Remote sensing of the xanthophyll cycle and chlorophyll fluorescence in sunflower leaves and canopies


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Summary. Sudden illumination of sunflower (Helianthus annuus L. cv. CGL 208) leaves and canopies led to excess absorbed PFD and induced apparent reflectance changes in the green, red and near-infrared detectable with a remote spectroradiometer. The green shift, centered near 531 nm, was caused by reflectance changes associated with the de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin and with the chloroplast thylakoid pH gradient. The red (685 nm) and near-infrared (738 nm) signals were due to quenching of chlorophyll fluorescence. Remote sensing of shifts in these spectral regions provides non-destructive information on in situ photosynthetic performance and could lead to improved techniques for remote sensing of canopy photosynthesis.

Key words: Chlorophyll fluorescence – Photosynthesis – Remote sensing – Sunflower (Helianthus annuus) – Xanthophyll cycle

The xanthophyll cycle is clearly implicated in the dissipation of absorbed PFD (photon flux density in the photosynthetically active waveband) in excess of the amount that can be used in photosynthesis. Conditions of excess PFD can occur under a variety of stress conditions when there is an imbalance between absorbed PFD and the rate of photosynthetic dark reactions. In response to excess PFD and the associated increase of the chloroplast thylakoid pH gradient, the xanthophyll pigment violaxanthin (V) is reversibly de-epoxidized to zeaxanthin (Z) via antheraxanthin (A) (Yamamoto 1979, Hager 1980). The quantity of zeaxanthin is closely correlated with the rate of dissipation of excess PFD as heat (Demmig-Adams et al. 1989). Furthermore, the relative amounts of the xanthophyll cycle pigments are closely related to the photon yield of photosynthetic electron transport in many species (Thayer and Björkman 1990). Thus, the xanthophyll cycle pigments are sensitive indicators of the efficiency of photosynthetic energy conversion.

At the leaf level, the xanthophyll cycle can be studied with optical techniques. Bilger et al. (1989) demonstrated that changes in xanthophyll cycle pigment levels can be monitored as leaf absorbance changes at 505–515 nm. If these absorbance changes could be detected at the canopy level, it should be possible to estimate excess PFD and the rate of photosynthetic electron transport at the canopy level with remote sensing.

Most remote vegetation indices (e.g. the “simple ratio”, SR, and “normalized difference vegetation index”, NDVI) detect the quantity and spatial arrangement of green vegetation and can be used to estimate canopy photosynthetic capacity and net primary productivity (Goward et al. 1985; Sellers 1987). However, they are poor indicators of daily to weekly variation in realized photosynthetic rates under certain stress conditions (Running and Nemani 1988). Because the epoxidation state (EPS) and pool size of the xanthophyll cycle pigments adjust rapidly to levels of excess PFD, they may provide good indicators of realized photosynthesis under a wide range of conditions.

Our objective was to establish that reflectance changes indicative of zeaxanthin formation due to excess PFD could be measured on intact canopies with passive remote sensing. We designed experiments to generate large changes in excess PFD over short time periods. Using a spectroradiometer positioned above a sunflower canopy, we demonstrated that spectral changes associated with excess PFD can be readily detected at the canopy level.

Materials and methods

Sunflower (Helianthus annuus L. cv. CGL 208) was used in all experiments. In late May, 1989, seeds were planted in rows, and seedlings were thinned to an average spacing of 55 cm (between rows) and
27 cm (within rows). At seeding time, the soil was supplemented with 204 kg Ha⁻¹ N, 113 kg Ha⁻¹ K, 59 kg Ha⁻¹ P, and 68 kg Ha⁻¹ fritted micronutrients. Field plants were irrigated every 4 to 5 days.

Field measurements were made in August, 1989. By this time, plants had attained complete canopy closure and were in their late flowering stages. Flower heads were removed on the day prior to experimental measurements to avoid possible complications associated with the large visible reflectance of the heads (unpublished data).

