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Response of Two Legumes to Two Ultraviolet-B Radiation Regimes

Daniel L. Levy and J.W. Skiles

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RESPONSE OF TWO LEGUMES TO TWO ULTRAVIOLET-B RADIATION REGIMES

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ABSTRACT

Depletion of the stratospheric ozone layer has been directly linked to increased levels of UV radiation at the earth's surface. The purpose of this study was to evaluate the responses of soybean (*Glycine max*) and alfalfa (*Medicago sativa*) to increased UV-B radiation (280-320 nm). Soybean and alfalfa were grown successively in a growth chamber that provided UV-B intensities 45% above nominal summer field levels. Mylar-D (UV-B opaque) and mono-acetate (UV-B transparent) films were used to establish the two UV-B treatments. Soybean grown under increased UV showed 21% smaller internodal lengths and higher concentrations of UV-B absorbing pigments (i.e. flavonoids) compared to plants grown under no UV. Significant results for alfalfa included 22% greater leaf flavonoid concentration under increased UV, 14% greater leaf chlorophyll concentration under no UV, and 32% greater above-ground biomass with no UV. These leguminous species possess mechanisms that protect against UV-B damage as indicated by increases in foliar concentrations of UV-B absorbing compounds. Alfalfa appears to be more sensitive to UV-B damage than soybean. Remote sensing of chlorophyll fluorescence may offer a means of monitoring UV-induced plant stress and damage.

INTRODUCTION

Increased UV-B radiation (280-320 nm) at the earth's surface is directly related to depletion of the stratospheric ozone layer. Quintern *et al.* (ref 1) performed a continuous biological dosimetry experiment for cytotoxic solar UV radiation in Antarctica and detected an increase in biologically harmful UV-B radiation due to reduced ozone concentrations. Between 1992 and 1993, Seckmeyer *et al.* (ref 2) noted increased levels of UV-B radiation accompanying the lowest recorded total ozone columns in Southern Germany. Similarly, Kerr and McElroy (ref 3) show that increased UV-B can be linked to ozone depletion.

Living systems have adapted to UV radiation mostly by filtering out the excesses with screening pigments (refs 4, 5). However, when these screening mechanisms fail, UV causes differing degrees of damage in plants, animals, and humans (refs 6, 7). Species-level studies quantify the effects of increased UV-B on plants, assess the risks, and produce reliable data for prediction of cropping systems. Results from such studies can have important ecological implications (ref 8).

The synthesis of UV-screening pigments (i.e. flavone glycosides and anthocyanins) in the epidermal cell layer of plant leaves is a natural mechanism of plant protection from UV-B stress (ref 9); the synthesis of enzymes involved in the flavonoid pathway is under the control of a UV-B responsive promoter (ref 10). Anthocyanins and flavonoids have as one of their major functions the absorption of UV radiation that might otherwise cause damage to plants (refs 4, 11, 12). Barnes *et al.* (ref 13) noted accumulation of UV-absorbing leaf pigments in certain arctic plant species that were exposed to supplemental UV-B radiation under a solar spectrum in the field. Searles *et al.* (ref 14) found increased foliar UV-B absorbing compounds in mahogany and cassava with exposure to solar UV compared with plants shielded from UV.

Studies have shown that increased exposure of plants to UV-B radiation can affect the photosynthetic and growth mechanisms. Latimer and Mitchell (ref 15) found that increased UV-B exposure of eggplant reduces leaf expansion and shoot dry weight. Sullivan and Teramura (ref 16) found a reduction in the growth and photosynthetic capacity of loblolly pine in response to UV-B. Ziska *et al.* (ref 17) obtained a significant decrease in cassava root weight with UV-B exposure. There exists considerable evidence that remote sensing of chlorophyll fluorescence can be used to evaluate plant photosynthetic capacity and health (ref 18). Rosema *et al.* (ref 19) find that laser-induced fluorescence (LIF) can be used to monitor ozone stress in vegetation. Subhash and Mohanan (ref 20) show that LIF spectra of rice leaves reveal nutrient stress and that these measurements represent a way of remotely sensing stress effects in plants. Furthermore, Valentini *et al.* (ref 21) show that water stress and carboxylation limitations can be detected by changes in the chlorophyll fluorescence spectra of leaves; this effect can also be measured by remote sensing. Thus, remote sensing of chlorophyll fluorescence spectra represents a potentially important means of monitoring UV-induced plant stress.

While previous studies have shown the harmful effects of increased UV-B radiation on certain plant species, many of these studies use extremely high intensities of UV-B. For example, Tevini *et al.* (ref 22) continuously irradiated test plants with UV-B for five to ten days, and Dubé and Bornman (ref 23) irradiated plants with 24-hours worth of biologically effective UV-B radiation in four hours.

