Chlorophyll Fluorescence Analysis and Imaging in Plant Stress and Disease

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**Chlorophyll fluorescence analysis
and imaging in plant stress and disease**

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Quantitative analysis of chlorophyll fluorescence transients and quenching has evolved rapidly in the last decade. Instrumentation capable of fluorescence detection in bright actinic light has been used in conjunction with gas exchange analysis to build an empirical foundation relating quenching parameters to photosynthetic electron transport, the state of the photoapparatus, and carbon fixation. We have developed several instruments that collect video images of chlorophyll fluorescence. Digitized versions of these images can be manipulated as numerical data arrays, supporting generation of quenching maps that represent the spatial distribution of photosynthetic activity in leaves. We have applied this technology to analysis of fluorescence quenching during application of stress hormones, herbicides, physical stresses including drought and sudden changes in humidity of the atmosphere surrounding leaves, and during stomatal oscillations in high CO₂. We describe a recently completed portable fluorescence imaging system utilizing LED illumination and a consumer-grade camcorder, that will be used in long-term, non-destructive field studies of plant virus infections.

In recent years, chlorophyll (Chl) fluorescence has become an increasingly useful means for determining the energy state of the photosynthetic apparatus (6, 17, 21, 23, 29) of plants, particularly in studies on environmental influences such as adaptation to light, extremes of temperature, drought, exposure to chemical agents or UV-B radiation, and disease (2, 9, 16, 18, 26, 27, 28, 31). This has been stimulated by improvements in methods for Chl fluorometry and our understanding of the mechanisms competing for the excitation energy trapped by photosynthetic systems. This paper describes the development of a quantitative approach to analyze spatial components of fluorescence emissions from leaves through the use of digital imaging. Recent reviews have examined the historical development of Chl fluorescence analysis (14, 17, 29), and methods of conventional fluorescence recording (15, 23). Reports of practical applications in limnology, forestry and agronomy are increasing in number (16, 17, 18, 26), and interest is mounting in the exploitation of remote fluorescence sensing (10, 16), as a “ground-truthing” technique to augment other forms of remote sensing.

Modulated light Chl fluorometers often “view” the leaf with non-imaging fiber-optic bundles, and average information over one to several square centimeters of leaf surface (25, 26). However, the discrete compartments defined by the leaf vasculature in many species of herbaceous plants and deciduous trees are much smaller than this. These compartments may isolate regions with distinct characteristics, such as stomatal diffusivity or degree of infection by a pathogen that could, in turn, create a mosaic of biochemical activity. This greatly complicates biochemical interpretation of gas-exchange from photosynthesis under stress conditions. We hypothesized that, if present, this heterogeneity would modulate fluorescence yield, and that differences might be sufficient to discriminate by digital imaging techniques. We have prepared images of nonphotochemical quenching (q_N) from video images of leaf fluorescence (8), and from these, derived images in which grey-levels represent the underlying rate of photosynthesis (7). In numerous cases the apparent photosynthetic activity was markedly non-uniform.

Fluorescence analysis and imaging techniques

Upon illumination, the fluorescence yield of dark-adapted leaves rises rapidly (200-500 ms) to a maximum (dark-adapted maximum fluorescence, $(F_M)_D$ or maximum variable fluorescence, $(F_V)_M$) and then falls, sometimes in an oscillating manner (variable fluorescence, F_V), as photosynthesis and fluorescence yield both approach a steady state (6, 14, 21). Bright flashes of light ($3\text{-}4000\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) delivered during the variable fluorescence yield phase produce a yield referred to as saturated variable fluorescence, $(F_V)_S$. Analysis of the kinetics of this phenomenon, known as the Kautsky effect (13), was well established by the time more advanced instruments permitted detailed examination of the mechanisms that modulate fluorescence yield (5, 11). Some commercial fluorometers measure a very rapid transition in fluorescence excited only by a weak, modulated-light measuring beam (F_0 ; 25,26). F_0 has been reported to be useful for analysis of stress responses (26), but this transition is so rapid (<0.5 ms) that it cannot be resolved by conventional video framing rates (~ 16 ms). We will not address video methods for F_0 estimation.

