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REVIEW ARTICLE

Chlorophyll fluorescence—a practical guide

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Abstract

Chlorophyll fluorescence analysis has become one of the most powerful and widely used techniques available to plant physiologists and ecophysiologists. This review aims to provide an introduction for the novice into the methodology and applications of chlorophyll fluorescence. After a brief introduction into the theoretical background of the technique, the methodology and some of the technical pitfalls that can be encountered are explained. A selection of examples is then used to illustrate the types of information that fluorescence can provide.

Key words: Chlorophyll fluorescence, electron transport, photoinhibition.

Introduction

In recent years, the technique of chlorophyll fluorescence has become ubiquitous in plant ecophysiology studies. No investigation into the photosynthetic performance of plants under field conditions seems complete without some fluorescence data. This trend has been fuelled to a large degree, by the introduction of a number of highly user-friendly (and portable) chlorophyll fluorometers. In spite of the simplicity of the measurements, however, the underlying theory and the interpretation of data remains complex and, in places, controversial. A number of excellent reviews exist that discuss the theoretical background of both measurement and analysis, however, these are typically written from a biophysicist's or a molecular plant physiologist's point of view (Horton and Bowyer, 1990; Krause and Weis, 1991; Govindjee, 1995). The aim of this review is to provide a simple, practical guide to chlorophyll fluorescence for those beginners who are interested in applying the technique in both field and laboratory situations. Whilst the principles behind the measurements will be discussed briefly, the emphasis will be on the applications and limitations of this technique in plant ecophysiology.

The basis of chlorophyll fluorescence measurements

The principle underlying chlorophyll fluorescence analysis is relatively straightforward. Light energy absorbed by chlorophyll molecules in a leaf can undergo one of three fates: it can be used to drive photosynthesis (photochemistry), excess energy can be dissipated as heat or it can be re-emitted as light—chlorophyll fluorescence. These three process occur in competition, such that any increase in the efficiency of one will result in a decrease in the yield of the other two. Hence, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be gained.

Although the total amount of chlorophyll fluorescence is very small (only 1 or 2% of total light absorbed), measurement is quite easy. The spectrum of fluorescence is different to that of absorbed light, with the peak of fluorescence emission being of longer wavelength than

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Abbreviations: F_v/F_m, ratio of variable to maximum fluorescence—the quantum efficiency of open photosystem II centres; F_m, maximum fluorescence yield; F_o, minimum fluorescence yield; F_s or F_t, steady-state fluorescence yield; J, photosynthetic electron transport rate (μequiv m^{-2} s^{-1}); *NPQ*, non-photochemical quenching; PFD, photon flux density (400–700 nm); PSII, photosystem II, *qP*, photochemical quenching; *qE*, energy-dependent quenching; *qI*, photoinhibitory quenching; *qT*, quenching related to state transitions; Q_A, primary quinone acceptor of photosystem II; ΔpH, transthylakoid pH gradient; Φ_{PSII}, quantum yield of photosystem II photochemistry.

that of absorption. Therefore, fluorescence yield can be quantified by exposing a leaf to light of defined wavelength and measuring the amount of light re-emitted at longer wavelengths. It is important to note, however, that this measurement can only ever be relative, since light is inevitably lost. Hence, all analysis must include some form of normalisation, with a wide variety of different fluorescence parameters being calculated (see below).

One modification to basic measuring devices that has been instrumental in revolutionizing the application of chlorophyll fluorescence, has been the use of a 'modulated' measuring system (Quick and Horton, 1984). In such systems, the light source used to measure fluorescence is modulated (switched on and off at high frequency) and the detector is tuned to detect only fluorescence excited by the measuring light. Therefore, the relative yield of fluorescence can now be measured in the presence of background illumination, and, most significantly, in the presence of full sunlight in the field. Most modern fluorometers use such modulated measuring systems and anyone considering investing in a fluorescence system is strongly advised to select a modulated fluorometer.

Why does fluorescence yield change? The Kautsky effect and beyond

Changes in the yield of chlorophyll fluorescence were first observed as early as 1960 by Kautsky and co-workers (Kautsky *et al.*, 1960). They found that, upon transferring photosynthetic material from the dark into the light, an increase in the yield of chlorophyll fluorescence occurred over a time period of around 1 s. This rise has subsequently been explained as a consequence of reduction of electron acceptors in the photosynthetic pathway, downstream of PSII, notably plastoquinone and in particular, Q_A. Once PSII absorbs light and Q_A has accepted an electron, it is not able to accept another until it has passed the first onto a subsequent electron carrier (Q_B). During this period, the reaction centre is said to be 'closed'. At any point in time, the presence of a proportion of closed reaction centres leads to an overall reduction in the efficiency of photochemistry and so to a corresponding increase in the yield of fluorescence.