In this study, test plants were irradiated in a growth chamber with known, constant UV-B intensities evenly over the photoperiod. Although the UV regime of the growth chamber did not simulate daily solar fluctuations, an important advantage of conducting the experiment in a growth chamber versus the field is that field conditions include changing and uncontrollable variables (i.e. temperature, humidity, etc.) that affect plant health simultaneously with UV irradiation and consequently confuse assessment of UV response (ref 24). The average UV-B intensity to which the plants were exposed in the growth chamber was 45% above ambient, summer solar UV-B levels (Fig. 1); this percent increase in UV-B radiation is comparable to a 20% reduction of the stratospheric ozone layer (ref 25).

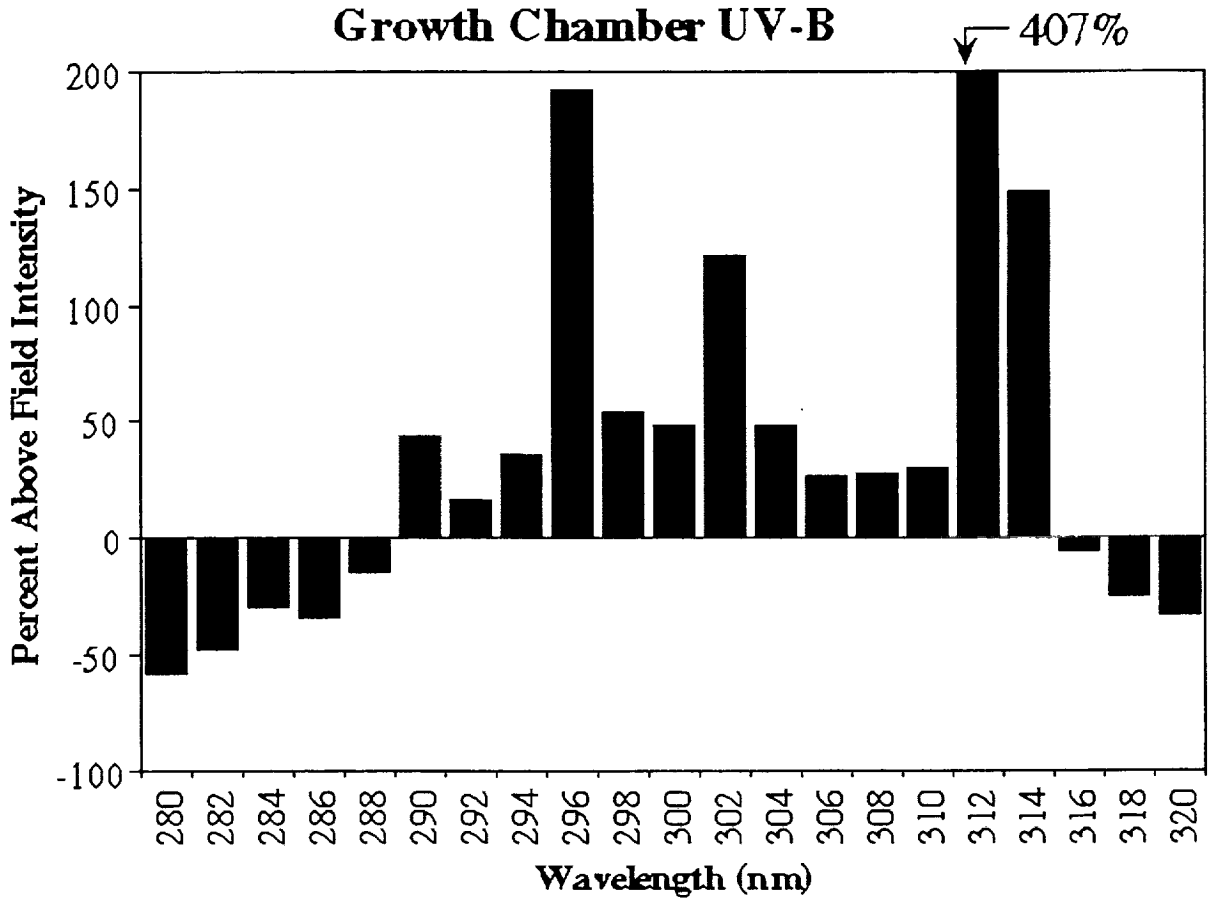

Figure 1. UV-B intensities in the growth chamber expressed as percentages above outdoor values measured in the field on September 15, 1995 (NASA Ames Research Center, Mountain View, CA).

