Increased Thermal Deactivation of Excited Pigments in Pea Leaves Subjected to Photoinhibitory Treatments

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ABSTRACT

The photoacoustic technique was used to monitor thermal deexcitation of the photosynthetic pigments in intact pea leaves (Pisum sativum L.) subjected to photoinhibitory treatments. When the leaves were exposed to photon flux densities above 1000 micromoles per square meter per second, the amplitude of the photothermal component of the in vivo photoacoustic signal strongly increased. This high-light-induced stimulation of nonradiative energy dissipation (heat emission) was accompanied by an inverse change in the O$_2$ evolution activity and in the steady state emission of 685 nanometer chlorophyll fluorescence. The time course of these effects was shown to be very rapid, with a $t_{1/2}$ of around 15 minutes. When high-light-treated leaves were readapted to the dark, the heat emission changes were reversed, following somewhat slower kinetics. A reversible increase in the rate of light energy dissipation via radiationless transitions could be a photoprotective mechanism eliminating excess excitation energy from the photosynthetic reaction centers. Interestingly, this process does not operate at temperatures below about 12°C.

Sudden exposure of leaves to incident PFD\(^1\) much higher than those experienced during growth causes a gradual decline in photosynthetic activity (photoinhibition), due primarily to reduction in the photochemical efficiency of PSII (8, 24, 29). This photoinhibition is accompanied by marked effects on Chl fluorescence. Measurements of fluorescence emission at room temperature from leaves previously exposed to excessive PFD show a reduced fluorescence yield (9). Such high-light treatments also result in a considerable reduction of the variable PSII-fluorescence emission (at around 692 nm) measured in liquid nitrogen (77 K) (1, 2, 11–13, 30). Using the model proposed by Kitajima and Butler (21), the observed changes in the characteristics of the 77 K Chl fluorescence have been interpreted as being the result of an inactivation of the PSII reaction center complex and increased nonradiative decay at this center (1, 11–13). As the high-light-induced changes in 77 K fluorescence yield are largely reversible (11), it has been suggested that Chl fluorescence quenching in leaves under photoinhibitory conditions is not necessarily the manifestation of a detrimental effect on PSII. On the contrary, it may be seen as a consequence of a regulatory process which provides some degree of protection to the PSII centers by nondestructively diverting excess excitation energy via increased nonradiative energy dissipation (11–13).

The photoacoustic study reported in the present paper was conducted in order to check this latter possibility. A detailed treatment of the theory of photoacoustic spectroscopy applied to photosynthesis has been previously published (25). In brief, absorption of intensity modulated light by the photosynthetic pigments and subsequent thermal deexcitation of the excited electronic states result in periodic heat flow from the chloroplasts to the extracellular air spaces. The resulting modulated changes in the gas pressure give rise to acoustic pressure waves which propagate through the leaf and which can be detected by a sensitive microphone in the sealed photoacoustic cell. In the case of plant leaves, the photoacoustic signal is complicated by the fact that the sample is photochemically active and that, at sufficiently low frequencies, modulated O$_2$ evolution also contributes to the formation of the acoustic waves (5, 28). The two contributions, heat emission and O$_2$ evolution, can be separated using the vectorial method of Poulet et al. (28) after saturation of photochemistry with a strong nonmodulated light.

The data presented here demonstrate that heat emission is strongly increased in pea leaves exposed to high PFDs, confirming thus the suggestions derived from 77 K fluorescence studies. Further, the increased thermal deexcitation of the excited pigments, which was paralleled by a decrease in the Chl fluorescence yield and O$_2$ evolution activity, was completely reversible, provided the high-light stress was not too severe.

MATERIALS AND METHODS

Plant Material

Plants of Pisum sativum L. (cv Doublette) were grown for 2 weeks in perlite at day temperature between 20 and 25°C in a glasshouse under natural sunlight. The midday PFD was about 350 $\mu$mol m$^{-2}$ s$^{-1}$. The photoacoustic/fluorescence measurements were performed on the third pair of leaflets.

