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Photoacoustic Measurements: Application in Plant Science

C. Buschmann

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Photoacoustic measurements: application in plant science

By C. Buschmann

Botanik II (Plant Physiology and Plant Biochemistry), University of Karlsruhe, Kaiserstrasse 12, *D*-7500 *Karlsruhe, F.R.G.*

By means of the photoacoustic effect light-induced heat can be detected, which is produced during non-radiative de-excitation following absorption of light. This paper gives an overview of the current knowledge of photoacoustic measurements. First, the principle of detection and the different instrumentations are outlined in general. Then the applications of photoacoustic measurements in plant science and in particular in photosynthesis research are described in detail. (*a*) The induction kinetics of intact leaves at high modulation frequencies (where the signal is only determined by heat production) are contrasted with those at low modulation frequencies (where the signal can be determined by photosynthetic oxygen evolution superimposed on the heat production). Examples for (*b*) the determination of time constants and for (*c*) the measurement of the time delay between the excitation and the detection of the signal (phase) are given. Finally, (*d*) photoacoustic spectra are described. These can be used to determine the pigment composition, the depth profile, the efficiency of energy transfer and the pigment activity of a sample.

1. Introduction

On 13 January 1881 J. Tyndall introduced the photoacoustic effect (PA effect) to the Royal Society of London in a report on the 'Action of an intermittent beam of radiant heat upon gaseous matter'. Independently of experiments by W. C. Röntgen, his observations were influenced by the discovery of the PA effect by A. G. Bell in 1880. However, few results were published and it was only in 1973 that this effect was rediscovered in the Bell Telephone Laboratories in Murray Hill. It was mainly the work of A. Rosencwaig that expanded the application of PA measurements in a wide variety of different technical and natural sciences (Rosencwaig 1980), the latest developments of which are now presented and discussed in an internal congress every two years. This report will be restricted to the application of PA measurements in plant science. Earlier reviews on this topic (Malkin & Cahen 1979; Balasubramanian & Rao 1981; Buschmann *et al.* 1984; Braslavsky 1986; Buschmann & Prehn 1986) are partly included.

With the measurement of the PA effect, in general only light-induced heat is detected. In photosynthetically active leaves light-induced oxygen evolution may be superimposed on the heat production. Light-induced heat is produced during the process of non-radiative de-excitation or decay (radiationless transition), which occurs when the molecules leave their excited state(s), reached during absorption of light. Light energy absorbed by chlorophylls, carotenoids and eventually other pigments (e.g. flavonoids) can be transferred into heat or emitted as fluorescence. A photosynthetically active leaf produces light-induced heat during the energy transfer in the antenna systems and during photochemistry in the reaction centres (Butler 1978). The general theory of the PA effect has been summarized earlier (see, for example, Prehn 1979; Rosencwaig 1980). A theoretical treatment with emphasis on photosynthesis is described by Malkin & Cahen (1979).

2. Measuring apparatus

(a) *Principle of detection*

The most sensitive and frequently used PA detector unit consists of a closed sample compartment (PA cell) with a microphone (figure 1). The sample is enclosed in the PA cell where it should fill almost the entire volume. When measuring leaves, segments have to be cut from the leaf tissue that fit into the sample compartment. Amplitude-modulated light, created in most cases by a mechanical chopper, illuminates the sample through a transparent window. The absorption of the modulated excitation light leads to the production of modulated (light-induced) heat, which induces a modulation of the temperature of the gas adjacent to the sample. The gas then expands in rhythm with the heat modulation. As the PA cell is tightly closed, pressure changes are produced that can be measured by the microphone. The signals of the microphone are transferred to a lock-in amplifier, which selectively amplifies the signals detected with the same modulation frequency as the excitation light. PA spectra are taken by illuminating the sample with monochromatic light, which is scanned over a chosen wavelength range. In most cases a one-beam system is used and the PA spectrum of carbon black (in which light is totally absorbed and fully transferred into heat) serves as a reference.

