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Technical communication

Measurement of chlorophyll fluorescence within leaves using a modified PAM Fluorometer with a fiber-optic microprobe*

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Abstract

By using a fiber-optic microprobe in combination with a modified PAM Fluorometer, chlorophyll fluorescence yield was measured within leaves with spatial resolution of approximately 20 μm. The new system employs a miniature photomultiplier for detection of the pulse-modulated fluorescence signal received by the 20 μm fiber tip. The obtained signal/noise ratio qualifies for recordings of fluorescence induction kinetics (Kautsky effect), fluorescence quenching by the saturation pulse method and determination of quantum yield of energy conversion at Photosystem II at different sites within a leaf. Examples of the system performance and of practical applications are given. It is demonstrated that the fluorescence rise kinetics are distinctly faster when chloroplasts within the spongy mesophyll are illuminated as compared to palisade chloroplasts. Photoinhibition is shown to affect primarily the quantum yield of the palisade chloroplasts when excessive illumination is applied from the adaxial leaf side. The new system is envisaged to be used in combination with light measurements within leaves for an assessment of the specific contributions of different leaf regions to overall photosynthetic activity and for an integrative modelling of leaf photosynthesis.

Introduction

Within a leaf there is anatomical and physiological differentiation of tissues and chloroplasts for optimization of photosynthesis. Corresponding to the light gradient within a leaf there is a gradient in photosynthetic properties of chloroplasts (Vogelmann et al. 1989). In a typical dorsiventral leaf, the palisade chloroplasts near the upper leaf side show '*sun*' characteristics whereas the chloroplasts in the spongy mesophyll at the lower leaf side display '*shade*' characteristics (Terashima and Inoue 1985a,b). In principle, the different light adaptation states can be readily demonstrated by chlorophyll fluorescence measurements from the adaxial and abaxial leaf surfaces (Schreiber et al. 1977).

In recent years there has been substantial progress in instrumentation and methodology of chlorophyll fluorescence measurements (for reviews see Krause and Weis 1991; Schreiber and Bilger 1993; Schreiber et al. 1994). The pulse-amplitude-modulation (PAM) technique has proven successful for selective and sensitive assessment of chlorophyll fluorescence yield at largely varying ambient light intensities (Schreiber 1986, 1994). In particular, this technique allows the application of strong light pulses for transient saturation of photosynthetic electron flow, which is the basis of fluorescence quenching analysis (Schreiber et al. 1986; Weis and Berry 1987; Genty et al. 1989; Walters and Horton 1991).

* This paper is dedicated to Ulrich Heber on the occasion of his 65th birthday, with great respect for his outstanding achievements in photosynthesis research.

Fiber-optic microprobes have been useful for measurements of light gradients and spectral properties in plant tissues (Vogelmann and Björn 1984; Vogelmann et al. 1989; Cue et al. 1991; Martinez v. Remisowsky et al. 1992) and microbial mats (Jørgensen and Des Marais 1988; Kühl et al. 1994). Recently, fluorescence based fiber-optic microsensors were developed (Klimant et al. 1995). Bornman et al. (1991) used a fiber-optic microprobe to measure chlorophyll fluorescence within leaves of *Medicago sativa*, which was excited by strong blue light from outside. In this case, fluorescence intensity was determined by a number of factors, including the gradient of blue actinic light, the reabsorption of 688 nm fluorescence and chlorophyll concentration. Using this approach, however, it is not possible to assess photosynthetic properties of the different chloroplast layers on the basis of chlorophyll fluorescence.

Here, we report on a new measuring system, based on a modified PAM fluorometer, which is capable of detecting chlorophyll fluorescence yield with high accuracy and spatial resolution within leaves using a fiber-optic microprobe. It will be shown that the sensitivity and selectivity is sufficiently high for recordings of dark-light induction transients (Kautsky effect) and to carry out quenching analysis (Schreiber et al. 1986), with determination of the maximum quantum yield, ΔF/Fm (Kitajima and Butler 1975; Björkman and Demmig 1987), and of the effective quantum yield of energy conversion at PS II during actinic illumination, ΔF/Fm′ (Genty et al. 1989).

