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In vivo measurement of spectroscopic and photochemical properties of intact leaves using the 'mirage effect'

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This paper describes a new, highly sensitive, method for in vivo studies of photosynthesis based on the 'mirage effect' in which thermal energy dissipation from intact leaves, illuminated with intensity-modulated light, is sensed through the periodic deflection of a laser beam propagating along the leaf surface. The photothermal deflection technique allows one to rapidly estimate the gross efficiency of photochemical energy storage by comparing the heat emission signal with and without an additional strong, photosynthetically saturating, non-modulated light. In pea leaves, the maximal storage efficiency at low light intensities was shown to approach 55%. The general utility of this simple photothermal method is illustrated by examining the variation of the deflection signal under different conditions. The spectral resolution of this new method is shown to be much higher than that of the photoacoustic method.

Photothermal deflection technique; Mirage effect; Photoacoustic technique; Photosynthesis; Intact leaf

1. INTRODUCTION

Mirages are common optical illusions in deserts and other areas of high temperature throughout the world. They result from the deflection of light beams due to unsteady refractive index distributions caused by dissipation into the air of the energy absorbed by the earth from intense sunlight. An application of the mirage effect has been proposed by Boccara et al. [1–3], consisting in the detection of the heat waves generated by the absorption of modulated light in a solid via the measurement of the refractive index gradient in the fluid in contact with the absorbing material. The index-of-refraction gradient is measured by the periodic deflection of a laser beam parallel to the sample surface. This photothermal deflection (PD) technique has proved to be very useful in characterizing various physical and chemical properties (absorption spectra, thermal diffusivity, etc.) of solid, liquid and gaseous materials [1,2,4–7].

Here, for the first time, this new photothermal method has been applied to the in vivo measurement of thermal deactivation of excited pigments in intact plant leaves. We showed that the PD method can be used to readily measure the in vivo absorption properties of leaves and to rapidly estimate the photosynthetic capacity of plants. PD measurements are closely related to photoacoustic (PA) measurements in which heat emission is detected as a pressure change using a microphone [8]. The preliminary results presented in this com-

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Abbreviations: DCMU, dichlorophenyldimethylurea; PA signal, photoacoustic signal; PD, photothermal deflection; PL, photochemical losses; S/N ratio, signal-to-noise ratio

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munication indicated that the PD technique has several advantages over the PA methods, amongst which a higher sensitivity and a much better spectral resolution.

2. MATERIALS AND METHODS

The experimental set-up is schematically depicted in fig.1. A detailed description of the system will be presented in a forthcoming paper. The light provided by a 1000 W xenon arc lamp (Schoeffel Instrument) was passed through a monochromator (Schoeffel), chopped using a mechanical chopper (Scitec Instruments) and focused upon the leaf sample. The samples were small rectangular pieces of leaves (around 0.5 cm^2) stuck and stretched on a small glass slide which was placed in the PD cell filled with filtered distilled water (50 cm^3). The index-of-refraction gradient generated in the fluid in contact with the sample deflected a low-power He-Ne laser probe beam (Uniphase, 4 mW) focused near the periodically irradiated sample. The diameter of the beam above the sample was around 40 μm. The glass slide and the leaf sample were fixed on a translational displacement system in order to precisely adjust the respective positions of the laser beam and the surface of the leaf (at around 20 μm from each other). The periodic deflection of the laser beam was monitored by the use of a position sensor (Optikon SD 380-23-21-051) coupled to a lock-in amplifier (Ithaco, model 393). The PD signals were analyzed by an Apple IIc computer or directly displayed on a chart recorder. The background non-modulated light, supplied by a d.c. operating halogen lamp, was transmitted onto the sample using a fiberoptic light guide. Light intensities were measured using a calibrated lightmeter (United Detector Technology, model 1223).

For the purpose of comparison, PA spectra were also measured in leaves with a laboratory-built photoacoustic spectrometer which has been detailed elsewhere [9,10].

The experiments reported here were performed on pea leaves (*Pisum sativum* L.), grown in a growth chamber at constant temperature, relative air humidity and light conditions.