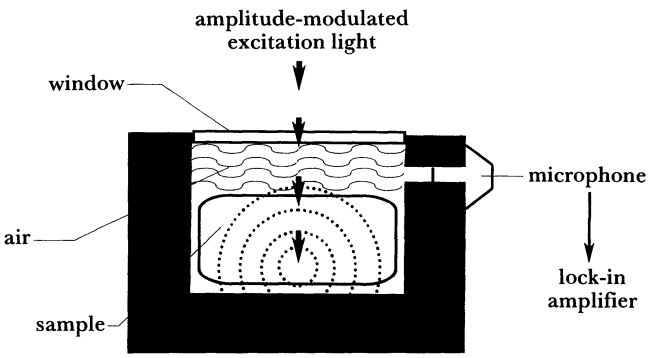

Figure 1. Scheme of a PA cell, the most sensitive and frequently used detector system for PA measurements. For a detailed description of the principle of detection see §2*a*.

In some cases, where a closed PA cell cannot be applied or when cutting of leaves is not desirable, detectors can be used that work in an open system (Tam 1986). At present, however, these alternative detectors cannot reach the sensitivity of a sophisticated closed PA cell. In all cases modulated light illuminates the sample and the PA signal is processed by a lock-in amplifier. The PA signal of leaves can be sensed by a pyroelectric detector, which directly measures the temperature modulation of the sample and therefore has to be in close contact with it (Kocsányi & Giber 1989). Piezoelectric detectors that sense the vibration caused by the temperature modulation of the sample have to be fixed in direct contact to the sample or via a contact medium, e.g. water (Jabben & Schaffner 1985). Highly sensitive infrared detectors have been used for the detection of the PA signal from leaves (Bults *et al.* 1982*b*; Kanstad *et al.* 1983). In these measurements the modulated, light-induced heat has to be separated from the high rate of non-modulated, thermal background radiation that is always present at temperatures above absolute zero. Probe-beam refraction detectors are rarely used for measuring the PA signal in leaves. They detect the diffraction of a probe light beam passed very

close and in parallel to the sample surface. This diffraction is a measure of the heat production in the sample because of the strong temperature dependence of the refractive index of the air above the sample. The PA signals of a leaf measured with the most sophisticated detector system still show a much lower signal:noise ratio than the signals of the chlorophyll fluorescence. A good signal:noise ratio of the PA signal is sometimes only achieved when the lock-in amplifier is set to integration time constants of 1 s or more, and these do not allow measurements of fast signal changes.

(*b*) *Factors influencing the height of the* PA *signal*

The height of the PA signal is mainly influenced by the following sample parameters: absorption coefficient, yield of non-radiative de-excitation, thermal characteristics (e.g. thermal conductivity, specific heat) and other basic physical properties, e.g. density. One unique feature of PA measurements is the fact that the modulation frequency determines the depth of the sample from where the signal is sensed, the 'thermally active layer'. By choosing a high modulation frequency the PA signal is detected only from close to the sample surface, because the heat waves created below are damped. At lower modulation frequencies, the PA signal also comes from deeper inside the sample. Thus the signal height in general decreases with increasing modulation frequency (figure 2). The formula of Rosencwaig & Gersho (1976) describes the dependence of the thermally active layer from the sample parameters and from the modulation frequency of a homogeneous sample. By a systematic variation of the modulation frequency a non-destructive 'depth profile' analysis of the sample can be made (see §3*c*(ii)).

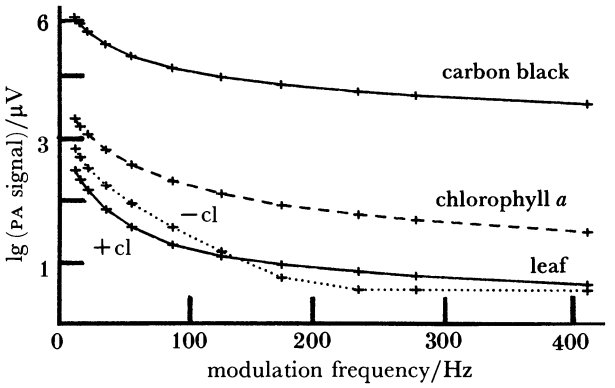

FIGURE 2. Dependence of the PA signal on the modulation frequency of excitation light for different samples. Carbon black gives the highest PA signal, because it is a totally light-absorbing substance that completely transfers absorbed light energy into heat. Chlorophyll *a* was measured from an ether extract on filter paper after evaporation of the solvent. The leaf (*Raphanus sativus* L.) was measured either without (−cl) or with (+cl) the addition of saturating, non-modulated white light (continuous light: 2000 μmol quanta m^{-2} s^{-1}). Up to the modulation frequency of 125 Hz the decrease of the PA signal caused by the non-modulated light indicates that the signal is determined by photosynthetic oxygen, whereas above 125 Hz this oxygen effect is missing (see §3*a*(i)). Modified from Buschmann (1987).