When a leaf is transferred from darkness into light, PSII reaction centres are progressively closed. This gives rise (during the first second or so of illumination) to an increase in the yield of chlorophyll fluorescence. Following on from this, however, the fluorescence level typically starts to fall again, over a time-scale of a few minutes. This phenomenon, termed fluorescence quenching, is explained in two ways. Firstly, there is an increase in the rate at which electrons are transported away from PSII; this is due mainly to the light-induced activation of enzymes involved in carbon metabolism and the opening of stomata. Such quenching is referred to as 'photochemical quenching'. At the same time, there is a increase in the efficiency with which energy is converted to heat. This latter process is termed 'non-photochemical quenching' (NPQ). In a typical plant, changes in these two processes will be complete within about 15–20 min and an approximate steady-state is attained, although the time taken to reach this state can vary significantly between plant species (Johnson *et al.*, 1990).

Deconvoluting fluorescence signals

In order to gain useful information about the photosynthetic performance of a plant from measurements of chlorophyll fluorescence yield, it is necessary to be able to distinguish between the photochemical and non-photochemical contributions to quenching. The usual approach is to 'switch off' one of the two contributors, specifically photochemistry, so that the fluorescence yield in the presence of the other alone can be estimated. *In vitro* this can be achieved by the addition of chemicals, such as the herbicide Diuron (DCMU), that inhibit PSII, reducing photochemistry to zero. This method is, however, both impractical and undesirable in a more physiological context. Instead, a method has been developed, the 'light doubling' technique, that allows the contribution of photochemical quenching to be transiently reduced to zero (Bradbury and Baker, 1981; Quick and Horton, 1984). In this approach, a high intensity, short duration flash of light is given. The effect is transiently to close all PSII reaction centres. Provided the flash is short enough, no (or a negligible) increase in non-photochemical quenching occurs and no long-term change in the efficiency of photosynthesis is induced. During the flash, the fluorescence yield reaches a value equivalent to that which would be attained in the absence of any photochemical quenching, the maximum fluorescence, F_m. Comparison of this value with the steady-state yield of fluorescence in the light (F_t) and the yield of fluorescence in the absence of an actinic (photosynthetic) light (F_o) gives information about the efficiency of photochemical quenching and by extension, the performance of PSII.

As well as changes occurring in the efficiency of photochemistry, the efficiency of heat dissipation (i.e. non-photochemical quenching) can change depending on various internal and external factors. Such changes are reflected as changes in the level of F_m. Unlike photochemistry, it is not possible to inhibit heat dissipation totally, so it is not possible to measure the yield of chlorophyll fluorescence in the absence of non-photochemical quenching. Hence, all estimations of non-photochemical quenching are strictly relative to some dark-adapted point (termed here F_m^o). For this reason, it is necessary to design experiments in such a way that a dark-adapted, non-stressed reference point can be estimated. This

requirement can be a major limitation in field conditions, where it is usual to estimate the pre-dawn value of F_m.

Quenching analysis

In the brief history of chlorophyll analysis, a large number of different coefficients have been calculated to quantify photochemical and non-photochemical quenching and the same parameter will often be referred to in many different ways. Despite attempts to normalize the terminology (van Kooten and Snel, 1990), the novice may well experience considerable confusion when reading the available literature. It is not the intention here to cover all the parameters that have been calculated. Instead, this study concentrates on the most useful and the most common (Table 1). The terminology used here is believed to be the consensus, however, other terms for the same parameters will almost certainly be found in the literature.

The calculation of fluorescence parameters is probably best explained by reference to a typical experimental trace (Fig. 1). Under field conditions, an experiment of this type may not always be appropriate, however, most of the parameters can still be calculated. The measurement is initiated by switching on the measuring light, giving a measure of the F_o (minimal) level of fluorescence. A saturating flash of light is then applied, allowing the measurement of F_m in the dark-adapted state (F_m^o). Following on from this, an actinic light is applied and, at appropriate intervals, further saturating flashes are applied. From each of these, a value for F'_m, the fluorescence maximum in the light, can be measured. The steady-state value of fluorescence immediately prior to the flash is termed F_t. After a flash, removal of actinic light (preferably whilst simultaneously giving a far-red light) allows measurement of F'_o.

Table 1. *Commonly used fluorescence parameter*

Fluorescence levels are as defined in Fig. 3 and in the text.