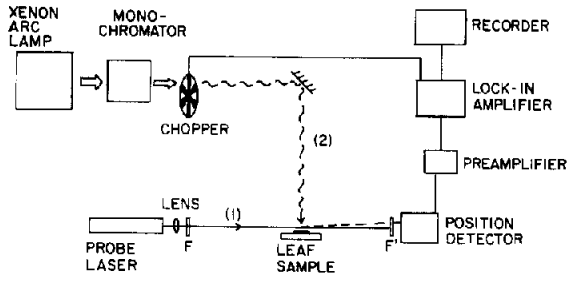

Fig.1. Scheme of the photothermal deflection (PD) system used to measure heat emission from leaves. (1) Probe laser beam; (2) intensity-modulated light. F, 2% transmittance red filter; F', neutral density filter (10% transmittance).

3. RESULTS AND DISCUSSION

Fig.2 shows the PD signal generated by a pea leaf illuminated with a 680 nm light modulated at 12 Hz. Upon illumination, the deflection signal was seen to immediately rise to a constant and stable level with a satisfactory signal-to-noise (S/N) ratio (>80). The PD traces were consistently reproducible provided the measurements were done in the useful S/N range. Indeed, as the amplitude of the PD signal drastically decreased with increasing modulation frequency (not shown), useful measurements (i.e. acceptable S/N ratios) were restricted to a rather small frequency range between 10 and around 50 Hz. In fig.2 is also shown the effect of a non-modulated high-intensity background light. This continuous light is used to self-reference the sample in a way similar to that commonly used for the in vivo high-frequency photoacoustic signals of leaves [11]. In the presence of the strong background light, photochemistry is saturated and almost all the absorbed light energy is dissipated as heat (radiative energy dissipation can be neglected), resulting in a rise in the amplitude of the photothermal signal to its maximal level. The comparison of the PD signal in the presence and in the absence of the background light provides then an estimation of the portion of absorbed light energy which is stored in the intermediates of the photosynthetic process (photochemical losses, PL). Being non-modulated, the background light does not induce any measured deflection signals. The inset of fig.2 shows the progressive saturation of the photochemistry with increasing intensity of the background light. A typical light-saturation curve was obtained, showing a clear linear relationship in the low light intensities range and a plateau at higher intensities. From this saturation curve, it appears that (young) pea leaves are already photochemically saturated at light fluence rates close to 80–100 $W \cdot m^{-2}$. This value is probably limited to the plant species studied and the growth conditions used in this study. PD signals qualitatively similar to the signal shown in fig.2 were obtained with leaves of a variety of plant species. The major limitation was, however, the fact that the sample surface necessarily had to be very flat in order to insure a good alignment of the laser beam and the sample. So far, we were not

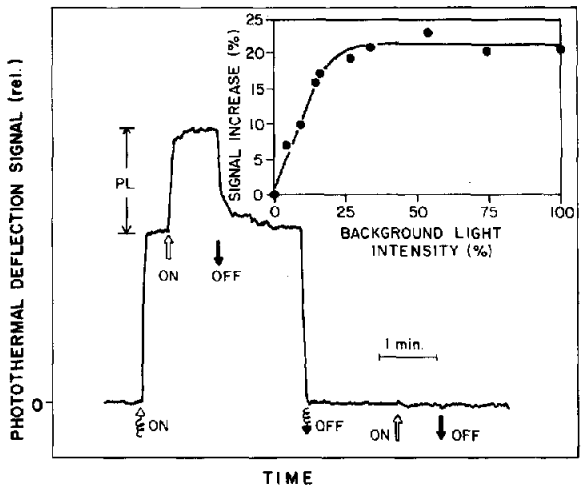

Fig.2. Typical PD signal obtained from a pea leaf illuminated with a 680 nm light modulated at 12 Hz. (⇕ , ⇕) Modulated light (14 $W \cdot m^{-2}$), on and off; (⇑ , ⇓) strong non-modulated white light (270 $W \cdot m^{-2}$), on and off. Lock-in amplifier time constant was 1.25 s. The photochemical losses (PL) are the percentage difference between the photothermal signal in the presence and in the absence of the saturating background light. (Inset) Increase in the PD signal induced by different intensities of the background light. Maximal intensity (100%) of the background white light was 270 $W \cdot m^{-2}$.