3. MEASUREMENTS

For plant science the main advantage of PA measurements lies in the new possibility of detecting light-induced heat, which represents the part of the energy lost in the non-radiative decay. This method made it possible to show by direct measurement that in P_{700}-enriched particles 80% of the absorbed energy is used for photosynthesis whereas 3% is lost in

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fluorescence emission and 17% in heat production (Vacek *et al.* 1979). Changes of the PA-signal caused by addition of substances affecting the photosynthetic electron transport (Katoh & Yamagishi 1984; Beauregard *et al.* 1987), or photophosphorylation (Katoh & Yamagishi 1984) have been studied to increase knowledge about non-radiative de-excitation processes involved in photosynthesis.

(*a*) *Kinetics*

(i) *Induction kinetics*

The PA signal of a photosynthetically active leaf predarkened for about half an hour undergoes a characteristic induction kinetic. As is well known from the chlorophyll fluorescence signal (Kautsky effect), this induction marks the gradual onset of photosynthetic activity over a few minutes. Simultaneous measurements of the induction kinetics of the PA signal and that of chlorophyll fluorescence were first described by Inoue *et al.* (1979). The fluorescence signal can easily be measured and may help in the interpretation of the PA signal.

Induction kinetics of heat production at high modulation frequencies. At higher modulation frequencies (in radish cotyledons: above 125 Hz, see figure 2) the PA signal is determined only by heat production. The induction kinetic of the PA signal then has a similar appearance to that of the chlorophyll fluorescence. The fast rise to the kinetic maximum is followed by a slower decrease to a final steady state (figure 3). The increase of the high-frequency PA signal due to the addition of non-modulated saturating light has been proposed as being a measure of the

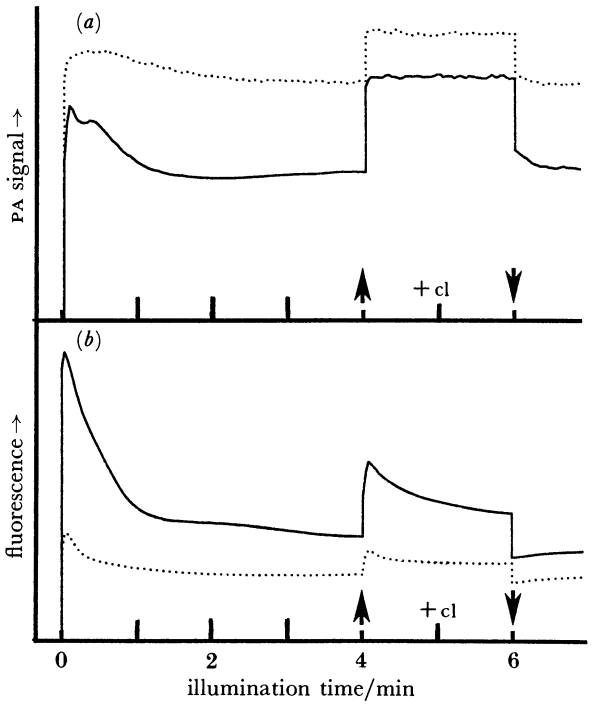

FIGURE 3. Induction kinetics of (*a*) the PA signal and (*b*) the simultaneously measured 680 nm chlorophyll fluorescence of a green *Raphanus* cotyledon before (——) and after (· · · · ·) photoinhibition (15 min at 600 W m^{-2}). Before the measurement the leaves were dark-adapted for 15 min. The excitation light of a 5 mW HeNe laser was modulated by a chopper with a frequency of 279 Hz. Four minutes after the onset of illumination saturating, non-modulated white light (cf. figure 2, continuous light (+cl)) was added for 2 min. Modified from Buschmann (1987).