Photochemical quenching parameter:		
Φ_{PSII}	Quantum yield of PSII	$(F'_m-F_t)/F'_m$
qP	Proportion of open PSII	$(F'_m-F_t)/(F'_m-F'_o)$
F_v/F_m	Maximum quantum yield of PSII	$(F_m-F_o)/F_m$
Non-photochemical quenching parameters:		
NPQ	Non-photochemical quenching	$(F_m^o-F'_m)/F'_m$
NPQ_F	Fast relaxing NPQ	$(F_m^o/F'_m)-(F_m^o/F_m^r)$
NPQ_S	Slowly relaxing NPQ	$(F_m^o-F_m^r)/F_m^r$

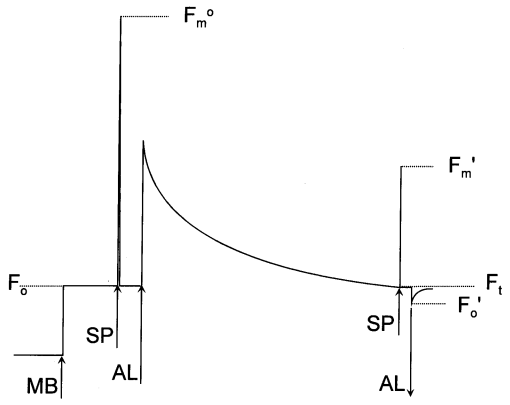

Fig. 1. Sequence of a typical fluorescence trace. A measuring light is switched on (↑MB) and the zero fluorescence level is measured (F_o). Application of a saturating flash of light (↑SP) allows measurement of the maximum fluorescence level F_m^o. A light to drive photosynthesis (↑AL) is then applied. After a period of time, another saturating light flash (↑SP) allows the maximum fluorescence in the light (F'_m) to be measured. The level of fluorescence immediately before the saturating flash is termed F_t. Turning off the actinic light (AL), typically in the presence of far-red light, allows the zero level fluorescence 'in the light' to be estimated.

Photochemical processes

Photochemical quenching parameters always relate to the relative value of F'_m and F_t. The most useful is the parameter that measures the efficiency of Photosystem II photochemistry, Φ_{PSII} (Genty *et al.*, 1989). This is calculated as:

$$\Phi_{PSII}=(F'_m-F_t)/F'_m \quad (1)$$

This parameter measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry. As such, it can give a measure of the rate of linear electron transport and so an indication of overall photosynthesis. Under laboratory conditions, there is a strong linear relationship between this parameter and the efficiency of carbon fixation, however, a discrepancy between these two parameters may occur under certain stress conditions, due to changes in the rate of photorespiration or pseudocyclic electron transport (Fryer *et al.*, 1998). Since Φ_{PSII} is the quantum yield of PSII photochemistry, it can be used to calculate linear electron transport rate (J) and, therefore, overall photosynthetic capacity *in vivo* (Genty *et al.*, 1989) as described in Equation 2

$$J=\Phi_{PSII}\times \mathrm{PFDa}\times(0.5) \quad (2)$$

where PFDa is *absorbed* light (μmol photon m^{-2} s^{-1}) (measured using an integrating sphere) and 0.5 is a factor that accounts for the partitioning of energy between PSII and PSI. There are a number of assumptions in this calculation, however, most notably that excitation energy is distributed evenly between the two photosystems. It is generally not practical to measure the light absorbed by a leaf, however, and provided that similar samples are being compared (i.e. that absorption of light is constant), *relative* changes in J can usefully be monitored by simply multiplying Φ_{PSII} by incident light (but see discussion below). Most usefully of all, this is the simplest fluorescence parameter to measure. A measurement can be made by simply pointing a fluorometer at a leaf and flashing a light.

Another widely used fluorescence parameter, measuring photochemistry, is 'photochemical quenching', qP. This is calculated as:

$$qP = (F'_m - F_t)/(F'_m - F'_o) \quad (3)$$

Although, superficially very similar to Φ_{PSII}, the significance of this parameter is somewhat different. Whilst Φ_{PSII} is the proportion of absorbed energy being used in photochemistry, qP gives an indication of the proportion of PSII reaction centres that are open. An alternative expression of this is $1-qP$, the proportion of centres that are closed and is sometimes termed the 'excitation pressure' on PSII (DP Maxwell *et al.*, 1994). Φ_{PSII} and qP can be interrelated by a third parameter, F_v/F_m (Genty *et al.*, 1989). This is a measure of the intrinsic (or maximum) efficiency of PSII (i.e. the quantum efficiency if all PSII centres were open). F_v/F_m is given by the equation:

$$F_v/F_m = (F_m - F_o)/F_m = \Phi_{PSII}/qP \quad (4)$$

(where F_m and F_o can be either $F'_{m/o}$ or $F^o_{m/o}$). So, whilst Φ_{PSII} relates to achieved efficiency, qP and F_v/F_m provide information about the underlying processes which have altered efficiency. A change in qP is due to closure of reaction centres, resulting from a saturation of photosynthesis by light. A change in F_v/F_m is due to a change in the efficiency of non-photochemical quenching. Dark-adapted values of F_v/F_m reflect the potential quantum efficiency of PSII and are used as a sensitive indicator of plant photosynthetic performance, with optimal values of around 0.83 measured for most plant species (Björkman and Demmig, 1987; Johnson *et al.*, 1993). Values lower than this will be seen when the plant has been exposed to stress, indicating in particular the phenomenon of photoinhibition.

