X at room temperature, with a final cold TE rinse. Samples were soaked 3X in UV endonuclease buffer (30 mM Tris-HCl, pH 7.6, 40 mM NaCl, 1 mM EDTA) over ice. Two similar plugs were placed in a single labeled Eppendorf centrifuge tube, placed in a water bath (70℃ – 72℃) and the re-melted DNA-agarose mixture pipetted (7 μL) to form new plugs. The new plugs were covered with UV endonuclease buffer and stored overnight (4℃). The buffer was replaced with 20 μL Reaction Mix (UV endonuclease buffer containing 1 mM dithiothreitol (DTT) and 0.1 mg ml^{-1} bovine serum albumin (BSA)), and the samples were kept on ice.

3. Plug preparation: Plugs were divided into two groups: the “Minus” plugs and the “Plus” plugs. The Reaction Mix was refreshed in the “Minus” plugs, removed from the “Plus” plugs, and then replaced with a solution of Reaction Mix and sufficient *Micrococcus luteus* UV endonuclease (MLE) enzyme to cut the DNA at all dimer locations. The preparation of MLE used in these experiments cleaved approximately 4×10^{15} CPDs $\mu L^{-1}h^{-1}$. The relative numbers of DNA fragments in each “Plus” plug (sample cut with MLE) and “Minus” plug (sample not exposed to the enzyme) were determined. All plugs were kept on ice for 30 minutes and then incubated at 37℃ for 60 minutes. Additional Reaction Mix (2 μL) was added to the “Minus” plugs, and an equal volume of Reaction Mix with 50% MLE was added to the “Plus” plugs. Samples were incubated at 37℃ for 30 minutes and held on ice until processed. All plugs were rinsed 2X with TE, covered with alkaline stop (0.5 M NaOH, 50% (vol/vol) glycerol and 0.25% (wt/vol) bromocresol green), and stored on ice at 4℃. The gels (agarose (SeaKem LE; FMC, Rockland, ME) (0.4% wt/vol) in 1 mM EDTA, 50 mM NaCl) were prepared and equilibrated with alkaline electrophoresis solution (2 mM EDTA, 30 mM NaOH), at room temperature. The molecular length standards from bacteriophages

G (750kb), T4 (170 kb), λ (48.5 kb), the HindIII digest of λ (23, 9.4, 6.6, 4.3, 2.1 kb) and BioLow (1.0, 0.5, and 0.2 kb) (BioMarker Low, BioVentures, Inc., Murfreesboro, TN) were prepared, and treated with alkaline stop for 60 minutes. All sample plugs were placed in a water bath (at 37°C) for 60 minutes to denature the DNA, rinsed with cold alkaline electrophoresis solution (30 mM NaOH, 2 mM EDTA), and the companion pair of "Plus" and "Minus" samples were loaded into adjacent gel wells.

4. Static field electrophoresis: The gel rig was placed in a 10°C water bath to provide temperature stability. The gel was subjected to a static field electrophoresis for 15 minutes at 3 volts/cm. Recirculation of the electrophoresis solution was begun, and the gel electrophoresed for an additional 25 minutes at 3 volts/cm.

5. Unidirectional pulsed-field electrophoresis (UPFE): This method improves the resolution of DNA molecules longer than 100 kilobases (kb), and increases the ability to measure low frequencies of lesions (Sutherland et al., 1987; Quaite et al., 1992; Quaite et al., 1994) that can occur in DNA exposed to ultraviolet light. Electrophoresis resumed with UPFE (15 volts/cm; 0.3 second pulse with 10 second interpulse period, 10°C with buffer recirculation) for 7 hours ± 1 hour. The DNA plugs were removed, and the electrophoresis continued for a total of 24 hours. The DNA fragments were dispersed in the gel according to their single-strand molecular lengths. The gel was rinsed, neutralized 2X with 0.1 M Tris-HCl pH 8.0, and stained with ethidium bromide (0.67 μg/mL). Gels were stored at 4°C until imaged.