Decline in fluorescence yield from maximum levels is generally referred to as quenching, and is caused by a suite of mechanisms that includes primary photochemical quenching (q_Q , associated with trapping of quanta and water splitting by photosystem II [LHCII]), and other secondary processes that appear to collectively drain excess excitation energy away from the reaction centers (non-photochemical quenching, q_N). These parameters can be estimated from fluorescence yield data (Fig. 1). Non-photochemical fluorescence quenching is correlated with the establishment of proton gradients between the different compartments of the chloroplast, that in turn may drive reversible synthesis of xanthophyll pigments, particularly zeaxanthin. Zeaxanthin formation may be responsible for quenching by resonant energy transfer (9). Other components of q_N include changes in the distribution of LHCII complexes; recent work points to functional linkages between these processes (24). q_N is measured during experiments in which the yield of fluorescence is recorded during light flashes that saturate the photoapparatus ($3\text{-}5000\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 5, 25). If photosynthesis is simultaneously measured by gas-exchange analysis, an empirical correlation can be

developed to predict photosynthetic activity under a broad range of conditions, based on parameters derived from fluorescence (32).

Imaging of whole-leaf Chl fluorescence has been achieved with a phosphoroscope and photography (4) and with video cameras (19). We have developed a series of bench top systems for imaging in a macro-photographic range of magnifications (Fig. 2), in order to resolve fluorescence emissions in the areoles of heterobaric leaves exposed to abscisic acid (ABA; 8, 30). Fluorescence excitation was accomplished with argon-ion lasers, continuous-burn xenon lamps, or fiber-optic illuminators using halogen lamps (7, 8). Light intensity was controlled with shutters or with filter wheels. Emissions were detected with monochrome or color charge-coupled device (CCD) cameras equipped with macro lenses and band-pass or long-pass filters in the 695-740 nm region (most CCD cameras are equipped with far-red filters that block beyond 690 nm, that must be removed for fluorescence imaging). In all cases the cameras had to be set for manual control of gain. Video signals were recorded on conventional analog videotape for later processing, or immediately captured (digitized) with "frame-grabber" boards for microcomputers.

We have more recently built a portable system based on a consumer grade Hi8 camcorder (S-video capable, 8-mm format; Canon L-1), and an illuminator utilizing a conical array of ultrabright red light-emitting diodes (LEDs; AND, Inc., Burlingame, California) with primary emission at 660 nm (Fig. 3). With twelve LED beams crossing in a ~2 cm diameter spot, plant tissues are uniformly illuminated with saturating pulses of actinic (steady state) illumination, with intensity up to $4000 \mu\text{E}\cdot\text{m}^2\cdot\text{s}^{-1}$. Precision of the light output is better than 2%. This system also includes an embedded single-board computer (Z-World Engineering, Davis, California), which controls both the LED array, and the camera via a serial interface (control-L or LANC; Sony, Inc.). Dark-adaptation of leaves in the field is accomplished with shuttered leaf clips developed for a portable, commercial fluorometer (Plant efficiency analyzer, PEA; Hansatech Instruments, Ltd., King's Lynn, Norfolk, UK). A detailed analysis of the performance of this portable system is in preparation.

Once images from any of these systems are digitized, each pixel is normalized against a frame taken during $(F_V)_M$, using an arithmetic scheme adapted from Schreiber (25, 26). Frames representing any of the derived fluorescence parameters in the grey-scale (e.g.: q , q_N , q_Q) can be prepared by appropriate manipulations of frames acquired during F_V or $(F_V)_S$, and the reference, $(F_V)_M$. Using this approach in parallel with an area-averaging system (PAM, Heinz Walz NA, FRG), a high correlation between CCD detection and conventional modulated-light fluorometers can be demonstrated (8; Fig 4).

In our original work (8), additional steps were required to properly scale the resulting values, owing to the lack of floating-point subsystems on the minicomputer used for processing. Since that time, software libraries have become available that support floating-point arithmetic processors on desktop microcomputers, in a graphical programming environment (Concept VI, Graftek, France; LabVIEW, National Instruments, Austin, Texas) that greatly facilitates development of custom image acquisition and analysis applications.

The derived images can be subjected to a variety of analyses or calibration operations particularly if imaging of non-stressed control leaves can be performed along with gas exchange analysis (12). This supports estimation of c_i (mesophyll CO_2 concentration) or photosynthesis rate on an area resolved basis during imposition of stress or disease (7).