A difficulty in the estimation of qP and F_v/F_m is the need to estimate the value of F_o at the time of measurement. In the laboratory, this is achieved by darkening the leaf and, usually, by applying far-red illumination (wavelength >680 nm) for a few seconds before and immediately after the end of illumination. The latter is important to ensure that all PSII reaction centres open rapidly after the end of illumination. Commercial portable fluorometers usually incorporate a far-red light source, however, the technical problems of darkening the leaf in the field remain. These are usually overcome by transiently covering the leaf with a black cloth whilst simultaneously providing far-red light. A method for estimating F'_o, without making direct measurements has recently been proposed (Oxborough and Baker, 1997). This method was developed specifically for use in fluorescence imaging techniques where estimation of F'_o is particularly problematic. Whilst this method seems to work well under specific laboratory conditions, it makes assumptions about the nature of the processes contributing to fluorescence quenching. As such, it does not necessarily apply under other conditions, especially where the plant is exposed to stress and where significant amounts of photoinhibition may occur. For this reason, the application of this approach is probably best avoided under field conditions.

An alternative expression that is sometimes used and that is essentially identical to F_v/F_m is F_v/F_o (Krause and Weis, 1991). Although perhaps not such an intuitive parameter (it does not give a direct measure of efficiency), it has the advantage of being more sensitive to changes in efficiency at high values and so, in some circumstances, may be a better way of expressing data.

Non-photochemical processes

The problem of needing to dark-adapt leaves also applies when quantifying non-photochemical quenching. Here, however, the problems are exacerbated by the need to measure a 'dark-adapted' value of F_m. This value would typically be measured, in the laboratory, after between a full night and 24 h of dark-adaptation. There is also the implicit assumption in such experiments that the plants have not been exposed to any stress prior to the start of the experiment. The aim of such procedures is to attain a reference level of F_m in which photochemical efficiency is at its maximum and heat dissipation is at a minimum. In field experiments, it is possible to measure the pre-dawn value of F_m and use this as a reference point, however, this may be sensitive to the prehistory of the plant. Changes in the dawn F_v/F_m may, however, give important information concerning the effect on the plant of environmental stress (see below).

The most straightforward way of quantifying non-photochemical quenching is by measuring the ratio of a change in F_m to the final value of F_m:

$$NPQ = (F^o_m - F'_m)/F'_m \quad (5)$$

(Bilger and Björkman, 1990). NPQ (sometimes referred to as SV_N) is linearly related to heat dissipation and lies on a scale 0–infinity. In a typical plant, values might be expected in the range 0.5–3.5 at saturating light intensities, however, this varies markedly between species and on the previous history of the plant. An older term for quantifying non-photochemical quenching, qN that requires measurement of F_o, is still sometimes used (van Kooten and Snel, 1990). This parameter falls on a scale of 0–1 and is, therefore, very insensitive to changes in quenching at higher values. It is important to note that NPQ and qN measure changes in heat dissipation *relative to* the dark-adapted state. The same increase in heat dissipation will appear as a smaller increase in quenching in the case where the reference point has higher quenching. This means that direct comparisons between leaves with different histories or leaves of different species can be ambiguous. Generally, if the dark-adapted value of F_v/F_m is

markedly different, direct comparison of values of *NPQ* should be avoided.

In order to appreciate the physiological significance of non-photochemical quenching, it is necessary to understand the processes that contribute to the quenching. Any change in *NPQ* measures a change in the efficiency of heat dissipation, relative to the dark-adapted state. Broadly, such an increase can occur as a result either of processes that protect the leaf from light-induced damage or of the damage itself. An alternative way of looking at these processes is in terms of the rate at which they relax following a period of illumination. Different processes have different relaxation times ranging from a few minutes to several hours. The kinetics of relaxation of these different processes can, therefore, be used to distinguish them.