The study undertaken was to place two legume species, soybean (*Glycine max* Merrill cv. Lammer) and alfalfa (*Medicago sativa* L.) in two different UV-B environments and evaluate the plants' responses to increased UV-B. Several indicators of response were selected, including foliar concentration of UV-B absorbing pigments and chlorophyll concentration, to determine if UV-irradiated plants responded differently from control plants screened from UV-B. Soybean was chosen because it is a major agricultural crop and has become the main source of edible vegetable

oils and of high protein feed supplements for livestock (ref 26). Alfalfa was selected as an important forage crop for livestock (ref 27) and representative of a broad class of dicotyledonous plant species.

This study is part of a research project previously outlined by D'Antoni *et al.* (ref 28). They propose research that will quantify the responses of terrestrial vegetation to UV radiation by studying pigment concentrations, photosynthesis, and spectral parameters of selected plant species in the field and in greenhouses. The research project currently consists of a field experiment at NASA Ames Research Center (in California, USA) and the growth chamber experiment described in this paper.

MATERIALS AND METHODS

Soybean

Soybean plants were grown in an indoor growth chamber. Plants were grown one seed per pot in a potting soil of peat moss and perlite; they were watered as needed, and no fertilizer was used.

The light source was a bank of ten General Electric[1] "high output" F48T12/CW/HO, 40-watt fluorescent tubes supplemented with four clear, 60-watt incandescent bulbs plus one Philips F40UVB fluorescent tube as the UV-B source. Plants were exposed to a 14-hour photoperiod and received adequate levels of photosynthetically active radiation (ref 29). A black wooden frame was constructed to divide plants into two treatments and to suspend screening films 40 cm above soil level. Films were also draped 25 cm along the length of the frame to reduce plant exposure to reflected radiation while allowing for sufficient air circulation. Mylar-D film (UV-B opaque) was suspended over one group of plants, and mono-acetate film (UV-B transparent) was suspended over another group. Mylar is opaque in the 280-316 nm range, and acetate absorbs most radiation from 280-290 nm and then reduces intensities in the 290-320 nm range by roughly 15% (Fig. 2). Plants were germinated under their respective treatments. Coverings were changed every 20 days since mylar degrades under field conditions when exposed to solar light intensities. An Optronic Laboratories OL-752 spectroradiometer was used to measure solar irradiance and irradiance of the light source (Figs. 1 and 2).

[1] Use of trade names is for convenience only and does not imply endorsement by the SETI Institute, the National Aeronautics and Space Administration, California Institute of Technology, or the U. S. Government.

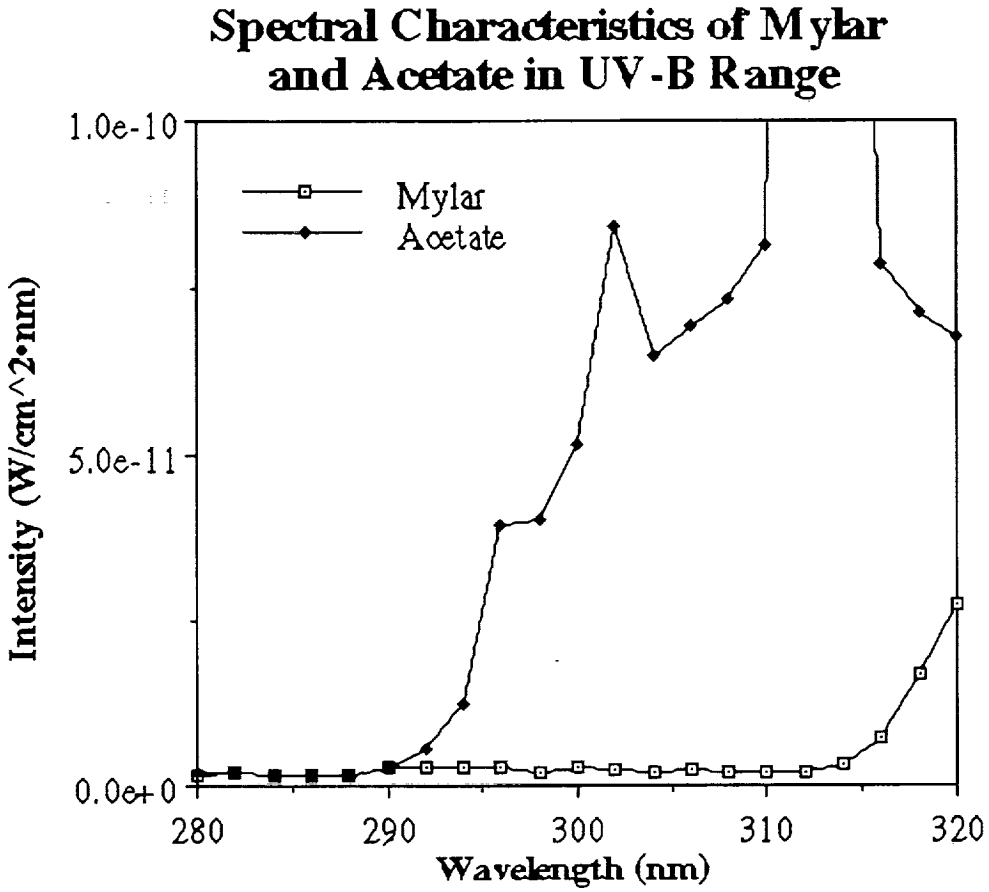

Figure 2. Spectral characteristics of mylar and mono-acetate films in the UV-B portion of the EM spectrum.