Photoinhibitory Treatment

Photoinhibition was induced in intact pea leaflets by exposing them to a greater PFD than that at which the plants were grown. White light produced by a 250 W halogen lamp was passed through two heat-reflecting filters and transmitted onto the leaf samples using a 1-cm-diameter fiberoptic light guide (Schott). The PFD of the light at the leaf surface was adjusted (from 0 to 8000 $\mu$mol m$^{-2}$ s$^{-1}$) using neutral density

\(^{1}\) Abbreviations: PFD, photon flux density; $A_{FR}$, amplitude of the photothermal component of the photoacoustic signal; $A_{OS}$, amplitude of the $O_2$ evolution-type photoacoustic signal.
filters (Schott). The photoinhibition experiments were conducted at 25°C except when the effects of temperature were examined. Leaf temperature was adjusted by circulation of water (from a Haake F4391 water bath) through a block of brass on which the leaf sample was placed during photoinhibition. Temperature measurements with a LM 335 integrated circuit temperature sensor (NS Corp.) showed an increase in leaf temperature of only a few degrees during the photoinhibitory treatments (for example, +3°C after 40 min at 4000 μmol m⁻² s⁻¹), indicating that heating of the tissues following absorption of high amounts of light was very limited. This observation dismisses the idea that heating could be responsible for the photoacoustic changes observed in this study. On the other hand, no wilting of the leaves was observed during the experiments, presumably due to the fact that the leaf samples were placed in a small volume. It should be noticed that, as previously shown in leaves sprayed with abscissic acid, stomatal closure has no influence on the photoacoustic signal (the acoustic waves are produced inside of the leaf).

**Photoacoustic Measurements**

After the high-light treatment, pea leaflets were placed in a photoacoustic cell, similar to that described in detail in Buls et al. (5). The photoacoustic measurements were performed at room temperature (25 ± 2°C). Modulated and nonmodulated lights were applied on the leaves using a polyfurred fiber optic system. The light produced by a 360 W D.C. operating halogen lamp (3M) was filtered through a 5-cm layer of water and a combination of two filters (Schott BG 18 and Ealing 35-5481). This 400 to 600 nm light was chopped at a frequency of 14 Hz (unless otherwise specified) using a Bentham 218 rotating sector chopper. The PFD of the modulated exciting light was 70 μmol m⁻² s⁻¹. The photoacoustic signals from the microphone (Knowles) were fed into a lock-in-amplifier (Stanford Research Systems, model SR530) working in the two phase mode in order to record simultaneously the in-phase and quadrature (90° out-of-phase) components of the signal.

Analysis of the photoacoustic data was done according to Poulet et al. (28) and as explained in several previous papers (15–17). At low frequency of modulation (below about 100 Hz), the photoacoustic signal can be described as a vector quantity which is the sum of two components arising from modulated O₂ evolution and modulated heat release (photothermal signal). The photothermal signal in a photochemically active sample is smaller than the maximum signal because a fraction of the absorbed light energy is stored in photosynthetic products ('photochemical losses,' PL). The maximum photothermal signal is used as a reference signal. The sample is self referenced by adding a strong nonmodulated background light (PFD of 3400 μmol m⁻² s⁻¹) to the modulated beam. A maximum photothermal signal is obtained in this case because the background light saturates photosynthesis by 'closing' the reaction centers, resulting in an almost complete conversion of absorbed modulated light into heat. Concomitantly, at photosynthetic saturation, the modulated component of O₂ evolution is eliminated.