Under most conditions, the major contributor to *NPQ*, is termed high energy state quenching (often referred to as *qE*) and is thought to be essential in protecting the leaf from light-induced damage (for review see Horton *et al*., 1996). This process requires the presence of a low pH in the lumen of the thylakoid and involves the light-induced formation of the carotenoid zeaxanthin (Demmig-Adams, 1990; Demmig-Adams and Adams, 1992). High energy state quenching relaxes within minutes when the leaf is placed in darkness. A second process that relaxes over a time scale of a few minutes is termed a state transition (*qT*) (Walters and Horton, 1991). State transitions involve the reversible phosphorylation of light-harvesting proteins and are thought to be important in balancing the distribution of light energy between Photosystems I and II at low light. These two forms of quenching cannot easily be distinguished from their relaxation kinetics, however, *qT* generally only makes a small contribution to overall quenching and is only present at low light. For most purposes, all processes that relax over a time-scale of a few minutes following the cessation of illumination can be regarded as photoprotective processes.

Processes that relax over a longer time-scale (hours) are usually collectively referred to as 'photoinhibition' (*qI*). It is important to note that this term, when applied to fluorescence analysis, generally refers to both protective processes and to damage to the reaction centres of PSII (Osmond, 1994), whilst in more molecular studies, it is specifically the latter that is referred to as photoinhibition. Photoprotective processes with long relaxation times have been related to the presence of zeaxanthin and are thought to occur in the light-harvesting antenna of PSII (Horton *et al*., 1996). Damage to PSII reaction centres results in quenching occurring within the PSII reaction centre. This distinction between antenna and reaction centre processes is important because, whilst the former cause changes in the F_o level of quenching, the latter do not and, as such, they can to some extent be distinguished, at least under laboratory conditions.

To attain a full understanding of the contribution of fast and slow relaxing quenching, under physiological conditions, it is necessary to perform relaxation analysis (Walters and Horton, 1991). In such an experiment, quenching is allowed to relax and F_m recorded at regular intervals. As each estimation of F_m involves giving a leaf a saturating flash, it is important that the interval between these is carefully selected, such that any effect of the flash has relaxed before the next is applied. Typically, an interval of 5 min is sufficient, with relaxation being followed over 45 min to 1 h. A graph is then produced of $\log(F_m)$ against time. Extrapolation of data points recorded towards the end of the relaxation back to the time where the actinic light was removed, allows the calculation of the value of F_m (F_m^r) that would have been attained if only slowly relaxing quenching had been present in the light. Slowly and rapidly relaxing quenching can then be calculated using the following formulae:

$$NPQ_S = (F_m^o - F_m^r)/F_m^r \quad (6)$$

$$NPQ_F = (F_m^o/F'_m) - (F_m^o/F_m^r) \quad (7)$$

The extent and composition of *NPQ* has been successfully used to explore differences in photoprotection and photoinhibition at the phenotype and genotype level. Whilst sun plants generally exhibit a high capacity for *qE*, coupled to high light use when exposed to high light levels, shade plants of the same species exhibit reduced light use, *qE* and an increased likelihood of photoinhibition (Demmig-Adams *et al*., 1995). Additionally, it has been demonstrated that genotypic differences exist in the composition of *NPQ* which relate to the ecology of the plant. In a study of 22 British native species, it was demonstrated that sun ecotypes exhibited a high capacity for photoprotective *qE*, independent of growth light intensity as compared to shade species (Johnson *et al*., 1993). The performance of such a complete analysis of quenching is unlikely to be practical under field conditions. A simpler approach that has been used to deconvolute fluorescence signals involves the application of a single flash 2–5 min after the end of illumination and using this as an estimate of F_m^r. After 5 min, however, there is likely to be a significant contribution of rapidly-relaxing quenching remaining and so this is likely to give a significant underestimate of NPQ_F and a corresponding overestimate of NPQ_S. A single flash after 30 min, may give a sufficiently accurate estimate of F_m^r under field conditions.

Alternative approaches to chlorophyll fluorescence analysis

In addition to the 'quenching analysis' approach outlined above, recent work has explored the possibilities of

analysing the kinetics of the fluorescence rise resulting from the transfer of a leaf from dark to light. One significant advantage of this approach is that it allows information to be attained using simple (cheaper) non-modulated fluorometers. Work by, in particular, Strasser and co-workers (Stirbet *et al.*, 1998) has suggested the derivation of a number of different parameters, based on the details of the kinetics of fluorescence rise. This analysis remains, however, controversial and is probably best avoided until it has been placed on a firmer theoretical footing.

What can chlorophyll fluorescence do for you?