Leaf chlorophyll concentrations were determined with a Minolta SPAD-502 chlorophyll meter. (See Appendix A.) The primary advantage of the SPAD meter over traditional chlorophyll determination methods is that samples being tested need not be destroyed. As explained by Wood (ref 30), the SPAD meter is a nondestructive, hand-held instrument that measures red and infrared light transmittance in plant leaves; this light intensity is directly proportional to leaf chlorophyll content.

Because the units returned by the SPAD meter do not directly yield leaf chlorophyll concentration, it was necessary to calibrate the SPAD meter. N,N-dimethylformamide (DMF) extractions were performed based on the methods of Moran and Porath (ref 31), Moran (ref 32), and Inskeep and Bloom (ref 33). Two SPAD readings were taken per leaf, one on each side of the main vein. Using a 12 mm diameter cork borer, two disks were then removed from the leaf, one on each side of the vein. Chlorophyll was extracted with 3 ml of DMF per sample. The extractions were stored in the dark at 4°C for one week to allow the chlorophyll to elute from the leaf disks, and absorbance readings were taken at 647 nm and 665 nm on a Hitachi U-1100 spectrophotometer. Using the following equation from (ref 33), chlorophyll concentration was calculated in mg/l:

$$\text{chlorophyll } a+b = 17.90*OD_{647}+8.08*OD_{665} \quad (1)$$

where OD is absorbance at the indicated wavelength. After calibration, the SPAD meter was used to find chlorophyll concentration of the soybean leaves grown under the two treatments.

To determine leaf flavonoid concentration, samples were frozen with liquid nitrogen and ground to a powder. Flavonoids were extracted with 20 ml of a solution of methanol, water, and 2M hydrochloric acid in the proportions 79 : 20 : 1 after Mirecki and Teramura (ref 34); hydrochloric acid preserves anthocyanins (ref 35). The extract was then centrifuged and syringe filtered. Absorbance of the extract was measured from 200 nm to 700 nm, at 5 nm increments. Absorbance readings were normalized to leaf sample fresh weight. For comparing concentrations of UV-B absorbing pigments between treatments, average absorbance was calculated in the UV-B range.

Chlorophyll and flavonoid readings were taken for plants in the third, fifth, and eighth weeks after germination; sample size was eight leaves per treatment per week. Internodal distances and plant fresh weight biomass (above-ground and below-ground) were determined in the fifth and eighth weeks; sample size was four plants per treatment.

Alfalfa

At the conclusion of the soybean experiment, a similar experiment was conducted with alfalfa. Alfalfa plants were established in the same growth chamber under the same irradiance regime. Plants were grown from seed, about 50 seeds per pot, and watered as needed. Each pot was given 50 ml of Hoagland's solution in the tenth and sixteenth weeks after germination. Again, the plants were germinated and grown under either the mylar or acetate treatment.

The SPAD meter was calibrated to measure alfalfa leaf chlorophyll concentration using DMF extractions. The procedure was identical to that used for soybean with the exception that six disks (7 mm diameter) removed from six alfalfa leaves from the same plant constituted one sample. Alfalfa leaf flavonoid concentrations were determined in the same manner as for soybean (see Appendix A).

In the nineteenth week after germination, all plants were harvested, and chlorophyll and flavonoid readings were taken on a per pot basis; sample size was 30 leaves per pot per treatment. Fresh weight above-ground biomass and average internodal distances were also determined on a per pot basis.

RESULTS

Spectral data

UV-B measurements were obtained for the growth chamber and for a field site at NASA Ames Research Center, Mountain View, CA on September 15, 1995. Figure 1 shows growth chamber UV-B intensities as percentages above field intensities. The average intensity across the UV-B portion of the spectrum emitted by the growth chamber is 45% above that of the field. (Because of a

characteristic spectral peak at 312 nm in the standard fluorescent tubes, intensity at this wavelength is four times the field level.) Figure 2 compares the spectral characteristics of mylar and mono-acetate in the UV-B range. Spectral measurements were also made for new mylar and mylar after 20 days of exposure to growth chamber light intensities. In both the visible and UV-B regions of the spectrum, negligible change was observed.