6. Electronic imaging: A quantitative (digital) electronic image was obtained using a charge-coupled device-based (CCD) system designed and built by J. C. Sutherland (Sutherland et al., 1987). The dual-camera system gave a linear response to the DNA-bound ethidium fluorescence. By obtaining the optical density profile in digital form of the DNA in each lane within the gel, median migration distances were calculated by the computer (Sutherland et al., 1990). The distribution of fluorescence was measured. Results were proportional to the quantity of DNA at each position within the lane (Sutherland et al., 1990).

7. Analysis and dimer quantification: The number of breaks in the DNA molecule was derived from the total number of DNA molecules in the MLE treated sample, less the number of molecules in the untreated sample. In general, molecules in the "Plus" sample are shorter than those in the "Minus" sample (Freeman et al., 1986). The standard sensitivity using this method is approximately 1 lesion/Mb, with a current limit sensitivity of about 1 lesion/5 Mb. In a preliminary action spectrum using very high irradiance levels ($0\,kW\,m^{-2} - 15\,kW\,m^{-2}$), nearly 120 dimers were measured (data not shown), showing that these isolines are capable of accumulating a very large amount of DNA damage (Pope, 2001). Samples containing a known quantity of dimers (~30 CPDs − 60 CPDs) were periodically run with the test samples to verify the analytic procedures, providing a positive control.

15.2.5 Determination of DNA Lesions—Monoclonal Antibody Method

Plant material was collected simultaneously with the gel-electrophoresed samples (samples collected in July 1999) and stored at −80℃ until processed. Samples were shipped on dry ice to the University of Tennessee at Chattanooga, Chattanooga, TN for processing. As described in A. E. Stapleton (1999) and the references therein, DNA was isolated from leaf tissue powdered in liquid nitrogen. Samples were processed by adding ~500 ng of DNA to TE buffer, followed by denaturation and slot-blotting to the membrane. CPDs were detected using a TDM-2 monoclonal antibody. This method detects the primary-bound antibody by an alkaline phosphatase-conjugated secondary antibody. By using a chemiluminescent substrate (CSPD), chemiluminescence was detected with autoradiographic film (or with a cooled CCD camera), and the counts were plotted as a function of time.

15.2.6 Experimental Design and Statistical Analysis

The experimental design was a completely randomized split plot design with two UV-B treatments (main plot) and two cultivars within each treatment as the subplot. Each treatment was replicated twice. Samples were analyzed using the mixed procedure of the Statistical Analysis System (SAS, Cary, NC), with separate models used for the ESS gel data, CPD antibody data, and for the methanol extract absorbance, as each of these used different samples, thus precluding a global multivariate analysis. The models tested for main effects of cultivar, UV-B treatment, sampling day and sampling hour, and their interactions. As several samples were measured on the gel system more than once, a nested term for gel within plant was included in the ESS data analysis. The SAS GLM procedure was used to estimate variance components for the ESS data that contained the nested term.

15.3 Results and Discussion

15.3.1 Leaf Phenolics

The first sampling collection day (July 9) was a clear, sunny day, while the second sampling day (July 22) was primarily overcast and rainy. This provided an opportunity to observe the occurrence of DNA damage and the levels of UV-absorbing compounds under contrasting conditions of PAR and UV radiation.

The absorption spectrum in the UV region of the extracts from the Clark-soybean line was typical of a common flavonoid spectrum (e.g., Mabry et al.,

1970). However, the spectrum from the magenta isoline was suggestive of a lack of flavonoids. The absorption peak in the 280 nm − 290 nm range did not exhibit the classic bathochromic shift indicative of flavonoids (data not shown). It is likely that the UV absorbance in the magenta line was due to the presence of hydroxycinnamates (HCAs) which also strongly absorb in the UV-B waveband. Analysis with HPLC confirmed that the primary flavonoids accumulating in the Clark cultivar were the flavonols quercetin and kaempferol, with quercetin accounting for a large proportion of the total flavonols present (Fig. 15.1). Only minute traces of quercetin were observed in the extract from the Clark-magenta plants.