Materials and methods

Plant material

Nicotiana rustica was grown in the green house at approximately 200 μE m^{-2} s^{-1} photosynthetic active radiation. The experiments were carried out with 350 μm thick leaves of an 8-week-old plant. Microscopic observation of a leaf cross-section showed the following anatomical features: adaxial epidemis, 25 μm; palisade parenchyma, 175 μm; spongy mesophyll, 135 μm; abaxial epidermis, 15 μm.

Leaves of *Syringa vulgaris* were obtained from plants grown in the Würzburg Botanical Garden under favorable environmental conditions, without experiencing excessive illumination. Leaf-thickness was approximately 200 μm, with the following tissue contributions: adaxial epidermis, 20 μm; double layer of palisade cells, 100 μm; spongy mesophyll, 65 μm; abaxial epidermis, 15 μm.

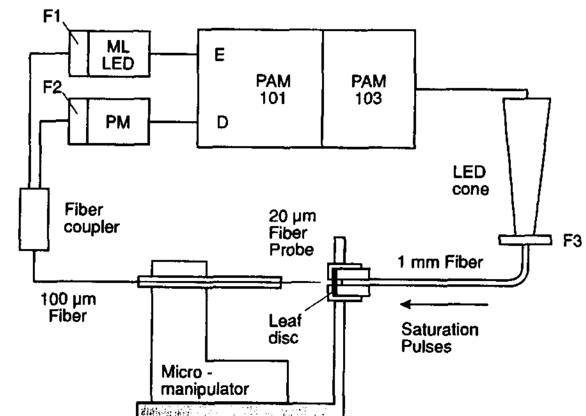

Fig. 1. Block diagram of the experimental set-up of measurement of chlorophyll fluorescence yield within leaves using a fiberoptic microprobe. Modulated fluorescence is measured with a PAM fluorometer, consisting of the Main Control Unit PAM-101 and the accessory module PAM-103, using a special emitter-detector unit with a highly sensitive photomultiplier and special fiberoptics. See text for further details.

Measuring system

The measuring system is depicted schematically in Fig. 1. Chlorophyll fluorescence was measured with a standard PAM Fluorometer (PAM-101 and Flash Trigger Control Unit PAM-103) (H. Walz GmbH, Effeltrich, Germany) which was adapted to the purpose of this work by special emitter and detector units, fiberoptics and a high intensity light source for saturation pulses. The fiber microprobe was made from a multimode silica/silica step index fiber with 100 μm core and 140 μm cladding diameter (Radiall, Germany). A fiber taper was formed while heating a bare fiber end in a small flame of a gas burner and fiber tips of 10–30 μm in diameter were prepared by cutting the taper with a small knife. The tapered fiber was mounted in an injection needle, as described earlier (Kühl and Jørgensen 1992), to improve the mechanical stability. The samples were enclosed in form of 10 mm leaf discs in a holder connected to a micro-manipulator, on which the fiber microprobe was mounted. The leaf disc holder has a central 1 mm bore hole for entering the fiber probe from the front and for insertion of a single 1 mm plastic fiber carrying the saturation pulse light from the rear. The micro-manipulator was operated manually with a resolution of 10 μm for microprobe advancement. The position of the fiber tip with respect to the leaf surface

was visually controlled with the help of a binocular microscope.