'photochemical loss' (Malkin & Cahen 1979), because it represents the amount of energy with which part of the modulated light induced photosynthesis before the addition of non-modulated light. During the induction kinetic, the fluorescence signal precedes the PA signal (figure 4). This is more clearly seen by calculating the ratio between the PA signal and the fluorescence signal during simultaneous measurement. This indicates that the yields of fluorescence and heat do not change in a parallel manner during the induction kinetic. This finding would restrict the close relation of fluorescence and photosynthetic activity, which has up to now been the basis for the interpretation of fluorescence induction kinetics. When the induction kinetic of the PA signal is compared to those of the photochemical quenching, q_Q, and of the non-photochemical quenching, q_E (measured simultaneously with the pulse amplitude modulated (PAM) fluorometer), a fast change of the light-induced heat emission can be distinguished from a slow change obviously more related to the q_E-quenching (figure 5). The partial parallelism between heat production and q_E-quenching would confirm the assumption that the establishment of the proton gradient across the thylakoid membrane results in heat

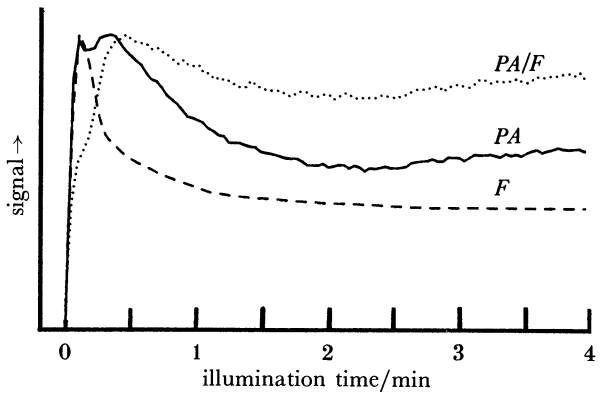

FIGURE 4. Induction kinetics of the PA signal (——) and of the simultaneously measured 680 nm chlorophyll fluorescence (---) of a green *Raphanus* cotyledon. The modulation frequency of the excitation light was set to 279 Hz. The ratio between the PA signal and the fluorescence was calculated during the measurement and is plotted (· · · · · ·). C. Buschmann & L. Kocsányi, unpublished results.)

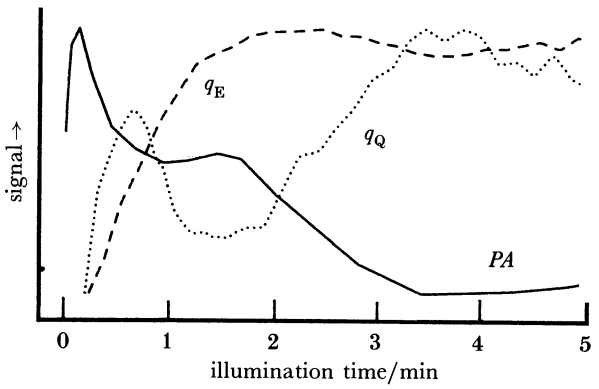

FIGURE 5. Induction kinetics of the PA signal (——) and of the simultaneously measured non-photochemical quenching, q_E (----), and photochemical quenching, q_Q (· · · · · ·), of the chlorophyll fluorescence. The light of a 5 mW HeNe laser was modulated by a chopper with a frequency of 279 Hz. This laser light served for the detection of the PA signal and for the induction of the transient. Fluorescence and its quenching components were measured with a PAM fluorometer. (C. Buschmann & L. Kocsányi, unpublished results.)

production. During a photoinhibitory treatment, the high-frequency PA signal rises parallel to the decrease of the fluorescence signal (Buschmann & Prehn 1988). This demonstrates that the absorbed light energy is increasingly transferred into heat. After photoinhibition the high-frequency PA signal remains high and, similarly to the decreased fluorescence, shows only a low decline from the kinetic maximum (figure 3). The development of photosynthetic functions in a leaf during greening in the light can also be followed by simultaneously measuring induction kinetics of fluorescence and heat production (Buschmann & Prehn 1985).

Induction kinetics at low modulation frequency. At low modulation frequencies the microphone of the PA cell is also able to detect pressure changes induced by modulated, light-induced gas evolution or gas consumption (Gray & Bard 1978). It has been postulated (although the final proof is still awaited) that in leaves, modulated evolution of photosynthetic oxygen is superimposed on the heat signal (Bults *et al.* 1982*a*). This is, however, only valid at low modulation frequencies, as at higher modulation frequencies photosynthetic oxygen evolution becomes homogeneous, as was shown earlier with an oxygen-rate electrode (Joliot & Joliot 1968). Because of special tissue structures this oxygen effect may be absent (e.g. in the green alga *Bryopsis maxima* (Katoh & Yamagishi 1984)). When a sample, such as bundle-sheath cells of C_4 plants (Beauregard *et al.* 1987) or cyanobacterial heterocysts (Carpentier & Mattijs 1986) does not evolve photosynthetic oxygen, heat emission can also be sensed at low modulation frequencies.