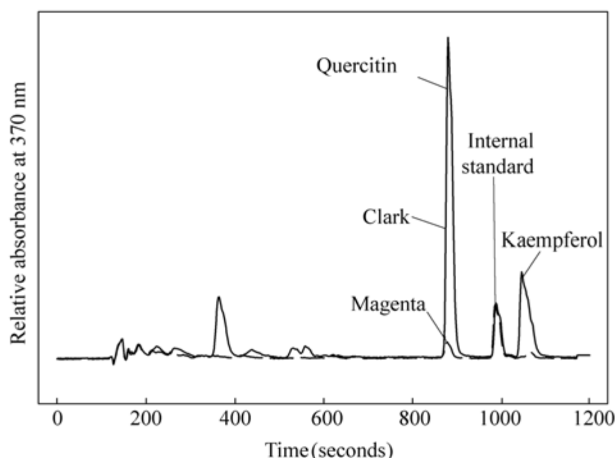

Figure 15.1 HPLC trace from methanol extracts of Clark and Clark-magenta soybean lines grown in the field under ambient levels of UV radiation. The primary flavonol in Clark was found to be quercetin, and only trace amounts of this compound were found in the magenta line

The absorbance at 300 nm of the methanol extracts varied between the two sampling days with higher absorbance values found on the sunny day compared to the cloudy day ($P = 0.02$). There was also a significant difference between the Clark and the Clark-magenta lines on the sunny, but not the cloudy, day. Overall, however, there was little response in these measurements to supplemental UV-B radiation (Fig. 15.2).

It has been suggested (e.g., Searles et al., 2001) that the most common response to increasing levels of UV-B radiation is the increase in UV-absorbing compounds. Several studies with soybean have found that this is a common response (Murali and Teramura, 1986; Sullivan and Teramura, 1990; Gitz et al., 2005). Since the levels were higher on the sunny compared to the cloudy day, the short-term (day-to-day) variations observed in this study may have been linked with ambient levels of UV-B, UV-A, and/or PAR. However, Cosio and McClure (1984) found

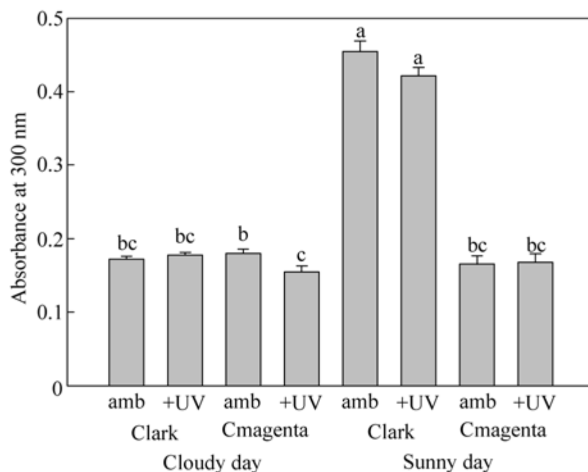

Figure 15.2 The absorbance at 300 nm of a methanol extract from soybean grown in the field under either ambient or ambient plus supplemental levels of UV-B radiation. Each bar is the mean of 5 replicate samples from 2 treatment replicates plus 1 SD. Different letters over a bar indicate a significant difference between the bars

that activities of key enzymes in the flavonoid biosynthetic pathway were greatly reduced following the completion of leaf expansion. Only exposure to UV-C, not found in sunlight reaching the earth's surface, resulted in renewed enzyme activity. Therefore, it seems unlikely that new synthesis of flavonoids on the sunny day would have explained the differences. It is more likely to have been due to environmental conditions (self shading, ambient UV levels, etc.) present during the development of these particular leaves, which led to leaf-to-leaf variations in flavonoid levels. Sullivan et al. (2007) found that soybean responded to short-term changes in ambient levels of both UV-A and UV-B during the initial phases of leaf development and that the spectral sensitivity varied with the species and soybean cultivar. Ultraviolet-A also had a greater effect than did UV-B in soybean lines that were lacking flavonoids. Therefore, it is unclear just how flavonoid synthesis is regulated and further studies on the spectral and fluence response are needed before we have a complete understanding of this process. It is important that we understand the regulation of this process in order to fully understand how plants have adapted to UV-B radiation and how plants and ecosystems will respond to potential changes in solar UV radiation reaching the earth's surface.