Pulse modulated measuring light was generated by a light-emitting-diode (LED) with peak emission at 655 nm (Type H-3000, Stanley, Tokyo, Japan). The measuring light passed a short-pass filter (Calflex-X special, Balzers, Liechtenstein) which eliminates wavelengths above 700 nm (F1) and was coupled by a collimator lens system (Spindler & Hoyer, Göttingen, Germany) into a multimode fiber coupler (Gould Inc.) with step index fibers (HCS 110/125 Ensign Bickford Corp., USA). The fiber coupler is a fiber-optic analogue to a beam splitter and was used for separation of the fluorescence signal from the excitation light (Klimant et al. 1995). Standard ST-Fiber connectors were used. The fluorescence from the fiber tip passed a long-pass filter (F2) consisting of 1 mm RG9 (Schott, Mainz, Germany) in order to eliminate excitation light, before reaching the photodetector. A miniature photomultiplier with high far-red sensitivity was used (type H5700-50, Hamamatsu, Japan). The obtained signal was further amplified by a laboratory-built pulse amplifier before being processed within the PAM-101 unit by the selective window amplifier of the PAM Fluorometer. Saturating pulses of red light were triggered under the control of the PAM-103 unit. They were generated by a laboratory-built LED-array cone, which was based on 64 LEDs with peak emission at 660 nm (type HLRA 180 AP, Toshiba, Tokyo, Japan). The light from the LED cone passed a short-pass filter (Calflex-X special, Balzers, Liechtenstein), which eliminated wavelengths beyond 700 nm, before entering the 1 mm fiber connecting to the sample holder. Light-intensity at the fiber exit was 3000 $\mu E\ m^{-2}\ s^{-1}$.

The 655 nm pulse-modulated measuring light was applied either at 1.6 kHz or 100 kHz frequency. In the latter case it also served as actinic light for induction of the Kautsky effect and for driving photosynthesis. The frequency was automatically switched to 100 kHz during application of saturation pulses, in order to increase the signal/noise ratio. At the same time, by this approach the measuring light at 100 kHz contributed to light saturation, providing at the exit of the 20 μm fiber tip 370 $\mu E\ m^{-2}\ s^{-1}$ or 1240 $\mu E\ m^{-2}\ s^{-1}$ at measuring light intensity settings 6 or 10 at the PAM-101, respectively.

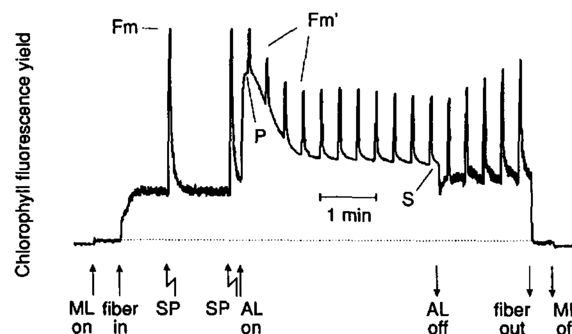

Fig. 2. Recording of a dark-light fluorescence induction curve with saturation pulse quenching analysis using a 20 μm fiberoptic microprobe positioned at 100 μm depth within a tobacco leaf. The measuring light (ML) was turned on before the fiber approached the adaxial leaf surface. Its intensity (setting 6 at PAM-101) was 6 $\mu E\ m^{-2}\ s^{-1}$ at the fiber tip and sufficient to induce some increase of fluorescence yield. Simultaneous with the application of 2 s saturation pulses (SP), modulation frequency was increased, raising integrated measuring light intensity to 370 $\mu E\ m^{-2}\ s^{-1}$, which was also used as actinic light (AL). The characteristic fluorescence levels Fm, Fm′, P and S are denoted. Damping setting 10 at PAM-101.