Conditions of excess PFD were obtained by suddenly increasing the PFD incident upon leaf or canopy surfaces. Previous leaf-level work had indicated that sudden exposure to high light after prolonged low light exposure would maximize spectral changes in the green (Bilger et al. 1989). Field plants were shaded with black cloth the evening before experimental measurement. Near solar noon the following day, the cloth was abruptly removed, causing a sudden increase in PFD from approximately 10 to approximately 1900 μmol m⁻² s⁻¹. During the next 40 min, we monitored spectral reflectance, xanthophyll pigments and Rubisco (Ribulose-1,5-bisphosphate carboxylase-oxygenase) activity on the previously shaded portion of the canopy.

Spectral reflectance was determined with a spectroradiometer outfitted with a 15° FOV lens (Spectron Engineering, Inc., Denver, Colorado) suspended approximately 4 m above the canopy surface on a horizontal boom. The spectroradiometer was held in a fixed, nadir (vertical) view during the entire experiment, allowing periodic spectral scans of the same 1 m² of canopy surface through time. Individual scans were triggered from a microcomputer (Toshiba3000), completed within 2-3 s, and data was saved to disk within 10 s for later analysis. Due to the slight time lag inherent in the spectroradiometer, "time zero" spectral scans represent measurements made 2-3 s after shade removal.

Apparent reflectance was calculated by dividing the radiance detected from the canopy by the radiance from a horizontal, halon reflectance standard. Field results were routinely expressed as apparent reflectance (Fig. 1-3) to normalize for any changes in solar irradiance during the experiment. The reflectance standard was a 60 cm x 60 cm panel with a 1 cm thickness of halon powder pressed to an average density of 1 gm cm⁻³ as recommended by Weidner and Hsia (1981). Reflectance standard measurements were made immediately before and after the experiment by removing the spectroradiometer from the boom and mounting it in a nadir orientation 1 m above the standard panel. Canopy radiance was then divided by the reference radiance, estimated from linear interpolation of the two measurements of the halon panel. Cloudless conditions prevailed during the field experiments, resulting in almost constant irradiance during each experiment.

Spectroradiometer scans were interspersed with frequent sampling of leaf tissue for Rubisco and xanthophyll pigment assays. Leaf samples were collected from 3 leaves in the spectroradiometer view port. These leaves were near full expansion and had been selected prior to shade removal because their near horizontal orientation and canopy position ensured that they would be similarly illuminated upon shade removal. Furthermore, these 3 leaves were typical in appearance and size to "average" leaves near the top of the canopy. These top-canopy leaves probably contributed most of the radiance detected by the spectroradiometer.

Leaf samples were collected by rapidly punching three 0.9 cm² disks (xanthophyll) or three 2.0 cm² disks (Rubisco) from each of the three leaves, and then rapidly freezing these disks in liquid nitrogen. Leaf disks were stored in the dark at -60°C for later analysis.

Xanthophyll pigment extraction and analysis followed the HPLC procedure of Thayer and Björkman (1990). Each data point consisted of three samples extracted and assayed together, and thus represents the mean of three top-canopy leaves. Relative concentrations of the xanthophyll pigments violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) were used to calculate the epoxidation state (EPS), where

**EPS = 100*(V + 0.5*A)/(V + A + Z)**

Initial and total Rubisco activities were determined by the procedure of Seemann (1986). Percent Rubisco activity was calculated as the initial divided by the total activity. Each data point consisted of the mean of the same three top canopy leaves used for xanthophyll pigment assays. In some cases, the three leaf disks were assayed separately and the mean and standard deviation of the three leaves were calculated for these samples.

Measurements of net CO₂ assimilation were made with a portable gas exchange system (LI-6200, LI-COR Inc., Lincoln, Nebraska) shortly before and 40 min after shade removal on three top-canopy leaves similar in size and orientation to the three leaves used for xanthophyll and Rubisco analysis. Additional "control" measurements were made on 5 nearby leaves that were not shaded during the experiment.

To confirm the identity of spectral features observed in the field upon shade removal, lab experiments were conducted in September and October, 1989, on south- or southwest-facing leaves excised from field plants or potted plants. Potted plants were grown in 3.75 l containers in a potting mix consisting of 1:1:1 (v:v:v) perlite, mushroom compost and commercial potting mix, and received frequent tap water irrigation alternating with a balanced commercial nutrient solution (Plantex 20-20-20 with micronutrients, Plantco Inc., Brampton, Ontario, Canada). These plants developed under full sun in a glasshouse and were 6-8 weeks old at the time of measurement.