15.3.2 DNA Damage

An assessment of how plants differing in flavonoid composition and quantities are affected at the DNA level may provide key information regarding UV tolerance mechanisms and the importance of flavonoids in this process. No significant

difference was noted between blocks on July 9th, and the data were combined for all further analysis. Replicate blocks were also sampled on July 22, but only samples from block 1 were processed using the gel electrophoretic method. Samples taken from both blocks were processed with the monoclonal antibody method.

Statistically significant differences in DNA integrity were observed using both methods of analysis. In the gel method, a significant effect was found for cultivar ($P=0.0064$), UV treatment ($P=0.0103$), day ($P=0.002$), and the interaction between UV treatment and cultivar ($P=0.0293$). On the cloudy day, supplemental UV-B induced more DNA dimers in the Clark-magenta line than in the ambient UV-treatment, and more than in either treatment in the Clark wild type with a normal flavonoid complement (Fig. 15.3). The Clark-magenta line showed a trend of increasing dimers on the sunny day, but this was not statistically significant. In the CPD analysis, significant effects were cultivar ($P=0.0001$), time of day ($P=0.015$), and sunny vs. cloudy day ($P=0.0001$). Also, when considering the diurnal changes in CPDs, the Clark-magenta line had increased CPD amounts at 13:00 h on the cloudy day (Fig. 15.4). Overall, the Clark line generally had less DNA damage than the Clark-magenta line, but the difference was only significant on the cloudy day (Fig. 15.5).

The level of dimers tended to increase early in the day and then subside in the afternoon. This suggests that at some points, the number of dimers formed over the course of the day exceeds the capacity of repair mechanisms. This short term accumulation of damage might be responsible for some of the deleterious responses of plants to increased levels of UV-B radiation.

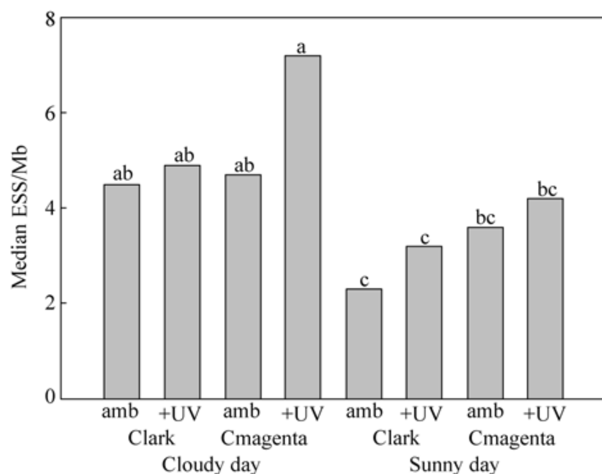

Figure 15.3 The number of ESS (measure of DNA lesions formed) in soybean grown in the field under either ambient or ambient plus supplemental levels of UV-B radiation. Each bar is the mean of 5 replicate samples from 2 treatment replicates plus 1 SD. Different letters over a bar indicate a significant difference between the bars

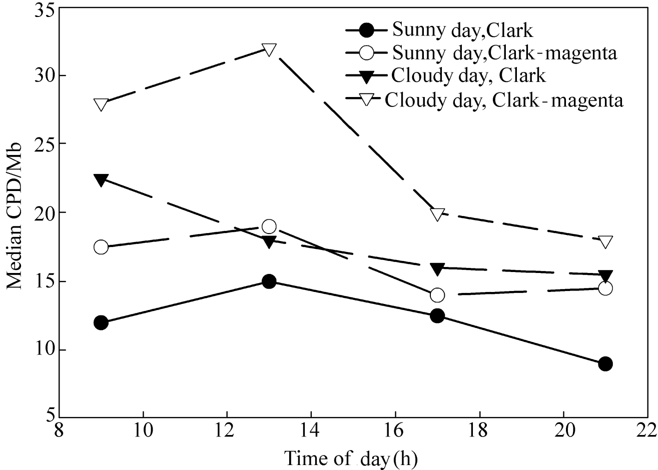

Figure 15.4 Diurnal changes in the number of CPDs formed in 3 soybean lines grown under ambient or ambient plus supplemental levels of UV-B radiation. Each point is the mean of 5 samples collected each 2-hour period (see text for details on sampling protocols)