Soybean

Leaf concentrations of UV-B absorbing pigments were consistently higher in plants exposed to UV (Table 1). UV-B absorbing flavonoid concentrations for soybean grown under increased UV in weeks three, five, and eight were 26%, 28%, and 44% greater, respectively, than for plants under no UV.

TABLE 1. SOYBEAN RESULTS FOR WEEKS 3, 5, AND 8 AFTER GERMINATION: FLAVONOID CONCENTRATION, CHLOROPHYLL CONCENTRATION, AVERAGE INTERNODAL DISTANCES, AND BIOMASS.

Time After Germination	**Week 3**			**Week 5**			**Week 8, Final**		
Treatment	+ UV-B	– UV-B	% difference	+ UV-B	– UV-B	% difference	+ UV-B	– UV-B	% difference
Flavonoid concentration (g^{-1})	7.64	5.66	26% *	4.92	3.52	28% *	6.25	3.53	44% *
Chlorophyll concentration (mg/l)	30.81	28.81	6% †	22.25	17.93	19% †	16.68	16.12	3% †
Internodal distance (mm)	‡	‡	‡	72	87	17% †	80	101	21% †
Above-ground biomass (g)	‡	‡	‡	0.95	0.92	3% †	1.10	0.96	13% †
Below-ground biomass (g)	‡	‡	‡	0.26	0.27	4% †	0.31	0.28	10% †

* Statistical analysis could not be performed because of insufficient data

† Nonsignificant difference as determined by Student's t-test

‡ Not determined

Calibration of the SPAD meter was accomplished with a sample size of 63. Using the SPAD meter and DMF chlorophyll extractions, the following leaf chlorophyll prediction equation was obtained:

$$\text{chlorophyll } a+b \text{ (mg/l)} = -6.78 + 0.888*\text{SPAD units} \quad (2)$$

An r^2 value of 0.910 was obtained for this equation which correlates to a 95% degree of confidence. With this r^2 value, it is appropriate to use the SPAD meter and equation (2) to accurately predict chlorophyll concentration in soybean leaves.

Chlorophyll readings were obtained at the same time flavonoid extractions were done. Differences in chlorophyll concentration between the two treatments were statistically negligible (Table 1). In each week, chlorophyll concentration was higher in plants grown under increased UV-B. Between the third and eighth weeks, leaf chlorophyll concentration decreased significantly in both treatments. This overall decrease in the apparent health of the plants can be attributed to aging of the plants. SPAD readings and flavonoid extractions were performed only on healthy leaves.

In the fifth and eighth weeks, internodal distances were found to be 17% and 21% smaller for plants exposed to UV-B; this finding is consistent with the idea that UV-B is damaging to the growth mechanism of the plant (refs 15, 16, 17). Biomass differences between treatments in both weeks were negligible.

Alfalfa

Calibration of the SPAD meter was done with a sample size of 221, six 7 mm leaf disks per sample (1326 leaf disks total). The alfalfa leaf chlorophyll prediction equation was:

$$\text{chlorophyll } a+b \text{ (mg/l)} = -16.44 + 0.960*\text{SPAD units} \quad (3)$$

An r^2 value of 0.758 was obtained for this equation. This 87% correlation allows the use of the SPAD meter and equation (3) to predict chlorophyll concentration in alfalfa leaves.

Chlorophyll and flavonoid readings were obtained for the same leaf samples under both treatments in the thirteenth, fifteenth, and seventeenth weeks after germination. Fifteen samples were selected per treatment per week with the sample size being six leaves chosen from the same plant. The goal of this procedure was to determine a correlation between leaf chlorophyll and flavonoid concentrations and to determine the time dependence of this correlation with further UV-B exposure. Data from each treatment and each week were graphed as chlorophyll versus flavonoid concentration. All graphs yielded very low r^2 values when fitted with simple as well as polynomial curves. The conclusion is that no predictable correlation exists between chlorophyll and flavonoid concentrations in alfalfa leaves, a conclusion supported by Sullivan *et al.* (ref 36) who noted that there does not seem to be a simple correlation between UV-B sensitivity and the concentration of methanol-extractable UV-B absorbing compounds.

Final measurements were taken in the nineteenth week (Table 2). Concentrations of UV-B absorbing pigments were found to be significantly higher in plants grown under increased UV-B as determined by t-test analysis. The fact that chlorophyll concentration, above-ground biomass, and internodal distances were less for plants grown under the acetate treatment (UV-B transparent) supports earlier findings (refs 15, 16, 17) that UV-B is damaging to plant photosynthetic and growth mechanisms.

TABLE 2. ALFALFA FINAL RESULTS IN WEEK 19 AFTER GERMINATION FOR FLAVONOID CONCENTRATION, CHLOROPHYLL CONCENTRATION, AVERAGE INTERNODAL DISTANCES, AND BIOMASS.