The photothermal signal and the oxygen signal may have a different phase with respect to the modulated light. In order to separate each component, the following procedure is performed as in (28). In the presence of the photosynthetically saturating background light, only the maximum photothermal signal is present. A rigid axis rotation is performed around the origin by changing the phase in the lock-in amplifier so that the total amplitude of the photothermal signal (A₉) is projected only along the in-phase mode. Upon switching off the saturating light, the modulated oxygen signal will appear, if present. The oxygen vector can have projections on the in-phase mode where it will now add up to the photothermal signal and on the quadrature mode where it will be now the only component. The total amplitude of the O₂ evolution signal (Aₒx) is obtained as a vectorial sum (square root of the sum of the squares) of its in-phase and quadrature components. However, the photochemical energy storage yield (PL) must be taken into account (5, 28). It is estimated separately at high frequency of modulation (329 Hz):

\[ PL = (A_+ - A_-)/A_+ \]

where \( A_+ \) and \( A_- \) are, respectively, the amplitude of the high-frequency photoacoustic signal in the presence and in the absence of the background saturating light. Thus, if \( Q_{ox} \) is the amplitude of the quadrature signal and \( I_{ox} \) is the amplitude of the oxygen component of the in-phase signal (appearing when the background light is switched off), \( A_{ox} \) can be calculated using the following formula:

\[ A_{ox} = \sqrt{(Q_{ox})^2 + (I_{ox} + [A_{PT} \cdot PL])^2} \]

One of the main advantages of the photoacoustic technique is the fact that it allows the quantum yield of O₂ evolution (on a relative basis) to be determined very rapidly (within a few seconds) using the ratio of the amplitudes \( A_{ox}/A_{PT} \). The relation of photoacoustic O₂ evolution measurements to the ordinary rate measurements of photosynthesis has been theoretically and experimentally examined in detail in Poulet et al. (28): in brief, \( A_{ox} \) is proportional to \( \phi_i \) where \( \phi \) is the quantum yield and \( i \) the absorbed modulated light intensity and the photothermal signal \( A_{PT} \) is proportional to \( h\nu i \) where \( h\nu \) is the photon energy. Thus, the ratio \( (A_{ox}/A_{PT}) (\lambda^{'} \nu \iota) \) is proportional to the quantum yield of oxygen evolution \( \phi \). Light saturation of O₂ evolution (cf. Fig. 4) was measured photoacoustically by monitoring the decrease in \( A_{ox}/A_{PT} \) caused by increasing PFD of the background light (28).

**Chl Fluorescence Measurements**

Modulated PSII-Chl fluorescence was measured simultaneously with the photoacoustic signal using one branch of the polyfurred fiber optic device. The fluorescence signal was detected by a photomultiplier tube (EMI 9558B) driven by a high voltage power supply (Bradenburg model 457 R) at 300 V. The photomultiplier was shielded by a 685 nm-interference filter (Balzers) and a RG 620 filter (Schott). The modulated 685 nm-fluorescence signal was analyzed by a lock-in amplifier (Ortec Brookdeal 9503-C) and displayed on a chart recorder.

**Polarographic Determination of O₂ Evolution**

For purposes of comparison, O₂ evolution was also measured in pea leaves using a Clark-type O₂ electrode (Hansatech, model LD2). White light produced by a 100 W halogen lamp...
was filtered through a 15-cm layer of water and focused on the leaf placed in the O2 electrode cuvette. The PFD of the light was adjusted using neutral density filters. Circulation of water around the cuvette ensured that the leaf temperature was permanently maintained at 25°C. O2 exchange measurements were made according to the technique of Delieu and Walker (10). All the PFDs were measured using a Li-Cor lightmeter (LI-188B).

RESULTS

Effect of Exposing Leaves to a High PFD on the Photoacoustic Signals

Figure 1 shows the effects of a photoinhibitory treatment (4000 μmol m⁻² s⁻¹ for 15 min) on both the in-phase and quadrature components of the photoacoustic signals generated by pea leaves illuminated with a blue-green exciting light modulated at low (14 Hz) or high (329 Hz) frequency. At low modulation frequency, the modulated O2 evolution-related photoacoustic signal and modulated heat emission are separated by applying a photosynthetically saturating nonmodulated light in addition to the modulated exciting light. In the presence of the saturating background light (which does not create any pressure waves detected by the microphone), the quantum yield of O2 evolution approaches zero and modulated O2 evolution is eliminated. Under these conditions, the photoacoustic signal is then purely photothermal. As illustrated in Figure 1, the phase has been adjusted in the lock-in amplifier so that the photothermal signal appears only on the in-phase mode. The amplitude of the photothermal signal APT can then be directly derived from the height of this in-phase signal. The determination of APT was done as fast as possible in order to avoid possible photoinhibitory effects which could be induced by the background light itself. The high-light treatment resulted in a drastic increase (around +50%) in the amplitude of the photothermal signal. Concomitantly, the O2 evolution signal, which appeared when the background light was switched off, was significantly reduced after photoinhibition.