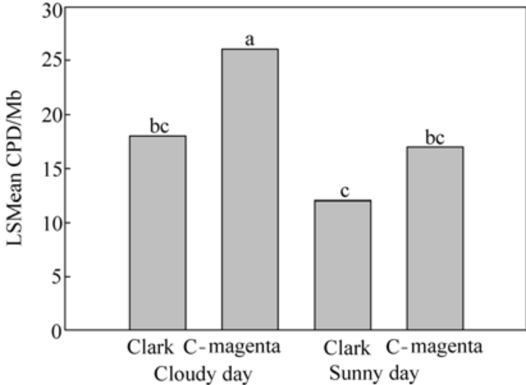

Figure 15.5 CPDs found in two soybean lines grown in the field under either ambient or ambient plus supplemental levels of UV-B. LSMeans of CPDs formed over the course of the day. UV-B treatments are combined since there was no statistical difference between treatments. Each bar is the mean of 5 samples collected each 2-hour period (see text for details on sampling protocols). Different letter over a bar indicates a statistically significant difference in the means at $P = 0.05$

Damage to DNA by UV-B radiation is one factor that could lead to abnormal plant growth and development, altered biochemical pathways and metabolism, inability to reproduce successfully, and even death. Therefore, plants have developed a suite of photorepair and dark repair processes. Ultraviolet-screening mechanisms, such as that afforded by flavonoids, is another protective response. In this study, minimal DNA damage was observed in all cases, but there was some indication

that the absence of flavonoids increased damage at certain times. It is also possible that screening by compounds other than flavonoids, per se (e.g., HCAs), may also be important. The minimal damage that occurred on the cloudy day in Clark-magenta did not persist at the end of the day (20:00 h), so we may assume that the repair of lesions was sufficient to mitigate the majority of damage.

Several studies have shown that the repair of UV-induced damage is mediated by photolyase in mature leaves (Dany et al., 2001). Since photolyase requires a photon of light, preferentially UV-A or blue light (Stapleton et al., 1997) to reverse the damaged DNA, this process must be completed before the energy from the sun dissipates late in the evening, as was the case in our samples. It was primarily on the cloudy day that lesions accumulated in the afternoon in Clark-magenta. The higher ratio of UV-B to PAR was such that minor increases in CPDs occurred periodically. Perhaps PAR levels were not sufficiently high enough to prevent these accumulations. Alternatively, it is possible that the absence of flavonoids, which are more efficient in the absorption of UV-A (Mabry et al., 1970) may lead to increased penetration of UV-A into the leaf mesophyll and therefore, lead to more effective repair systems.

These measurements quantify net dimer levels, and an increased rate of dimer formation might possibly have been countered by enhanced repair capability. Xu et al. (2008a) also noted that the rate of oxidant production appeared to be increased in these same soybean lines in ambient compared to reduced UV-B levels, but observed an increase in the antioxidant system in the magenta soybean line. It is well-known that the balance of repair mechanisms vs. those that provide protection is important (Jansen et al., 1998; Frohnmeyer and Staiger, 2003), and that an increase in UV-absorbing compounds is a common response to UV-B radiation (Searles et al. 2001). However, the results of this study do not allow us to quantify if it was protection (in the case of Clark) and repair (in the case of Clark-magenta) that maintained the low levels of CPDs. Clearly, the effects of UV-A on plant metabolism and the interactions between PAR, UV-A, and UV-B, is not fully understood. This is an area in which continued research may lead to a better understanding of how plants are affected by the various components of sunlight.