In these laboratory experiments, leaves were held in air at room temperature below the common base of a 4-parted fiber optic bundle (PAM 101F, Walz, Effeltrich, FRG), allowing simultaneous excitation and measurement of the same leaf portion. Conditions of excess PFD were induced by suddenly increasing leaf surface PFD from darkness (<1 μmol m⁻² s⁻¹) to high levels (1600-2100 μmol m⁻² s⁻¹; see figure legends) with light from one arm of a 500-W xenon arc lamp. The common end of the fiber optic was fitted with a pair of small lenses to collimate the light beam. The light source was a halogen lamp (50 W multi-mirror model ENL, General Electric, Cleveland, Ohio), attenuated by filter combinations that varied depending upon the experiment (see below). The radiance from the leaf upon increased illumination was measured by fitting another arm of the fiber optic to the spectroradiometer used in the field. Two remaining arms led to a fluorometer (PAM 101, Walz, Effeltrich, FRG), which detected chlorophyll fluorescence at wavelengths longer than 700 nm.

In the determinations of leaf reflectance in the green (Fig. 4), the light source was attenuated by a hot mirror filter (OCLI, Santa Rosa, CA; 50% transmittance at 735 nm). In this case, reflectance was calculated by dividing the radiance of the leaf by the radiance of a 2.4 cm diameter halon standard pressed to the same density as the field standard. In experiments examining leaf fluorescence in the red and near infrared (Figs. 5 and 6), the hot mirror was replaced by a water-filled glass jar and the PFD was adjusted with neutral density filters (FNG series, Melles-Griot, Irvine, CA). In determining the fluorescence spectrum (Fig. 7) the light was transmitted through a hot mirror (OCLI), a cyan dichroic filter (OCLI; blocking light > 650 nm) and a blue glass filter (BG38, Schott, Mainz, FRG), providing an actinic PFD of 500 μmol m⁻² s⁻¹. Since fluorescence signals in the red and near-infrared are not reflected radiation, we expressed spectral data in these wavelengths as spectral irradiance or as the difference in spectral irradiance between two scans (Figs. 5-7). Spectral irradiances were calculated from the spectroradiometer signals by calibrating the relative responses of each spectroradiometer waveband against a light source of known spectral irradiance (LI-COR 1800-02L).

In some experiments we examined the effect of the inhibitors dithiothreitol (DTT) and 3,4,5-dichlorohenyl-1,1-dimethylurea (DCMU) on the altered spectral features of excised leaves upon suddenly increased PFD. These leaves were cut under water with a sharp razor blade and the petioles were placed in a solution containing 3 mM DTT or 3 x 10⁻⁴ M DCMU, or in deionized water (controls). These leaves were kept under fluorescent lights at a
Results

The apparent spectral reflectance of sunflower canopies at various times following increased irradiance is illustrated in Fig. 1A. Apart from a slight decline in apparent reflectance above 700 nm with time, there was little visible difference between spectral scans taken at time zero and 40 minutes after shade removal. However, when expressed as difference spectra (apparent reflectance at time n minus apparent reflectance at time zero), three distinct troughs emerged, representing wavebands of unstable apparent reflectance (Fig. 1B). Two troughs, one centered near 685 nm and the other near 738 nm, were readily visible within 1/2 min of increased illumination. A third trough, centered near 531 nm, became visible within two minutes of increased canopy illumination. The center of this third trough varied between experiments from 525 to 534 nm (not shown).

These changes in apparent reflectance stabilized after several minutes (Fig. 2A, 2B). Xanthophyll cycle pigment levels and epoxidation state in top canopy leaves paralleled the changes in apparent reflectance (Fig. 2C).