Time After Germination	**Week 19 Final**		
Treatment	+ UV-B	– UV-B	% difference
Flavonoid concentration (g^{-1})	12.59	9.77	22% §
Chlorophyll concentration (mg/l)	21.45	25.03	14% †
Internodal distance(mm)	19	21	10% *
Above-ground biomass (g)	4.12	6.06	32% ‡

* Nonsignificant difference as determined by Student's t-test

†, ‡, § Significant difference at $p \leq 0.02$, 0.01, or 0.001, respectively, as determined by Student's t-test

DISCUSSION

Results indicate that increased UV-B radiation leads to increased production of UV-B absorbing compounds (i.e. flavonoids) in both soybean and alfalfa leaves (Tables 1 and 2). This implies that these legumes possess protection mechanisms against UV-B damage. In response to increased UV-B, greater concentrations of UV-B screening compounds are produced that absorb the relatively high intensities of UV-B and consequently decrease their potentially damaging effects on the plant. These results support findings by Beggs *et al.* (ref 4) and Schmelzer *et al.* (ref 10) that flavonoids absorb UV-B radiation and that flavonoid synthesis in plant leaves is triggered by UV-B. This study also shows that UV-B intensities 45% above ambient solar levels provided evenly throughout the photoperiod are sufficient to elicit a protection response against UV-B in both soybean and alfalfa.

T-test calculations for the soybean results show no significant differences between treatments for chlorophyll, biomass, and internodal distance readings, all measures of growth or photosynthetic capacity. However, statistical evaluation of alfalfa results reveals significant differences between treatments for leaf chlorophyll concentration and above-ground biomass. These findings indicate that the growth and photosynthetic mechanisms of alfalfa are damaged by UV-B intensities 45% above ambient solar levels. Reductions in plant growth may also be a result of photo-oxidation of indoleacetic acid and/or of direct DNA damage by UV-B (ref 37). Although leaf flavonoid concentration was significantly greater in alfalfa grown under increased UV, it was apparently not sufficient to prevent UV-B damage.

Krupa and Kickert (ref 38) surveyed the literature and reported the compiled responses of certain crop species to elevated levels of carbon dioxide, UV-B, and ozone. They found that soybean is sensitive to and alfalfa is tolerant of enhanced UV-B. Contrary to the findings of Krupa and Kickert

and for the variables selected, results of this study indicate that soybean is more tolerant of UV-B, whereas alfalfa shows some damage.

Soybean and alfalfa exhibited photosynthetic damage and reduced growth by at least one of the variables measured in this experiment in response to increased UV-B. This indicates that the photosynthetic and carbon fixation rates of these legume species are affected by UV-B exposure. Because the rate of photosynthesis is closely linked to productivity, crop yields may be affected by decreased stratospheric ozone and increased UV-B flux (refs 8, 17, 38). Both alfalfa and soybean are important agricultural crops (refs 26, 27), and a decrease in their yields would have important economic implications.

Finally, it seems reasonable to assume that UV stress can be remotely sensed in plants based on chlorophyll fluorescence spectra. SPAD measurements revealed that chlorophyll concentration in soybean and alfalfa leaves is affected by UV-B exposure. These differences can presumably be detected by changes in chlorophyll fluorescence. Remote sensing of UV-induced plant stress offers an important means of monitoring biologically active levels of UV radiation at the earth's surface and, as discussed by Lichtenthaler *et al.* (ref 39), of detecting plant UV stress and damage at an early stage.

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APPENDIX A
CALIBRATION OF THE MINOLTA SPAD-502 METER

INTRODUCTION

For collecting chlorophyll data for plants grown in the field, it is advantageous to have a means of gathering a large amount of data both quickly and accurately. It is also sometimes desirable to take chlorophyll readings and to measure some other parameter of leaf physiology or biochemistry for the same leaf samples. The Minolta SPAD-502 meter allows for obtaining chlorophyll readings in these situations. While the SPAD meter was developed for rice farmers as an easy way to assess crop health, it is a useful tool to the scientist conducting other kinds of plant research.

The primary advantage of the SPAD meter over traditional chlorophyll determination methods is that the sample being tested need not be destroyed. As explained by Wood *et al.* (ref 41), the SPAD-502 chlorophyll meter is a nondestructive, hand-held instrument that measures green color intensity in plant leaves *in vivo*; transmitted light intensity is directly proportional to leaf chlorophyll content. The detector is powered by two AA batteries, has a 2-second interval between measurements, and can store up to thirty data points. Leaf color is measured by closing two sensor arms around the sample. The sensor is composed of a light source (two light-emitting diodes) and a receptor (a silicon photodiode).