Results and discussion

Quenching analysis and dark-light fluorescence induction curves

Figure 2 shows a typical recording of chlorophyll fluorescence yield within a tobacco leaf using a 20 μm fiberoptic microprobe. When the measuring light (ML) is turned on while the fiber tip is not yet within the leaf, there is only a small signal. Where indicated, the fiber is rapidly advanced 100 μm deep into the leaf and a biphasic fluorescence rise is observed. As will be shown below the second phase is due partially to some actinic effect caused by the measuring light. Saturating light pulses of 2 s duration are applied for determination of the maximal fluorescence yield, Fm, of the sample in the 'dark-state'. It is apparent that this determination is very accurate and reproducible. When actinic illumination is turned on, there is induction of the so-called Kautsky effect (Kautsky and Hirsch 1931), with the fluorescence yield (F) first rising to a peak level, P, and then slowly declining to a steady-state level, S. Saturating light pulses are applied every 20 s in order to determine Fm′, which is lowered with respect to Fm by non-photochemical quenching. This is the basis of fluorescence 'quenching analysis' which results in determination of the coefficients of photochemical quenching, qP, of non-photochemical quenching, qN, (Schreiber et al. 1986) and of the effec-

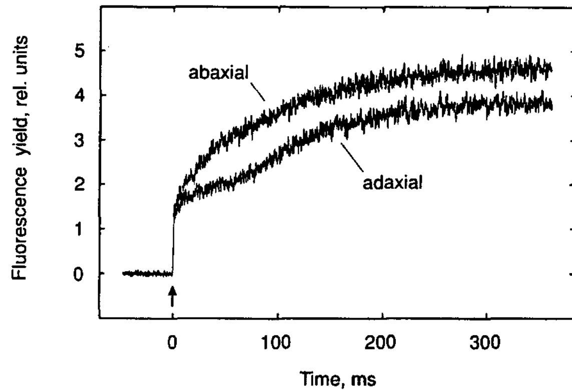

Fig. 3. Comparison of rapid dark-light induction kinetics measured at 60 μm depth from the adaxial and abaxial surfaces of a *Syringa vulgaris* leaf. The recordings where triggered by turning on the measuring light (setting 6, 100 kHz, corresponding to 370 μE m^{-2} s^{-1}) at time zero. Damping setting 4 at PAM-101. Four curves each were averaged with the help of a Nicolet-420 signal averager.

tive quantum yield of energy conversion at PS II, Φ_{II} (Genty et al. 1989). Actinic illumination is given by increasing the measuring light modulation frequency from 1.6 kHz to 100 kHz, by which way also the signal/noise ratio is substantially increased. When actinic illumination is turned off again, there is a rapid increase of photochemical quenching and a slower relaxation of non-photochemical quenching. Eventually, when the fiber tip is rapidly pulled out of the leaf again, the signal returns to the original low level.

The fluorescence recording presented in Fig. 2 is very similar to numerous previously published recordings obtained with the standard PAM Fluorometer. The difference is that normally a fiber-optic probe with a 14 mm active diameter is used and fluorescence is measured at some distance from the leaf surface. Here, we applied a 20 μm fiber, which is equivalent to a 49×10^4 smaller active area, and the fluorescence was measured within the leaf. Obviously the obtained signal/noise is sufficient for satisfactory fluorescence quenching analysis. It could be further increased by raising the measuring light intensity, as in the given example a relatively low setting (ML 6 at the PAM-101) was used. On the other hand, this would further increase the actinic effect of the measuring light.

In order to record the rapid induction kinetics at a time resolution of 10 ms (damping setting 4 at the PAM-101) signal averaging is advantageous. Examples of such measurements are shown in Fig. 3, where the fluorescence rise kinetics at two different sites within the leaf are compared. In A. the fiber tip was advanced 60 μm deep into the palisade parenchyma of a leaf of *Syringa vulgaris* from the adaxial surface, whereas in B. the tip was positioned in the spongy mesophyll 60 μm from the abaxial surface. In both cases 4 curves were averaged with 1 min dark periods between the 2 s recordings. The curves are normalized at the initial fluorescence yield (Fo-level). It is apparent that the fluorescence rise is distinctly faster in the spongy mesophyll than in the palisade parenchyma. This can be explained by the known differences in 'sun' (palisades) and 'shade' (spongy mesophyll) characteristics of the chloroplasts differentiated at these different sites within the leaf (Terashima and Inoue 1985a,b). Similar observations can be also made by large area measurements from upper and lower leaf surfaces (Schreiber et al. 1977). However, there is a distinct difference in that the assessed type of chloroplasts and the effective actinic intensity are better defined in the present approach using the fiber microprobe.