Following shade removal, Rubisco activity increased rapidly (Fig. 2D), indicating rapid induction of carboxylation. Mean (±SD) net CO₂ assimilation rates before and 40 minutes after shade removal were 0.6 (±1.2) and 33.9 (±2.2) μmol m⁻² s⁻¹, respectively. The 40 minute rate was identical to the rate of adjacent control leaves that had not been shaded (32.4±1.1 μmol m⁻² s⁻¹), confirming that full induction of carboxylation had occurred.

Canopy reflectance at 531 nm and epoxidation state of top canopy leaves were closely correlated, particularly during the first 10 min of increased exposure (R²=0.99; solid circles, Fig. 3). Data from 19 and 40 minutes after exposure began to depart from this linear relationship (open circles, Fig. 3), possibly due to changes in leaf

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**Fig. 1.** A Apparent spectral reflectance of an intact sunflower canopy determined at various times following sudden exposure to full sun upon shade removal. The PFD on a horizontal plane at the canopy surface before and after exposure was approximately 10 and 1900 μmol m⁻² s⁻¹, respectively; B Difference spectra determined from data illustrated in A.
Fig. 3. Canopy reflectance at 531 nm versus epoxidation state of three top-canopy leaves. Solid symbols and regression line represent data collected within 10 minutes of shade removal. Open symbols indicate data collected at 19 and 40 minutes. Data from the same experiment as shown in Fig. 1.

Fig. 4. Difference spectra between reflectance 9.5 minutes after sudden irradiance increase and reflectance at time zero, for DTT-treated and control leaves (solid lines). The two spectra have been offset by a $\Delta$ reflectance of 0.01 for clarity. The PFD before and after the irradiance increase was 0 and 2100 $\mu$mol m$^{-2}$ s$^{-1}$, respectively. Curves A and B show the reversible and irreversible components, respectively, obtained by subsequent darkening and reillumination of the control leaf. See "Results" for further details.

Fig. 5. Difference spectra between relative spectral irradiance 6 minutes after a sudden irradiance increase and radiance at time zero, for DCMU-treated and control leaves. The PFD before and after the irradiance increase was 0 and 1600 $\mu$mol m$^{-2}$ s$^{-1}$, respectively. Results from two DCMU Experiments are shown.

The fluorescence spectrum from an intact leaf was similar to the difference spectrum (spectral irradiance at time zero minus spectral irradiance at 6 minutes) of the intact canopy following shade removal. Both spectra showed peaks near 740 nm with shoulders near 685 nm (Fig. 7). Similar fluorescence spectra have been reported for intact leaves of a wide range of angiosperms (e.g., Virgin 1954; Chappelle et al. 1985).
Discussion

Passive remote sensing clearly detected changes in apparent reflectance in the green associated with excess PFD induced by rapidly increased illumination. The green shift was readily visible at both the canopy and the leaf level.

The strong short-term correlation between spectral reflectance at 531 nm and the xanthophyll pigment epoxidation state indicates that the xanthophyll cycle is closely associated with the green shift. Inhibition of this shift by DTT and DCMU, and the temporal stability of the major component of this shift, further implicates the xanthophyll cycle. DTT is a potent inhibitor of the xanthophyll de-epoxidase in vitro (Yamamoto and Kamite 1972) and of violaxanthin deepoxidation in intact leaves (Bilger et al. 1989). DCMU also inhibits violaxanthin deepoxidation, presumably because it prevents the build-up of a trans-thylakoid pH gradient.

Although Bilger et al. (1989) reported that zeaxanthin formation induces a maximal change in leaf absorbance at 505–515 nm, the peaks of both the total green shift and its irreversible component occurred at longer wavelengths (525–534 nm) in our experiments. This difference may be related to several factors. Firstly, the spectral dependence of the green shift, measured as Δ reflectance in our experiments, might differ when expressed as absorbance derived from measurements of transmitted light, as done by Bilger et al. (1989). These spectral differences could be caused by different path-lengths of reflected and transmitted radiation. Secondly, varying degrees of chloroplast movement, which was prevented in the study of Bilger et al. (1989) but not in our experiments, could cause slightly different spectral peaks.