The photothermal signal measured at low frequency in the presence of the strong background light is actually the maximal photothermal signal obtained when photochemistry is saturated. At high frequency (for example, 329 Hz), the modulated O2 evolution signal is completely damped and the photothermal part of the photoacoustic signal is the only component which persists. In this case, application of the saturating light increases the heat emission signal to its maximal level (Fig. 1, right). The percentage of difference between the high frequency-signal obtained in the presence and in the absence of the background light can be used to estimate the extent of photochemical energy storage, which has to be taken into account in the calculation of the total amplitude of the O2 evolution signal, Aox (for details, see “Materials and Methods” and also Refs. 5, 29). It can be seen in Figure 1 that high-light treatment resulted in a 1.5-fold increase in the amplitude of the high-frequency photothermal signal, thus confirming the low-frequency data.

Time Course of the High-Light Effects on the Photoacoustic Signals

Figure 2 shows the time course of the photoinhibition-induced changes in the amplitude of the (low frequency) photothermal signal (APT) as well as in the amplitude of the O2 evolution photoacoustic signal (Aox) and the steady state level of modulated Chl fluorescence measured simultaneously in the same sample. Exposure to a PFD of 4000 μmol m⁻² s⁻¹ caused a strong and, initially, rapid rise in the heat emis-

![Figure 1](https://example.com/image1.png)

**Figure 1.** Photoacoustic signals (quadrature and in-phase components) generated by pea leaves illuminated with a 400 to 600 nm light modulated at low (14 Hz) or high (329 Hz) frequency. C, Photoacoustic traces of control pea leaves; T, photoacoustic traces of photo-inhibited leaves (treated for 15 min with a strong white light of 4000 μmol m⁻² s⁻¹). ( ), Modulated exciting light on (400-600 nm, 70 μmol m⁻² s⁻¹); ( ), background saturating light on (3400 μmol m⁻² s⁻¹); ( ), background light off.

![Figure 2](https://example.com/image2.png)

**Figure 2.** Relative changes in the amplitude of the photothermal signal (APT), the amplitude of the O2 evolution component of the photoacoustic signal (Aox), and the steady state Chl fluorescence yield (FLUO) in pea leaves exposed to a strong white light (4000 μmol m⁻² s⁻¹) for different periods of time. Modulated exciting light: 400 to 600 nm, 14 Hz, 70 μmol m⁻² s⁻¹.
Light Intensity Dependence of the Heat Emission Increase

Figure 3 shows the effects of exposing pea leaves for 15 min to different PFDs ranging from 0 to more than 8000 μmol m\(^{-2}\) s\(^{-1}\). No significant changes were observed at PFDs below about 1000 μmol m\(^{-2}\) s\(^{-1}\). Above this, \(A_{PT}\) increased almost linearly with PFD. Concomitant with this heat emission rise, \(A_{OX}\) and the fluorescence yield were decreased, with \(A_{OX}\) being proportionally more affected than Chl fluorescence particularly in the very high PFDs range. It is interesting to note that the changes in the different photosynthetic parameters appeared at PFDs below the level necessary to saturate photosynthetic electron transfer. Indeed, as shown in Figure 4, saturation of O\(_2\) evolution, measured either photoacoustically or polarographically, was observed at PFDs close to 3000 μmol m\(^{-2}\) s\(^{-1}\). The exact photoinhibitory and saturation conditions are probably restricted to the plants used here and depend on the light intensity used during growth.