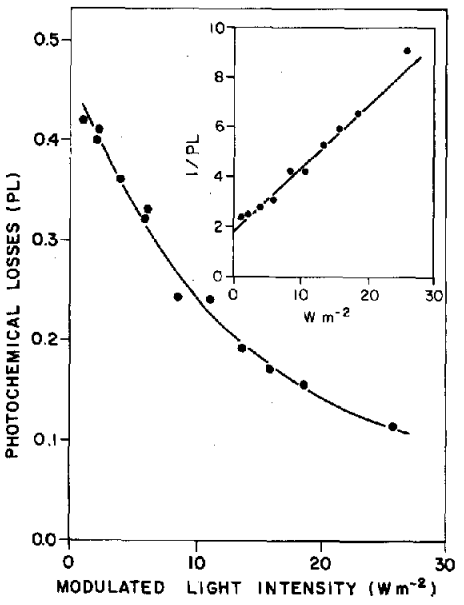

Fig.3. Effect of the intensity of the modulated light (35 Hz) on PL in a mature pea leaf. (Inset) Plot of the reciprocal of PL as a function of the modulated light intensity.

able to obtain PD signals with a tolerable S/N ratio from hairy (maize) or rough (bean) leaves.

An interesting characteristic of the in vivo PD signal is that the background light saturates the photochemistry almost instantly (within the time constant of the lock-in amplifier, i.e. between 1.25 s and 125 ms) whereas, when the saturating light was switched off, the return to the initial level was markedly slower, taking around 25 s. Although this delay in the recovery was not studied in detail, it might be related to the pool size capacity of the rate-limiting step of the photosynthetic electron transport chain. This phenomenon probably deserves further studies in the future.

The PD data presented in fig.2 were obtained with a modulated light of relatively high intensity (~ 14 $W \cdot m^{-2}$) which could consequently have an actinic effect and result in a partial (steady-state) closure of the reaction centers, thus possibly leading to a reduction of the apparent PL. This possibility was examined in an experiment, shown in fig.3, in which PL was measured at different intensities of the modulated light. It can be seen that photochemical energy storage was strongly influenced by the modulated light intensity, showing a hyperbolic relationship. It should be noted that we were able to measure the PL in the very low light intensities range (1–2 $W \cdot m^{-2}$) which, up to now, was difficult to explore by high-frequency PA measurements. The plot of the reciprocal of PL as a function of the modulated light intensity was linear (fig.3, inset) as expected from theoretical considerations [12]. Extrapolation of the linear plot to the zero fluence rate gives a measure of the maximal photochemical energy storage. In the case of mature pea leaves (fig.3), the extrapolated PL approached values as high as 55%. We observed that this value was dependent on the physiological and phenological state of the plant (not shown). For example, young pea leaves were characterized by a noticeably lower storage efficiency at low light intensities (around 35%) than older, mature leaves.

As shown in the inset of fig.2, photochemically saturated pea leaves behaved as passive absorbers relative to the modulated light. This means that the monitoring of the PD signal in the presence of the background light at different wavelengths of the modulated light could provide a measure of the in vivo absorption spectrum of the leaf. The PD spectrum of a mature pea leaf is shown in fig.4. This

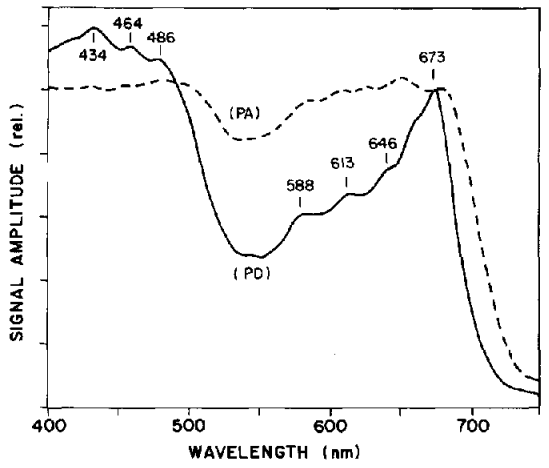

Fig.4. PD (——) and PA (– – –) spectra of a pea leaf taken at 18 Hz in the presence of continuous background light (160 $W \cdot m^{-2}$). The signals were measured every 2 nm at a speed of 50 $nm \cdot s^{-1}$. Spectra were smoothed out and normalized at 680 nm. Peak wavelengths are mean values of 7 separate experiments.