Measurement of maximum quantum yield and photoinhibition

There has been increasing evidence that the quantum yield of photochemical energy conversion at PS II can be satisfactorily estimated from the empirical fluorescence parameter (Fm-F)/Fm = ΔF/Fm (Kitajima and Butler 1975; Genty et al. 1989). The maximum fluorescence yield, Fm (or Fm′ with a preilluminated sample) is determined with the aid of a saturating light pulse. As was already shown in Fig. 2, in principle such quantum yield determinations within a leaf can be carried out with the new microprobe measuring system. In order to asses the *maximum* quantum yield at a particular site within the leaf, it is necessary to resolve the minimum fluorescence yield, Fo, in the dark-adapted state. It should be noted, that such measurements are complicated by the existence of so-called 'inactive PS II' (see e.g. Chylla and Whitmarsch 1989) which are closed at rather low light intensities.

In Fig. 4 a typical measurement of maximum quantum yield at 80 μm depth from the adaxial surface of a leaf of *Syringa vulgaris* is demonstrated. It is apparent that at the chosen time resolution (damping 7 at the PAM-101 unit), the instantaneous rise to the Fo-level, upon onset of the measuring light, can be well separated from the slower increase in fluorescence yield induced by the measuring light, which presumably involves the closure of 'inactive' centers.

When such measurements were made with the fiber tip being located at different depths within the leaf (measured from the adaxial surface), the observed

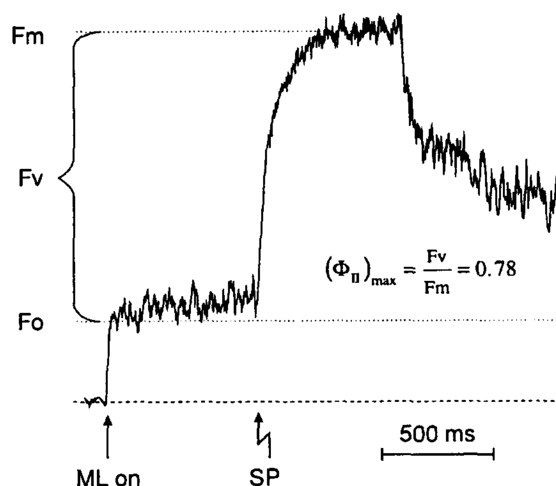

Fig. 4. Determination of maximal quantum yield Fv/Fm by the saturation pulse method at a depth of 80 μm within a leaf of *Syringa vulgaris*. Single recording at measuring light intensity setting 10 and damping setting 7 at PAM-101. The measuring light intensity at the fiber tip amounted to 20 μE m^{-2} s^{-1} at 1.6 kHz modulation frequency and 1240 μE m^{-2} s^{-1} at 100 kHz (during 0.6 s saturation pulse).