The discussion above has covered theoretical and technical considerations about chlorophyll fluorescence analysis. The rest of this review is intended to examine the question of how this theory can be applied to obtain useful information in field and laboratory ecophysiology studies. As has been stated already, chlorophyll fluorescence gives information about the state of Photosystem II. It can tell you the extent to which PSII is using the energy absorbed by chlorophyll and the extent to which it is being damaged by excess light. This may sound like fairly obscure information, of relevance only to those with a specialist interest in PSII, however, it is of much wider importance to the physiology of a plant. The flow of electrons through PSII is indicative, under many conditions, of the overall rate of photosynthesis. It gives us the potential to estimate photosynthetic performance, under conditions in which other methods would fail, in a manner that is almost instantaneous. PSII is also accepted to be the most vulnerable part of the photosynthetic apparatus to light-induced damage. Damage to PSII will often be the first manifestation of stress in a leaf. Although fluorescence is a powerful technique, it is also limited. It is easy to measure but, if experiments are not designed correctly, it can also be impossible to interpret. The most powerful and elegant applications of fluorescence do not use this technique alone, but combine it with other techniques, in particular, gas exchange measurements, to obtain a full picture of the response of plants to their environment.

PSII quantum yield as a proxy measure of photosynthesis

One of the main attractions of chlorophyll fluorescence is that it *appears* to give a measure of photosynthesis. This is, however, a great simplification and failure to appreciate this point can lead the user into difficulties. Fluorescence can be used to measure the *efficiency* of PSII photochemistry. As indicated above, this can be converted into a relative rate of linear electron transport by multiplying by the light intensity. Photosynthesis is usually thought of as being the gross rate of carbon fixation. Under laboratory conditions, PSII electron transport and CO_2 fixation can correlate very well (Genty *et al.*, 1989; Edwards and Baker, 1993). That correlation can (and does) break down under field conditions (Fryer *et al.*, 1998). Discrepancies can be caused by changes in the relative rates of CO_2 fixation and competing processes such as photorespiration, nitrogen metabolism and electron donation to oxygen (the Mehler reaction). Such discrepancies may be in themselves interesting (see below), but mean that accurate determination of CO_2 fixation is not possible with fluorescence alone. Further complication may arise due to heterogeneity between samples. Calculation of electron transport using $\Phi_{PSII} \times$ irradiance assumes that the light absorbed per PSII is constant. For leaves growing in different microclimates, this will typically not be true. This can be partially overcome if absorbance can be measured directly, using an integrating sphere, however, the problem of differences in photosystem stoichiometry remain. Thus, fluorescence can never be used to make comparative measurements of photosynthesis between different leaves or plants. For the above reasons, measurements of gas exchange, using infrared gas analysers, remain at the heart of studies in plant ecophysiology.

Provided the above reservations are borne in mind, Φ_{PSII} can provide useful information concerning photosynthetic performance in the field. In particular, fluorescence can give a good, rapid and minimally invasive measurement of changes in a particular sample (or in samples that can be considered to be homogenous) through time. For example, fluorescence has been used (Murchie *et al.*, 1999) to follow the electron transport rate in particular leaves of two rice cultivars throughout the day at different developmental stages. Individual leaves were marked and subsequent measurements made on the same leaves, so ensuring comparability between measurements. A similar approach was used in following diurnal changes in electron transport in loblolly pine trees exposed to elevated CO_2, using free-air CO_2 enrichment (FACE) (Hymus *et al.*, 1999). These authors observed that, whilst, in summer, elevation of CO_2 results in an increase in electron transport, in winter, electron transport is inhibited. A relatively greater increase in Φ_{PSII} was observed during sunflecks in a terrestrial CAM bromeliad, as compared to sympatric C_3 species within a Panamanian rainforest (Skillman and Winter, 1997). This reflects the greater energetic demand of decarboxylation of nocturnally accumulated organic acid in the CAM plant, relative to the costs of C_3 photosynthesis (including photorespiration), which supports light use under potentially damaging incident PFD. As in the previous example, a comparison is made *relative to measurements in the same leaf*, so that differences in absorbance are likely to be insignificant.

Another application where fluorescence may be useful is in examining the acclimation of plants to different microenvironments. By measuring the light dependency

of Φ_{PSII} it is possible to make simple and rapid estimates of the light saturation behaviour of different plants under field conditions. This does not allow a comparison of the absolute rates of photosynthesis between sites, but can be useful when studying samples, such as lichens and bryophytes, whose structure makes them difficult to study with conventional gas exchange.