spectrum was characterized by 7 distinct peaks which were consistently reproducible from one spectrum to the other: 2 main peaks at 434 and 673 nm which correspond to the absorption bands of chlorophyll *a* (Soret band and Qy(0–0) transition, respectively) and 5 additional, smaller bands detected at 464, 646, 588, 613 and 486 nm (average values of spectra from 7 different leaves) which could possibly correspond to respectively, the 2 main absorption bands of chlorophyll *b*, the Qx(0–0) and Qy(0–1) transition bands associated to chlorophyll *a* and a carotenoid band [13]. Fig.4 also shows the spectrum of the same pea leaf measured with the PA technique. Comparison of the PD and PA data clearly shows the much higher spectral resolution of the deflection technique. The PA spectra presented a rather flat shape with 2 large shoulders (400–500 and 600–700 nm), the little 'waves' observed in the spectra being attributable to the noise inherent to the technique. Our PA spectra of pea leaves were very similar to the in vivo spectra measured in leaves of various other species in previous studies [12,14,15].

In addition to a higher sensitivity, PD measurements have other obvious advantages as compared to PA spectroscopy. First, in contrast to PD signals, in vivo PA signals measured in leaves at low modulation frequencies (below about 100 Hz) have been shown to contain a gas exchange-related component which considerably complicates the interpretation of the results [14,16]. On the other hand, PD measurements are performed in an open cell (in liquid or gas) whereas PA measurements are done in a hermetically closed cell (under somewhat artificial conditions, probably at a very low CO_2 concentration). It should, however, be noted that the amplitude of the in vivo PD signals obtained in air were around 50-fold smaller than that of signals monitored in water (not shown), reducing considerably the accuracy of the measurements. The fact that in vivo deflection signals can be monitored in water could make the technique very suitable for studying in situ the effects of photosynthetic inhibitors such as herbicides or pollutants. This is illustrated in fig.5 which shows the effect of the herbicide DCMU on the PD signals. After a delay of around 15–20 min following the injection of DCMU in the cell (final concentration 10^{-4} M) which probably reflected the penetration of the herbicide inside of the leaf, the PL drastically decreased so that, after 100 min, the background light had no apparent effect on the amplitude of the PD signals (i.e. PL = 0), due to the complete blockage of the photosynthetic electron flow. This result also confirms that the above measurements were effectively related to the photosynthetic activity of the leaf.

In conclusion, from the present data, it appears that the beam deflection technique based on the

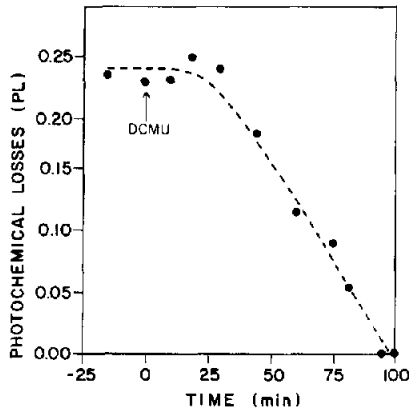

Fig.5. Effect of DCMU on the PL measured by the PD technique in a young pea leaf. At time zero, DCMU was injected into the PD cell so that the final concentration in DCMU was 10^{-4} M (in 2% alcohol). Modulated light: 35 Hz, 480 nm.

mirage effect is a very promising tool to probe both spectroscopic and photochemical properties of intact leaves. We think that, like other related photothermal methods [8,12,14,15,17], it could have various applications in plant physiology/biochemistry, in particular in the study of the regulation of leaf photosynthesis by the physico-chemical environment. Extension of the PD method to the investigation of photosynthesis in algae and chloroplasts is in progress.

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