In most studies at low modulation frequencies the PA cell is used like an oxygen electrode. In these measurements only the changes of oxygen concentration closely related to the light pulses are detected. The signals measured with the PA cell should thus reflect more the gross photosynthesis, whereas with oxygen electrodes only the overall net photosynthesis (including respiratory oxygen consumption) can be sensed. However, PA measurements of pulsed oxygen uptake during light-induced transients have also been reported (Malkin 1987). To measure only oxygen evolution a phase-separation method (see §3*b*) has been proposed that should suppress the contribution of light-induced heat to the PA signal. The induction kinetic of the PA signal of a radish leaf measured at a low modulation frequency (17 Hz) shows an increase, which is probably due to the increase of oxygen evolution during the first minutes of the induction period (figure 6). After photoinhibition the induction kinetic of the 17 Hz PA signal shows a slower kinetic rise (figure 6), which can be explained by the decreased rate of photosynthetic oxygen evolution. The gradual development of photosynthetic activity of a plant during greening in the light has also been studied by measuring the induction kinetic of the low-frequency PA signal simultaneously with that of fluorescence (Buschmann & Prehn 1985). An inhibition of oxygen evolution determined with the low-frequency PA signal was also demonstrated after chilling (Yakir *et al.* 1985), water stress (Havaux *et al.* 1986) or heat treatment (Havaux *et al.* 1987). Changes in oxygen evolution during Emerson enhancement and during state 1–state 2 transitions have been demonstrated (Canaani & Malkin 1984).

Additional illumination with non-modulated, photosynthetically saturating light ('background light'; Bults *et al.* (1982*a*)) leads to the decrease of the PA signal (figure 6.) This can be explained by the fact that oxygen evolution is no longer pulsed, and thus does not produce rhythmic pressure changes. Consequently oxygen cannot be sensed by the microphone. The PA signal is then determined only by the heat production, which must be increased compared with the signal measured without continuous light because the modulated light is fully transferred into heat (see above). In radish cotyledons, additional non-modulated light leads to a decrease

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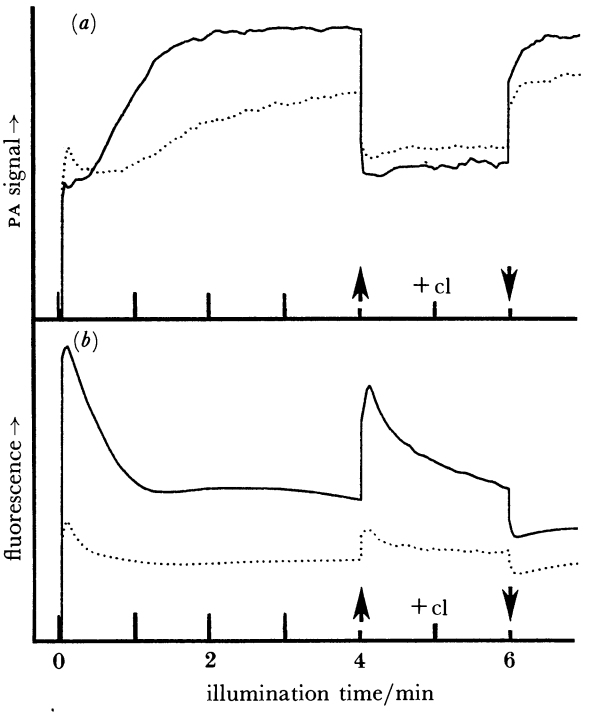

FIGURE 6. Induction kinetics of (*a*) the PA signal and (*b*) the simultaneously measured chlorophyll fluorescence of a green *Raphanus* cotyledon before (——) and after (· · · · ·) photoinhibition. Otherwise conditions as in figure 3, except for the modulation frequency of 17 Hz.

of the PA signal at modulation frequencies below 125 Hz (figure 2). This indicates that in radish cotyledons the PA signal below 125 Hz is determined by oxygen evolution, whereas above 125 Hz the oxygen effect can be neglected.