Applications to Plant Stress and Disease

Many plant pathogens cause obvious damage to photosynthetic tissues, as evidenced by lesions, chlorosis or diminished yield. It has been suggested that viruses such as tobacco mosaic virus (TMV) may be viewed as parasites of chloroplasts (3), and that they compete for nitrogen reserves otherwise utilized in the synthesis of ribulose 1,5-bisphosphate carboxylase (rubisco). This may also be true for a variety of other viral pathogens (22). Time course studies of these infections or their distribution within plants or communities is hampered by the labor intensive nature of virus isolation and characterization. Moreover, some virus infections may not be accompanied by visible symptoms, or symptoms may

develop only when the host is placed in an environment that places increased demands on scarce resources (20). We have observed that some virus strains may reduce photosynthesis, and cause patchy fluorescence quenching in host leaves, well before lesions become visible to the eye (20). We are planning experiments to evaluate the portable fluorescence imaging system for determining virus distributions within and between plants, through seasons and across different habitats.

Several examples of fluorescence images following experimentally applied stress (ABA, herbicides) have been reported previously (7, 8). We have also observed dramatic spatial variation in fluorescence quenching of low-light adapted tobacco leaves with moderate nitrogen stress, during stomatal oscillations. Oscillations were induced by placing a leaf in a dark, high CO₂ atmosphere (>2500 $\mu\text{l}\cdot\text{l}^{-1}$) for 10 min then illuminating in continuous high light ($\sim 2000 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for a 5-min recording period; images were recorded at 5-sec intervals. In this experiment, no saturating flashes were applied, but F_v images were normalized against a frame taken at $(F_v)_M$, to generate images of total quenching, q . Fig. 5 illustrates a trace from an area averaging fluorometer trained on the leaf; Fig. 6 shows this same trace with four frames selected from the image sequence at various times, along with surface plots of the q quenching frames (plots were generated using Transform 2.1, Spyglass, Inc., Champaign-Urbana, Illinois).

During the first 1.5 min, the overall fluorescence emission oscillated strongly, but was relatively uniform across the leaf surface (Frame 1). However, as the "primary" oscillation dampened, spatial oscillation was observed. This appeared as fronts of alternating quenching and relaxation (markedly higher in fluorescence yield) moving across the leaf. In many instances these fronts originated along veins, particularly near branch points in the vein structure (cf: Frame 25). Late in the experiment, when oscillation was no longer observable by the area-averaging fluorometer, a highly localized region of relaxed quenching emerged from a vein branch point (top center, Frame 53). This front of relaxed quenching migrated across the leaf for approximately the last 30 sec of the experiment (Frame 60).

The velocity and spatial aspect of this migrating region was striking, and reminiscent of dispersion of applied ABA in earlier experiments (8), although the trend during ABA application was always toward increased quenching and diminished gas exchange, but here there were oscillations above and below local average quenching levels. With the small regions involved, and speed of the response, a mechanistic explanation of this phenomenon will admittedly be difficult to develop.

Conclusions

Chl fluorescence imaging is a direct outgrowth of conventional fluorescence analysis, that can be shown to produce grey-scale images in which the values quantitatively represent the level of quenching attributable to photochemistry or “photoprotective” non-photochemical energy dissipation (24). Equipment required to produce these images is now modest in cost, and both capture and analysis of digital images can be performed easily with desktop microcomputers. Analysis of quenching kinetics from small regions is useful in addressing the distribution of photosynthesis activity across the surface of leaves, and can detect changes in the behavior of regions where virus infections have occurred (1), well before visible lesions have developed. In some cases, infections by low-virulence virus strains, that impaired photosynthesis but did not produce lesions, have been visualized with the imaging technique (20). Application of this approach to “scouting” for other plant stresses and disease agents may be possible with portable imaging systems now under development.

One possible drawback to the technique is the dependence on computer processing. Image processing is, in general, a memory and storage intensive operation. We typically store images only one quarter the size of typical displays (320 x 240 pixels), yet a 60 frame experiment such as that excerpted in Fig. 6 occupies about 4.6 megabytes on disk. Animating a dataset of these images produces a dramatic presentation that readily reveals subtle changes taking place during experiments, but for best performance animation requires holding all the

images in memory. Fortunately the rapid expansion of microcomputer capabilities has outpaced the emerging demands of fluorescence imaging.

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