SPAD measurements are based on differences in light attenuation at 650 nm (a spectral transmittance peak for chlorophyll *a* and *b* in the red part of the spectrum) and 940 nm (the near-infrared region of the spectrum where no chlorophyll transmittance occurs). A SPAD unit is calculated by a microprocessor in the device and is determined from the difference in light attenuation. Because the SPAD value returned by the meter is not a direct reading of chlorophyll concentration, it is necessary to find the numerical correlation between SPAD units and foliar chlorophyll concentration in order to use the instrument to predict chlorophyll amount in plant leaves when collecting data.

Osborn and DeBenedictis (personal communication) have calibrated the SPAD meter to measure total chlorophyll in grape leaves (*Vitis vinifera*) on a leaf area basis. Based on 359 leaf samples, they obtained the following equation:

$$\text{chlorophyll } a+b \text{ (mg/cm}^2) = -9.95\text{e-}3 + 1.61\text{e-}3*\text{SPAD} \qquad \text{(A1)}$$

with an r^2 value of 0.914 (96% correlation). For grape, they determined that cultivar and plant age do not significantly alter the SPAD-chlorophyll regression line.

Detailed below are SPAD calibration results for four plants species (alfalfa, *Medicago sativa*; soybean, *Glycine max* cv. Lammer; wheat, *Triticum aestivum* cv. Polk; and oat, *Avena fatua* cv. Ogle). (Equations and coefficients used in the main text above are included here for comparison.)

MATERIALS AND METHODS

Two methods for the extraction and measurement of chlorophyll amounts were tested in this study using alfalfa leaves. Both methods are widely used with the first technique dating to the 1940's.

The first method is based on the technique of Arnon (ref 40) which uses acetone as solvent. For each sample, SPAD readings for thirty alfalfa leaves from the same plant were obtained and averaged. The leaves were ground in a blender with 60 ml 80% acetone. The chlorophyll extract was vacuum filtered and centrifuged. Supernatant was stored in the dark at –20°C until spectrophotometric analysis was done. Absorbance readings for the extracts were obtained at 645 nm and 663 nm on a Hitachi U-1100 spectrophotometer using 1 cm cuvettes. Chlorophyll was calculated in g/l extract as follows:

$$\text{chlorophyll } a = ((45.6*OD_{663})-(9.27*OD_{645}))/3585.75 \quad \text{(A2)}$$

$$\text{chlorophyll } b = ((82.04*OD_{645})-(16.75*OD_{663}))/3585.75 \quad \text{(A3)}$$

$$\text{chlorophyll } a+b = ((20.2*OD_{645})+(8.02*OD_{663})/1000 \quad \text{(A4)}$$

Coefficients in equations (A2), (A3), and (A4) were taken from (ref 40). All chlorophyll concentration values were normalized to sample fresh weight (g). SPAD units versus chlorophyll was graphed.

The second method of chlorophyll extraction uses N,N-dimethylformamide (DMF) as the solvent and is based on the methods of Moran and Porath (ref 31), Moran (ref 32), and Inskeep and Bloom (ref 33). SPAD readings for six alfalfa leaves from the upper third of the same plant constituted one sample; these readings were averaged to obtain a single SPAD value. Using a paper punch (7 mm diameter), six disks were removed, one from each leaf. Chlorophyll was eluted from these disks in 3 ml DMF. Samples were stored in the dark at 4°C for one week. Absorbance readings were obtained at 647 nm and 665 nm in 1cm cuvettes, and chlorophyll concentration was calculated in mg/l:

$$\text{chlorophyll } a = 12.70*OD_{665}-2.79*OD_{647} \quad \text{(A5)}$$

$$\text{chlorophyll } b = 20.70*OD_{647}-4.62*OD_{665} \quad \text{(A6)}$$

$$\text{chlorophyll } a+b = 17.90*O_{D647}+8.08*OD_{665} \quad \text{(A7)}$$

Coefficients in equations (A5), (A6), and (A7) were taken from Inskeep and Bloom (ref 33). SPAD units versus chlorophyll was graphed.

For soybean, wheat, and oat, DMF calibrations were done in the same manner as for alfalfa with the following differences. Each soybean sample consisted of one leaf. Two SPAD readings were taken per leaf, one on each side of the main vein, and averaged. A cork borer (12 mm diameter) was used to remove two disks from the leaf in the approximate regions were SPAD values were obtained. For

wheat and oat, each sample was a 15 mm long leaf section. Six SPAD readings were made in the region of each leaf strip and averaged. In addition, a Cary 3 spectrophotometer was used for the oat and wheat calibrations.