Reversibility

The increase in heat emission as well as the decrease in fluorescence level and O\(_2\) evolution activity were shown to be reversible. After 12 min exposure to a PFD of 4000 μmol m\(^{-2}\) s\(^{-1}\), the leaf samples were readapted to the dark (Fig. 5). Dark adaptation resulted in a fast decrease in \(A_{PT}\) which, after about 40 min, reached the initial value measured before the photoinhibitory stress. Similarly, \(A_{OX}\) and fluorescence yield increased as soon as the strong light was switched off. The recovery of the latter two parameters was, however, noticeably slower than that of the photothermal signal.

As shown in Figure 6, the time course of the recovery was dependent on the photoinhibitory pretreatment. After a short high-light treatment of 6 min, the changes in heat emission were completely reversed within 20 min. Longer treatments resulted in slower recovery rates. After 15 min exposure to the strong light, 2 h were necessary for complete recovery, whereas pea leaves stressed for 25 min showed only a partial recovery (around 75%) after this dark adaptation time. Exposure of pea leaves to a PFD of 4000 μmol m\(^{-2}\) s\(^{-1}\) for 40 min (not shown) resulted in extremely slow rates of recovery (10% recovery after 2 h in darkness).
separated by in-phase/quadrature measurements. Under a high PFD of 4000 μmol m⁻² s⁻¹, the photoacoustically monitored yield of heat emission (ΔPT) in pea leaflets was almost doubled within around 30 min (Fig. 2). This increase in ΔPT was observed to be directly proportional to the PFD (above 1000 μmol m⁻² s⁻¹) of the light used to induce photoinhibition in the leaves (Fig. 3). The rise of the photothermal signal amplitude was accompanied by a concomitant decrease in the fluorescence yield and photosynthetic O₂ evolution. This inverse relationship is not surprising since the different pathways for pigment deexcitation (radiative and nonradiative decay, photochemistry) are interdependent. Blockage of one is expected to cause an increase in the other form of deexcitation and vice versa. An interesting aspect of this photoinduced change in the relative proportions of the different energy dissipation pathways is that it is rapidly reversed upon return to darkness. The dark-induced reversal in heat emission yield showed slower kinetics than the high-light-induced rise in ΔPT, however. From Figure 6, it can be estimated that the rate of the former process was roughly 7 times slower than that of the latter phenomenon. This reversibility suggests a physiological role for the high-light-induced stimulation of heat emission. Indeed, it can be argued that a large increase in thermal deexcitation of the excited pigments is useful since it can divert excess energy from the sensitive PSII reaction centers. It is generally supposed that photoinhibition occurs when the rate of excitation exceeds the dissipative capacity. In this study, it was shown that the ΔPT increase occurred well before light saturation of electron transport was reached (see Fig. 4; saturation was reached at around 3000 μmol m⁻² s⁻¹ whereas the ΔPT increase was already observed at 1000 μmol m⁻² s⁻¹). This fact reinforces the idea that increased heat emission is a regulatory and potentially protective mechanism which is triggered before damage to PSII occurs.

It is tempting to explain the high-light effects reported in this paper on the basis of the fluorescence quenching associated with establishing a ‘high energy state’ in the thylakoids. Indeed, it has been shown that the light-induced build-up of a trans-thylakoid pH gradient (ΔpH) causes a decrease in fluorescence emission (qE, ‘energy-dependent’ quenching)—an effect which has been assumed to result from increased thermal deexcitation (3, 4, 22, 23). Exactly how the proton gradient can influence radiationless transitions is still completely unknown. Ultrastructural alterations of the thylakoid membrane are usually evoked to explain the ΔpH effect (23). However, a recent study of the light induction of photosynthesis in dark-adapted leaves indicates that this suggestion is questionable since the slow quenching of induced fluorescence (partially attributable to qE) is apparently accompanied by a decrease in the (purely photothermal) high frequency photoacoustic signal instead of an increase (26). A similar discrepancy was also observed in (6). More work is necessary to clarify the problem and to understand the exact link between ΔpH and fluorescence emission. In addition, qE quenching has been reported to relax very rapidly (within seconds) upon darkening (22). Relaxation of the ΔPT increase was much slower, indicating that the phenomena reported here are probably not dependent on the existence of a ΔpH across the thylakoid membranes. Another explanation for the high-light-induced fluorescence quenching has been proposed recently

Effects of Temperature

As shown in Figure 7, temperature exerts a strong influence on the high-light-induced changes in heat emission. At temperatures below about 12°C, no significant increase in ΔPT (measured at 25°C) was indeed observed.