(ii) *Time constants of photochemical processes*

Time constants of photochemical processes have been measured with isolated phytochrome (Jabben *et al.* 1983) and in isolated purple membranes of *Halobacterium* (Cahen *et al.* 1978; Renard & Delmelle 1983). Fast adaptation of photosynthetic oxygen evaluation (determined by the low-frequency PA signal) to changing light intensities, varied between one change per second and one change per 2 h, has been determined by curve-fitting of the response to the frequency of the light changes (Dau & Hansen 1989). Assuming a linear response mechanism, the mathematical analysis reveals six different time constants, which lie in the range of milliseconds to minutes.

(*b*) *Phase*

The time delay between the excitation and the detection of the PA signal is termed 'phase'. This is measured by means of a two-phase lock-in amplifier and can be used to characterize the origin of the PA signal more effectively. In cases where there is no photochemistry, light-induced heat is produced nanoseconds after the light absorption. In a photosynthetically active leaf, however, because of the energy-transfer processes heat production is delayed. The PA signal should first be determined by the two photosystems separately, then by the two photosystems in series (Malkin & Cahen 1979). The part of the low-frequency PA signal effected by oxygen evolution shows a different phase from that effected by heat emission. This

is the basis for the phase-separation method, which should help to exclude heat production contributing to the low-frequency PA signal (Poulet *et al.* 1983). First, the phase of heat production is determined by measuring the PA signal during the addition of saturating, non-modulated light, where no oxygen effect occurs and only heat production is sensed. This heat-PA signal is adjusted to zero by turning the phase through about 90°. The PA signal determined at this particular phase should then contain no contribution from heat production.

(*c*) *Spectra*

PA spectra represent excitation spectra of the PA signal. They show in principle the same characteristics as the absorption spectra. The measurement of PA spectra allows measurement absorption spectra from nearly non-transparent samples (e.g. needles (Nagel *et al.* 1987)).

(i) *Pigment composition*

The composition of pigments *in vivo* has been measured by taking PA spectra from leaves (Buschmann & Prehn 1981, 1983; Veeranjaneyulu & Das 1982; Nagel & Lichtenthaler 1988), fruits (Kocsányi *et al.* 1989), flower petals (Li *et al.* 1983), isolated cells (Carpentier & Matijs 1986; Beauregard *et al.* 1987; Popovic *et al.* 1987), chloroplasts (Cahen *et al.* 1978), immobilized membranes (Thomasset *et al.* 1982) and pigment complexes (Dienstbier *et al.* 1984; Frackowiak *et al.* 1985). Figure 7 gives an example of a PA spectrum of an intact radish cotyledon. Because of the higher signal : noise ratio, most of the PA spectra have been measured at low modulation frequency. In photosynthetically active leaves oxygen evolution should then affect the PA signal (see figure 2). Illumination with saturating non-modulated light added

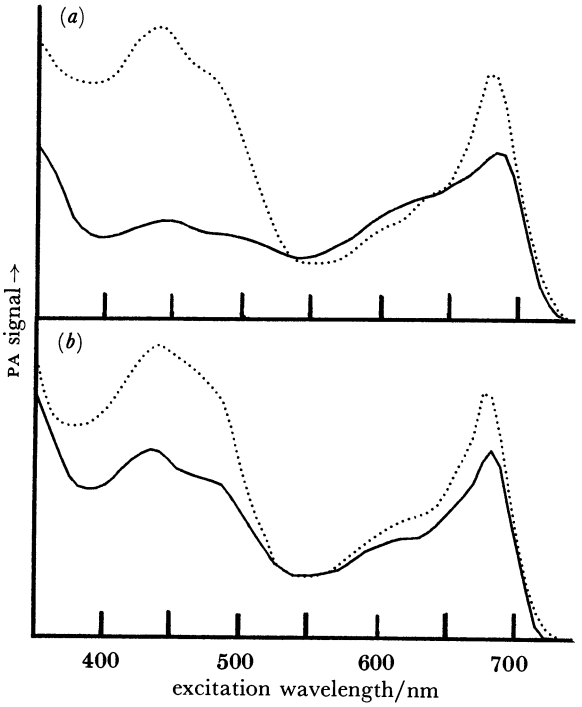

FIGURE 7. PA spectra of the upper and the lower leaf side of a green *Raphanus* cotyledon without (——) and with incubation with 0.1 mM DCMU (· · · · ·) measured at a modulation frequency of 20 Hz. Modified from Buschmann & Prehn (1981).