When dimer levels from this experiment were compared to maximum levels reached in a exposure response study (~120 CPD's/Mb, data not shown), it was clear that although there was a significant difference in dimers produced by the two cultivars, UV treatments, and irradiance levels, the difference was not biologically significant. Although Clark had fewer dimers on both days (3.46 ± 0.91 and 5.20 ± 1.67) as compared to Clark-magenta (4.39 ± 1.07 and 6.65 ± 1.66), the effects of the small size differences on the plants is probably minimal. This indicates that although flavonoids and phenolics do protect plants from UV-B exposure, in cases where they are not present, plants must provide protection to DNA integrity through other pathways. For example, additional repair enzymes may be produced in plants lacking phenolic protection.

15.4 Conclusions

In the meta-analysis of Searles et al. (2001), UV-induced changes in foliar chemistry, particularly that of production of epidermal screening compounds, were cited as one of the most common responses to UV-B radiation. In this and several related studies (Sullivan et al., 2007; Xu et al., 2008a, b), we evaluated the consequences of removal of one part of the screening system; namely, the flavonols of soybean, and found that they were important screening compounds. This attests to the role of these compounds in providing protection or at least minimizing damage. However, hypersensitivity was not found in the magenta line which suggests the existence of multiple screening pigments and repair processes that may be present in soybean and other plant species. In fact, studies have shown that although a wide range of plant responses to changes in UV-B levels may be observed, the fact remains that total plant growth is rarely severely impacted by small or moderate levels of UV-B when received under realistic field conditions (e.g., see Searles et al., 2001).

There is no question, however, that photons in this highly energetic waveband are quite biologically damaging, yet repair and protective process prevent much of the potential damage. For example, when evaluating the growth and morphology of these soybean lines (Table 15.1), there were some detrimental effects on growth and morphology, but survival and reproductive success were not compromised. Similar results were also obtained on studies with another soybean line, Harosoy, which also has a non-flavonoid isoline (Sullivan et al., unpublished data). In this case, Harosoy showed greater sensitivity which could have been related to differing flavonol composition. The Harosoy line primarily produces the flavonol kaempferol and only trace quantities of quercetin. This indicates that flavonoid composition, in addition to quantities and localization, may contribute to differing levels of UV protection. The importance of flavonoids as antioxidants, in addition to UV-screens, needs further evaluation (Xu et al., 2008b). Appreciable absorbance by the putative HCAs in the magenta lines may provide some UV protection as well. Finally, and for DNA damage in particular, the results of these and other studies indicate net dimer levels that are modulated by ongoing damage and repair processes. We still have a limited understanding of the induction of these repair processes. Clearly, if UV-screening by flavonoids was the only protection method, the results of this study would have been very different, and damage to the magenta line would have been severe at best. Therefore, the quantity and type of screening compounds, the localization and degradation of them, and other factors such as DNA repair processes, determine the sensitivity of plants to UV-B radiation. This aspect of the perception and response of plants to UV-B radiation needs further evaluation. Important topics for continued research are the evaluation of the spectral sensitivity and regulation of the induction and catalysis of these pigments and of the indirect effects of these photomorphogenic changes in leaf chemistry on plant development and ecological processes.

Table 15.1 The effects of supplemental UV-B radiation on the growth and morphology of soybean lines differing in flavonoid levels. Plants received either ambient levels of UV-B radiation (Controls or C) or ambient plus a daily maximum of 5.0 kJ of UV-B when weighted with the action spectra of Caldwell, 1971. An asterisk indicates a significant statistical difference ($P < 0.05$) between UV treatments as determined by the Student Newman-Keuls Multiple Range Test (SAS manual, 2000)

Cultivar	UV Treatment	Height (cm)	Plant Leaf Area (cm^2)	Plant Dry Weight (g)	RSR (root:shoot)
Clark	Control	153±15	2960±197	34.9±3.5	0.241±0.02
Clark	+UV	130±12	2400±210	32.0±3.7	0.301±0.03
$Clark_{wm}$	Control	139±13	2369±245	31.5±3.2	0.258±0.03
$Clark_{wm}$	+UV	106±11 *	1930±167	28.2±3.4	0.333±0.04

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