It is impossible to separate the two components of the green shift under field conditions. However, if our laboratory results are applicable to our field observations, the green shift observed in intact canopies should have included a minor component at 535–540 nm, attributable to increased light scattering associated with chloroplast conformational changes (Heber 1969). We conclude from these results that the bulk of the reflectance change at 531 nm is caused by the appearance of zeaxanthin. Thus, the 531 nm reflectance change may provide a useful signal for monitoring rapid declines in the efficiency of photosynthetic light reactions under conditions of excess PFD. It may also be a suitable probe for examining the regulation of photosynthetic light reactions under dynamic light environments (e.g. sunflecks and intermittent clouds).

The spectral irradiance changes in the red and near-infrared upon sudden illumination are caused by the quenching of chlorophyll fluorescence, as shown by kinetic and spectral comparisons of the spectroradiometer signal with known fluorescence signals (Figs. 6 and 7). This fluorescence is fully induced within a few seconds of increased illumination, and relaxes over the next several minutes through photochemical and non-photochemical quenching. Photochemical quenching would be stimulated by the increased Rubisco activity illustrated.
in Fig. 2D. One component of increased non-photochemical quenching is probably associated with the de-epoxidation of violaxanthin (Fig. 2C). Under the conditions of our experiments, large fluorescence changes were detectable because the "time zero" spectroradiometer scan occurred near the peak of fluorescence induction. Thus, the difference spectra obtained by subtracting this time zero scan from subsequent scans yield troughs in the red and near-infrared.

In our experiments, passive remote sensing of radiance shifts in the red and near-infrared due to fluorescence induction and quenching appeared to offer less potential than the green shift due to the extremely rapid kinetics and difficulties in obtaining a suitable reference level (e.g. maximal fluorescence yield in the absence of photochemical and non-photochemical quenching). Moreover, under most natural conditions, rapid stepwise increases in PFD from almost zero to very high values, which are required to obtain such a reference level, never occur. However, recent advances in field fluorometry using modulated measuring beams and saturating light pulses have largely overcome these limitations at the leaf level (Schreiber et al. 1986; Bolhar-Nordenkampf et al. 1989). Modulated measuring light allows the separation of the fluorescence signal against a background of reflected radiation, and saturating light pulses allow detailed analysis of non-photochemical fluorescence quenching, which can provide useful insights into reduced photosynthetic performance under a wide range of stresses (Schreiber and Bilger 1987). If saturating light pulses could be applied at the canopy level, e.g. with pulsed laser beams coupled with rapid, narrow field-of-view detectors, it might be possible to quantify dynamic photosynthetic processes at the canopy level with field fluorometry.

The relatively slow kinetics of the green shift allowed us to capture a "time zero" reference level within the first few seconds of shade removal. Alternatively, midday reflectances in the green could be referenced to reflectances at other wavebands, or to running canopy reflectances obtained under low ambient light levels (i.e. when the xanthophyll pigment epoxidation state is close to 100).

The changes in spectral irradiance in the green may lead to the development of nondestructive techniques for assessing several aspects of leaf and canopy function. Under appropriate conditions or with suitable corrections, this spectral signal may form the basis for a physiologically based remote index of photosynthetic electron transport and possibly of CO₂ fixation. In addition, the availability of a probe for excess radiation may yield techniques for assessing environmental stresses that depress realized photosynthesis below photosynthetic capacity. Operational techniques for the remote sensing of canopy function will likely require attention to solar angle, incident irradiance, canopy structure, leaf movement, soil reflectance, atmospheric effects and other factors, all of which affect canopy radiance (Sellers 1985; Choudhury 1987; Goel 1988). In the near future, these techniques may be most useful in monitoring canopies from ground-based systems, e.g. from a stationary platform a few meters above a canopy.

Conventional remote vegetation indices based on canopy structure and leaf display (e.g. NDVI and SR) do not always respond to the short term depressions of photosynthesis under fluctuating environmental conditions (Running and Nemani 1988). In contrast, the green shift closely follows changes in photosynthetic activity, and may be ideal for estimating the efficiency of photosynthetic processes under dynamic, natural settings. We are currently exploring the utility of reflectance signals in the green as general indicators of reduced photosynthetic performance in crops and wild plants.

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