DISCUSSION

Increased yield of thermal energy dissipation in photo-inhibited leaves has been hypothesized by several authors (1, 11–13, 29). Here, this effect has been measured using the photoacoustic technique. Our results are in agreement with a recent work of Buschmann (7) who also observed changes in the photoacoustic signal generated by radish cotyledons after photoinhibition. However, in this latter study, the heat and oxygen components of the photoacoustic signal were not

**Figure 6.** Reversibility of the high-light-induced changes in the amplitude of the photothermal signal (ΔPT) in pea leaves readapted to darkness after exposure to a strong white light of 4000 μmol m⁻² s⁻¹ for 6, 15, or 25 min. Modulated exciting light as in Figure 2.

**Figure 7.** Effects of temperature on the photoinduced increase in the amplitude of the photothermal component of the photoacoustic signal (ΔPT) in pea leaves. After exposing leaf samples for 15 min to a PFD of 4000 μmol m⁻² s⁻¹ at different temperatures, the photoacoustic signal was recorded at 25°C. ΔPT is expressed as a percentage of the value measured before the high-light treatment. See legend of Figure 2 for the characteristics of the modulated exciting light.
by Demmig et al. (12) who emphasized the protective role of carotenoids. They have observed that high-light treatments stimulate the formation of zeaxanthin which is directly correlated with the changes in 77 K fluorescence yield. Zeaxanthin could possibly act as an ‘alternative quencher’ competing with the reaction centers for excitation energy.

We cannot exclude the possibility that the dynamic regulation of heat emission in our experiments is combined with some photodestruction of PSII, especially after extended high-light treatments. Indeed, as shown in Figure 5, complete recovery of the ‘normal’ heat emission yield was associated with only partial recovery of photosynthetic O₂ evolution.

Irrespective of the exact mechanism leading to high Aₛᵣ in leaves under photoinhibitory conditions, the complex photoregulation of the relative yield of fluorescence and radiationless transitions shows that it is hazardous to use the amplitude of variable fluorescence as an indicator of photoinhibition damage, as is often the case (see, for example 9, 30). Before correct and unambiguous conclusions concerning photoinhibition can be drawn, it is necessary to estimate the different processes (i.e. rate constant of heat emission) which can contribute to the decrease in fluorescence yield in high-light treated leaves.

Change in heat emission yield is possibly only one of the biophysical mechanisms that allow plants to avoid or reduce photoinhibitory stress (29). The state regulation phenomenon which alters the excitation energy distribution between the two photosystems is possibly another process which could play a protective role (14). These biophysical mechanisms could be particularly important under stressful environmental conditions (14, 19). It is well known that photoinhibition can be enhanced when high light intensities are combined with other environmental constraints such as drought (2) or low temperatures (27). Although pea is a chilling-resistant species, chilling-induced blockage of the process leading to increased nonradiative deexcitation of the photosynthetic pigments (Fig. 7) could explain, at least partially, the sensitization to photoinhibition observed in chilled plants of other species, such as maize, whose electron transfer capacity has been shown to be rapidly light saturated at low temperature (18). Suppression of the photoinduced Aₛᵣ rise under conditions where photoinhibition is usually increased confirms the protective nature of the phenomenon. It would be worth knowing whether zeaxanthin accumulation is also suppressed at chilling temperatures. It is also interesting to note that extreme state 1, characterized by strong energy delivery to PSII, has been previously observed at low temperature in the same chilling-sensitive species, perhaps amplifying the adverse effects of light (20). The possible role of these different biophysical processes in chilling susceptibility/resistance will be examined more extensively in our future work.

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LITERATURE CITED