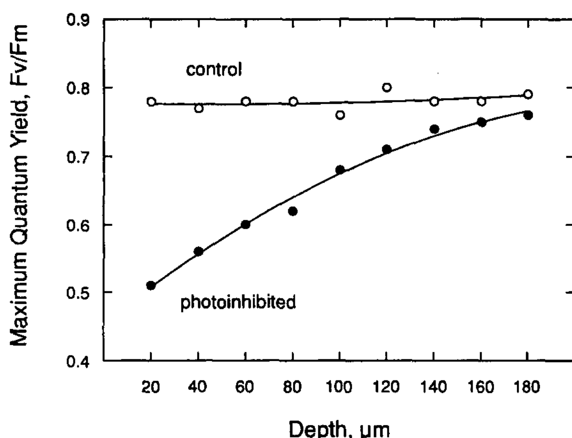

Fig. 5. Maximum quantum yield Fv/Fm measured within a leaf of *Syringa vulgaris* in dependence of the distance of the fiber tip from the adaxial leaf surface. A segment of the leaf was pretreated by 5 min exposure to 5000 μE m^{-2} s^{-1} white light from the adaxial surface. During the photoinhibitory treatment the leaf segment was submersed in cold water. Between the treatment and start of the measurements, 45 min dark-time was given for recovery of non-photochemical quenching which is not due to photoinhibition. Data were collected by advancing the fiberoptic microprobe either from the adaxial surface (20–100 μm) or from the abaxial surface (120–180 μm). Other measuring conditions as described for Fig. 4.

Fv/Fm was almost constant, showing high values of 0.77–0.80 (see control curve in Fig. 5). A different result was obtained when a leaf had been pretreated by excessive illumination from the adaxial side, which caused photoinhibition, associated with a loss in maximum quantum yield (Powles and Björkman 1982). As shown in Fig. 5 (photoinhibited curve), a substantial drop in Fv/Fm to a value of 0.51 was observed at 20 μm distance from the adaxial surface, whereas only minor effects were found when the fiber tip was at 60–20 μm distance from the abaxial surface (i.e. at 140–180 μM depth, measured from the adaxial surface). The data show a gradual decrease of photoinhibition within the leaf with increasing distance to the leaf surface at which the photoinhibitory illumination was applied. While these results are not unexpected in view of the fact that the intensity of the externally applied light drops rapidly within the leaf (Vogelmann and Björn 1984; Bornman et al. 1991), they demonstrate that the overall photoinhibition is likely to be overestimated, when, as has been common practice, it is assessed by fluorescence measurements from the leaf surface.

Conclusions

It may be concluded that detailed information on the photosynthetic performance of different tissue layers within a leaf can be obtained by a PAM Fluorometer combined with a fiber-optic microprobe and an ultrasensitive emitter-detector unit. With a 20 μm fiber tip the spatial resolution is similar to cell size. As the signal/noise ratio was very satisfactory, in principle still smaller tip diameters are possible. So far, however, in experiments with a 12 μm tip we encountered relatively frequently the phenomenon of an irreversible fluorescence increase, associated with the loss of quantum yield (data not shown). This presumably was due to local cell damage, loss of compartmentation and adhesion of free chloroplasts to the fiber tip. This was not a serious problem in the case of 20 μm tip used in the present study (see e.g. Fig. 2).

The new measuring system opens the way to a profound assessment of the specific contributions of various leaf regions to the overall photosynthetic activity. This may be considered an important step forward towards an integrative modelling of leaf photosynthesis, the theoretical basis for which has been advanced by Fukshansky and co-workers (Fukshansky and Martinez v. Remisowsky 1992; Fukshansky et al. 1992; Martinez v. Remisowsky et al. 1992). Fiberoptic microprobe measurements can provide information on the vertical gradient of photosynthetic activity within a leaf, while fluorescence image analysis provides information on the lateral distribution, as viewed from

the surface (Myers et al. 1994; Siebke and Weis 1995; Genty and Meyer 1995). Hence, these two approaches are complementary in creating an integrative three-dimensional image of the photosynthetic activity of a leaf.

Within the scope of this first communication we have addressed only part of the potential applications of the new measuring system. Work is in progress to combine the fluorescence measurements with light gradient measurements, using different wavelengths of actinic illumination. The combined information for the first time should allow calculation of photosynthetic activity at different sites within the leaf. As pointed out by Vogelmann (1993), it will be important to examine 'the relationship among leaf anatomy, light gradients, and photosynthetic performance at the whole leaf level as well as within the leaf'. Another important potential application relates to the study of photosynthetic microbial mats and sediments, which exhibit an intriguing vertical stratification of different photosynthetic microalgae and bacteria (Jørgensen et al. 1983; Jørgensen and Des Marais 1988; Kühl et al. 1994).

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