Problems may arise when measurements of electron transport rate are made in the open (especially in the laboratory), when fluctuating levels of CO_2 (often generated by the experimenter's exhalations) preclude in-depth analysis of the relationship between the light and dark reactions of photosynthesis. Therefore, specialized leaf cuvettes or gas-tight leaf chambers which incorporate ports which house the fibre optic cable(s) should be used where practicable. The system should limit self-shading whilst maximizing fluorescence yield, with the fibre commonly positioned at an angle of 45–60° to the leaf. Important information may be obtained if it is possible to control the gas supply to a leaf. The Hansatech leaf disc electrode chamber has been used to vary ambient CO_2 and O_2 (Lovelock and Winter, 1996) and to assess the capacity for electron flux through the photorespiratory and Mehler-ascorbate peroxidase pathway (pseudocyclic electron transport) and the relationship with photoinhibition and leaf senescence in an evergreen (*Ficus*) and deciduous (*Pseudobombax*) tropical tree species. In a different approach which exemplifies the novel kind of systems which can be used, diffusion limitations across the lichen thallus was investigated in *Lobaria scrobiculata*. The thallus was sealed in a custom-built gas-tight Petri dish and flushed with nitrogen, with CO_2 diffusion through different areas and thickness of the thallus assessed by tracking the recovery of Φ_{PSII} under low light when air was reintroduced to the system (Máguas *et al.*, 1997).

Relating electron transport to carbon fixation

Despite the fact that fluorescence emanates from only the top few layers of chlorenchyma, whereas gas exchange is integrated across the thickness of the leaf, simultaneous measurements have emerged as a powerful tool for investigating the relationship between light use efficiency, CO_2 fixation and photoinhibition. A relatively simple and widely used technique involves exploring the empirical relationship between electron transport and CO_2 fixation *in vivo*. Simultaneous measurements are made of CO_2 assimilation and Φ_{PSII} under non-photorespiratory conditions (elevated CO_2 or 1–2% O_2) at different light intensities. A linear plot of the quantum yield of CO_2 fixation (Φ_{CO_2}) and PSII photochemistry (Φ_{PSII}) allows the electron requirement per molecule CO_2 fixed to be quantified (Epron *et al.*, 1995). Assuming that this relationship holds under non-photorespiratory conditions, the extent of photorespiration may be estimated *in vivo*. In particular, this protocol has been used to explore the significance of photorespiration as a photoprotective maintenance mechanism during drought stress (Cornic and Briantais, 1991; Valentini *et al.*, 1995; Niinemets *et al.*, 1999).

Meyer and Genty used high resolution images of Φ_{PSII} to define leaf internal CO_2 concentration (*Ci*) following ABA treatment in leaves of *Rosa rubiginosa* (Meyer and Genty, 1998). Measurements of *Ci* made conventionally using gas exchange techniques during drought may be over-estimated as a consequence of both patchy stomatal response and an under-estimation of cuticular transpiration. This use of chlorophyll fluorescence has suggested that the primary effect of drought stress is one of stomatal closure with a resulting decreasing in internal CO_2 concentration which may limit carboxylation (Meyer and Genty, 1998, 1999; Sánchez-Rodríguez *et al.*, 1999). The technique does not require fluorescence imaging equipment and has been performed with combined measurements of gas exchange and fluoromtery. Sánchez-Rodríguez and co-workers used the empirical relationship between the ratio of Φ_{PSII}/CO_2 assimilation rate and *Ci* in control leaves subsequently to model *Ci* in droughted leaves (Sánchez-Rodríguez *et al.*, 1999). However, the methodology requires that this relationship does not change following the imposition of stress, that alternative electron flow (i.e. to the Mehler-peroxidase reaction) is negligible and that respiratory rates do not differ significantly between treatments. It is also necessary that *Ci* can be accurately determined by gas exchange in control leaves, which may preclude species with an inherently low stomatal or cuticular conductance.

Fluorescence analysis can also be applied to understanding the effects of low and high temperatures. For example, comparisons of the yield of CO_2 fixation (Φ_{CO_2}) and Φ_{PSII} were made to provide evidence that, when exposed to low temperatures, maize increases electron transport to alternative electron sinks, probably generating active oxygen species (Fryer *et al.*, 1998). Early in the growing season, the ratio of Φ_{PSII}/Φ_{CO_2} was higher than that seen in unstressed, fully developed leaves, suggesting that electrons were being used in pathways other than CO_2 fixation. This increase was accompanied by an increase in the capacity of antioxidation systems, implying that the leaves were suffering from oxidative stress at this time.