RESULTS

Thirty-three chlorophyll extractions for alfalfa were performed following the method of Arnon (ref 40). This method proved inadequate for the degree of precision necessary to use the SPAD as a reliable predictor of leaf chlorophyll content, yielding an r^2 value of 0.531 for chlorophyll concentration versus SPAD values. This corresponds to a 73% correlation.

Two hundred and twenty-one chlorophyll extractions were performed for alfalfa using DMF as the solvent. DMF extractions were less time consuming and gave data that were more consistent. A plot of SPAD units versus chlorophyll concentration produced an r^2 value of 0.758 (a correlation of 87%) and the following chlorophyll prediction equation:

$$\text{chlorophyll } a+b \text{ (mg/l)} = -16.44 + 0.96*\text{SPAD} \qquad \text{(A8)}$$

Table A1 lists the SPAD calibration results for alfalfa, soybean, wheat, and oat using DMF extractions.

TABLE A1. SPAD CHLOROPHYLL CALIBRATIONS FOR ALFALFA, SOYBEAN, WHEAT, AND OAT USING DMF EXTRACTIONS.

Species	Number of samples	Chlorophyll *a+b* (mg/l) prediction equation	r^2 value (percent correlation)
Alfalfa	221	chl = –16.44 + 0.96*SPAD	.758 (87%)
Soybean	63	chl = –6.78 + 0.89*SPAD	.910 (95%)
Wheat	80	chl = –1.11 + 0.22*SPAD	.856 (93%)
Oat	90	chl = –0.43 + 0.24*SPAD	.798 (89%)

DISCUSSION AND CONCLUSIONS

Considering data gathered for alfalfa, DMF chlorophyll extractions appear to be more efficient and accurate than acetone extractions; this same conclusion was reached by Moran and Porath (ref 31). One problem with the acetone extraction method, as discussed by Yoder and Daley (ref 42), is that grinding of the leaf sample causes large chlorophyll losses. The DMF method avoids mechanical

maceration and particle removal. Furthermore, smaller solvent volumes are required. Since using DMF as the solvent for determining foliar chlorophyll concentrations yields accurate data more quickly and consistently than using acetone, DMF extraction is the method of choice for SPAD calibration.

Comparing the four chlorophyll prediction equations given in Table A1, it is evident that separate SPAD calibrations are required for each plant species studied. Calibration will indicate whether or not the SPAD meter can determine chlorophyll concentration with the desired accuracy and reliability and will provide a direct conversion of SPAD units to foliar chlorophyll concentration.

When designing the SPAD calibration conditions for a particular plant species, several factors should be considered. It is necessary to select the leaf part that will constitute one sample (i.e. leaf disks, leaf strips, whole leaves). Sample size and DMF volume must be chosen so as to obtain satisfactory absorbance measurements on the spectrophotometer. Also, selection of the number of SPAD readings per sample is important. Smaller leaves often give less consistent SPAD readings; increasing the number of SPAD values collected for small leaves and averaging these values may yield a better regression line. In conjunction with DMF extractions, calibration of the SPAD meter allows its use in the field as a quick, easy, and reliable way to determine leaf chlorophyll content.

The Minolta SPAD-502 meter is a way to reliably determine foliar leaf chlorophyll concentration *in vivo*, especially in the field. Using alfalfa (*Medicago sativa*) as the test plant, DMF extractions proved more efficient and consistent than acetone extractions. Results for SPAD calibration by the DMF method are given for three other plant species, soybean, wheat and oat.

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13. ABSTRACT ***(Maximum 200 words)***

Depletion of the stratospheric ozone layer has been directly linked to increased levels of UV radiation at the earth's surface. The purpose of this study was to evaluate the responses of soybean (*Glycine max*) and alfalfa (*Medicago sativa*) to increased UV-B radiation (280-320 nm). Soybean and alfalfa were grown successively in a growth chamber that provided UV-B intensities 45% above nominal summer field levels. Mylar-D (UV-B opaque) and mono-acetate (UV-B transparent) films were used to establish the two UV-B treatments. Soybean grown under increased UV showed 21% smaller internodal lengths and higher concentrations of UV-B absorbing pigments (i.e. flavonoids) compared to plants grown under no UV. Significant results for alfalfa included 22% greater leaf flavonoid concentration under increased UV, 14% greater leaf chlorophyll concentration under no UV, and 32% greater above-ground biomass with no UV. These leguminous species possess mechanisms that protect against UV-B damage as indicated by increases in foliar concentrations of UV-B absorbing compounds. Alfalfa appears to be more sensitive to UV-B damage than soybean. Remote sensing of chlorophyll fluorescence may offer a means of monitoring UV-induced plant stress and damage.

14. SUBJECT TERMS
Key words: *Glycine max* (soybean), *Medicago sativa* (alfalfa), chlorophyll, flavonoids, UV-B, acetone, N,N-dimethylformamide (DMF)

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