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during the measurement of the spectrum (§3*a*(*i*)) or the application of the phase-separation method (§3*b*) should then only allow the measurement of heat emission. Obviously the contribution of oxygen evolution to the PA signal is not constant throughout the entire visible range, because the decrease of the PA signal due to the addition of saturating non-modulated light is higher in the red- than in the blue-light region (Nagel 1988; Szigeti *et al.* 1989).

(ii) *Depth–profile analysis*

Depth–profile analysis was carried out in different leaves. The principle of this technique is clearly demonstrated in the PA spectra of a *Tradescantia* leaf (figure 8). At higher modulation frequencies only the peaks of the anthocyanins contained in the epidermis are detected. By lowering the modulation frequency a chlorophyll peak in the red-light region appears, which is caused by the chlorophylls contained in the mesophyll below the epidermis. The choice of the depth from where the PA signal is detected is determined by selecting the appropriate modulation frequency. This enables the measurement of absorption spectra of inner sample parts, which otherwise would only be possible by cutting horizontal sections of the sample. At extremely high modulation frequencies (e.g. about 500 Hz) the PA spectrum more closely resembles a reflection spectrum of the leaf (Nagel *et al.* 1987). Obviously in this case, light reflected from the mesophyll contributes to the excitation of the PA signal in the outer epidermis.

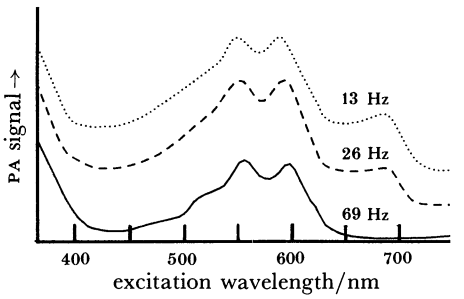

FIGURE 8. PA spectra of the lower side of a fully developed *Tradescantia* leaf measured at the modulation frequencies indicated. Modified from Buschmann & Prehn (1983).

(iii) *Energy transfer*

The effect of magnesium on the PA spectrum of isolated chlorophyll–protein complexes has been interpreted in terms of changes in energy transfer (Dienstbier *et al.* 1984). Energy transfer processes in antenna pigments of the thylakoids have been studied in isolated pigments fixed in polyvinyl alcohol films (Frackowiak *et al.* 1985). The spectrum measured with excitation light polarized either parallel or perpendicular to the direction of film stretching shows specific differences in the efficiency of energy transfer between pigments oriented in different directions, which can be identified by their absorption characteristics.

(iv) *Pigment activity*

The PA spectra are also influenced by the photosynthetic activity of the leaf. Incubation with the herbicide 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU) (Buschmann & Prehn 1981) or damage effects in needles affected by forest decline (Nagel *et al.* 1987) show a specific increase of the signal in the blue-light region as compared with the red-light region. The effect

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due to DCMU treatment was detected as being higher at the upper leaf side than at the lower (figure 7), indicating that the palisade parenchyma at the upper leaf half must have a higher photosynthetic activity than the spongy parenchyma at the lower leaf half. The ratio between the PA signal in the red-light region at 675 nm and that in the blue-light region at 475 nm has been used to evaluate damage effects in needles (Nagel *et al.* 1987). A specific spectral decrease of heat dissipation measured with isolated photosystem II particles, in which the electron flow was suppressed or activated by the addition of an electron acceptor and electron donor, led to the assumption that pheophytin might be involved in an energy storage process (Fragata *et al.* 1987). Specific reactions of photosynthetic electron flow were investigated by measuring photoacoustic spectra of isolated bundle-sheath cells of maize after addition of different reaction agents (Popovic *et al.* 1987).

4. Conclusion

PA measurements have already found a wide range of different applications. In plant science this method is mainly used in photosynthesis research. There is a need for further development of detector systems that allow measurement of PA signals with a higher signal:noise ratio and that are better adapted to different specific scientific problems. The main advantage of the PA method is the possibility of detecting light-induced heat emission during non-radiative de-excitation. This unique feature should help to increase our knowledge about photosynthesis *in vivo*.

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