Measuring stress and stress tolerance

Although fluorescence measurements may sometimes provide a useful measure of the photosynthetic performance of plants, its real strength lies in its ability to give information that is not readily available in other ways. In particular, fluorescence can give insights into the ability

of a plant to tolerate environmental stresses and into the extent to which those stresses have damaged the photosynthetic apparatus. The advent and refinement of portable fluorometers has led to an upsurge in the use of chlorophyll fluorescence measurements under field conditions. Measurements made over a diurnal course can yield information pertaining to *NPQ*, electron transport rates, quantum efficiency, and the extent of photoinhibition in response to light, temperature and other single or combined environmental stresses (Bilger *et al*., 1995). Early measurements used a sustained decrease in dark-adapted F_v/F_m and increase in F_o to indicate the occurrence of photoinhibitory damage in response to high temperature (Gamon and Pearcy, 1989), low temperature (Groom and Baker, 1992), excess PFD (Ögren and Sjöström, 1990), and water stress (Epron *et al*., 1992). Despite improvements in technology and the evolution of modulated systems, these observations remain valid and changes in F_v/F_m and F_o are still accepted and widely used as reliable diagnostic indicators of photoinhibition (He *et al*., 1996; Valladares and Pearcy, 1997).

However, over recent years, fluorescence techniques have become increasingly refined and dark-adapted measurements made in combination with those in the light now allow extremely detailed analyses of photosynthetic performance *in situ*. A very elegant example of the use of chlorophyll fluorescence in the field, comes from studies made on the cactus species *Opuntia macrorhiza* during September in the Rocky Mountains of North America (Barker and Adams, 1997). A thorough investigation was made of photochemical efficiency, photosynthesis and xanthophyll cycle activity in differently oriented cladodes, which received contrasting PFD and temperature over the course of the day. The study confirmed that photosynthetic organs which are subject to a high integrated PFD (in this example south- and west-facing cladodes) usually have a large xanthophyll cycle pool, with de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin strongly related to high *NPQ* (Demmig-Adams, 1990; Demmig-Adams and Adams, 1992). In all cases, high *NPQ* occurred early in the photoperiod during periods of low temperatures, whilst all cladodes exhibited a midday reduction in *NPQ* coincident with decarboxylation of malic acid. The south- and west-facing cladodes were subject to a second reduction in photosynthetic efficiency during the afternoon, when incident PFD was maximal on these organs. These cladodes exhibited a sustained photoinhibitory depression of F_v/F_m during the night which was associated with retention of zeaxanthin and antheraxanthin, in a photoprotective strategy which effectively primes the photosynthetic apparatus for non-photochemical quenching at dawn. Additionally, the study examined the interplay between light use and dissipation over the diurnal course, as quantified using chlorophyll fluorescence.

Recently, it has been demonstrated that the expression of xanthophyll-cycle dependent *NPQ* may be fine-tuned to changes in the environment (Adams *et al*., 1999). This observation was made for two vine species growing in an understorey of a *Eucalyptus* forest in Australia. High xanthophyll pigment de-epoxidation levels were observed following an initial sunfleck, which were maintained over the majority of the day, whilst *NPQ* fluctuated dependent on incident PFD. In contrast, exposed plants of the same species exhibited more closely related light-dependent changes in the de-epoxidation state and the rate of thermal dissipation. It is suggested that *NPQ* is modulated by transthylakoid ΔpH, mediated by subtle changes in electron transport rate which can effectively promote immediate quenching during sunflecks, but which would not significantly limit carbon gain in the periods between flecks (Adams *et al*., 1999).

A similar approach has been adopted to quantify the fates of light energy in exposed and shaded leaves of the tropical hemi-epiphyte *Clusia minor* (Roberts *et al*., 1998). In this example, a combination of both high photosynthetic light use and thermal dissipation minimized the accumulation of excess excitation energy and the probability of photodamage. In contrast, responses of slow-growing epiphytic bromeliads with a low photosynthetic capacity were dominated by extremely high levels of *NPQ* and minimal light use (Griffiths and Maxwell, 1999).

Although, as stated above, full deconvolution of *NPQ* is not normally practical in the field, simple measurements of relaxed F_m can provide insights into plants' stress responses. For example, epiphytic bromeliads are extremely slow growing and carboxylation is limited throughout the day, and all the species studied have a capacity for high levels of *NPQ* in response to high light (C Maxwell *et al*., 1994; Skillman and Winter, 1997). A detailed experiment, undertaken using a single pulse to determine F_m' in the field, revealed that shade-tolerant plants exhibited a higher proportion of sustained photoinhibitory *NPQ*, as compared to plants experiencing higher light which exhibited the same instantaneous level of *NPQ* (Griffiths and Maxwell, 1999).

Conclusion

In this review, an attempt to give a taste of the ways in which chlorophyll fluorescence analysis can be applied in ecophysiological studies and the complications that can arise has been made. Because it is so easy to generate data with chlorophyll fluorescence, it is also easy to generate large amounts of meaningless data and care must always be taken in the design and execution of experiments to ensure that data are informative. That said, fluorescence remains a powerful technique, the use of which looks set to continue rising in years to come.

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