Chlorophyll thermoluminescence of leaf discs: simple instruments and progress in signal interpretation open the way to new ecophysiological indicators

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Abstract
Luminescence from photosynthetic material observed in darkness following illumination is a delayed fluorescence produced by a recombination of charge pairs stored in photosystem II, i.e. the back-reaction of photosynthetic charge separation. Thermoluminescence (TL) is a technique consisting of a rapid cooling followed by the progressive warming of a preilluminated sample to reveal the different types of charge pairs as successive emission bands, which are resolved better than the corresponding decay phases recorded at constant temperatures. Progress in thermoelectric Peltier elements and in compact light detectors made the development of simple, affordable and transportable instruments possible. These instruments take advantage of multifurcated light guides for combined TL, fluorescence and absorbance/reflectance measurements. Meanwhile, experiments on unfrozen leaf discs, with excitation by single turn-over flashes or far red light and infiltration by specific inhibitors/uncouplers, have led to a better understanding of in vivo TL signals. Much like chlorophyll fluorescence and in a complementary way, TL in the 0–60 °C temperature range not only informs on the state of photosystem II in leaf tissues and its possible alterations, but also gives a broader insight into the energetic state inside the chloroplast by probing (1) the light-induced or dark-stable thylakoid proton gradient through the protonation of the Mn oxygen-evolving complex, (2) the induction of cyclic/chlororespiratory electron flow towards the plastoquinone pool, (3) the [NADPH+ATP] assimilatory potential. By a different mechanism, warming above 60 °C without preillumination reveals chemiluminescence high temperature thermoluminescence (HTL) bands due to the radiative thermolysis of peroxides, which are indicators of oxidative stress in leaves.

Key words: Delayed fluorescence, instrument, luminescence, photosynthesis, photosystem II.

Introduction
Oxygenic photosynthesis is endowed with the unique property of a variable fluorescence emission, $F_v$ spanning from a minimum level $F_0$ to an approximately 5-fold higher maximum level $F_m$. It originates from chlorophyll $a$ in the photosystem II antenna. In the mid 1980s, the introduction of light-doubling experiments, then of chlorophyll fluorimeters using a modulated excitation and saturating light pulses, allowed the separation of the two mechanisms of $F_m$ quenching that contribute to this variable emission by (i) a photochemical quenching, due to the ability of ‘open’ PSII centres to trap the energy of an absorbed photon conveyed to them through the antenna to create a +/− charge pair: these centres become temporarily ‘closed’, so they cannot trap the incoming energy which has to be dissipated in the pigment antenna partly as fluorescence emission, and (ii) a non-photochemical quenching, which reduces the excess excitation energy in
the antenna in situations where absorbed light overcomes the photosynthetic capacity (e.g. on a cold sunny day).

At the onset of a dark period following a strong illumination, a weak ‘luminescence’ emission, approximately 1% of the fluorescence intensity, can be detected with an emission spectrum similar to that of chlorophyll fluorescence (for reviews, see Lavoie, 1975; Jursinic, 1986; Tyysjärvi and Vass, 2003). At a constant temperature, this emission follows (in theory) a multiexponential decay kinetics. It results, with a low yield, from the recombination of charge pairs generated by prior illumination. This recombination recreates a singlet excited state in the chlorophyll antenna, which is partly dissipated as luminescence, also called ‘delayed’ fluorescence by reference to ‘prompt’ fluorescence emission observed during an illumination (the term ‘phosphorescence’ used for triplet state emissions is not relevant here). Photosynthesis is possible because light-separated charge pairs are stabilized on electron carriers by activation energy barriers, which limit the back-reaction. However, the sum (stabilized energy+activation energy) corresponds to the quantum energy of red photons, so the height of the activation barrier is necessarily limited and can be considered optimized for the physiological temperature domains of various photosynthetic organisms. Thus, charge recombination, reflected by luminescence emission, is highly temperature-dependent and grows exponentially according to an Arrhenius law up to 50–60 °C, a temperature range where the heat-induced breakdown of PSII occurs. As a consequence, thermoluminescence is a technique particularly well-adapted to study luminescence: it consists of cooling the sample before or immediately after an actinic illumination to make the recombination rate negligibly slow, then in warming it progressively in order to reveal the various types of luminescence-emitting charge pairs as TL elementary bands. This technique has a better resolving power than S1 and S3 remain stable during a dark adaptation whilst S2 and S3 are converted to S1, resulting in a 1/4 S0 3/4 S1 distribution in dark-adapted material. In leaves, approximately 40% of Qb is reduced (Rutherford et al., 1984a), so that the Qb/Qa ratio weakly oscillates with a period of 2 ± according to signal number.

In isolated thylakoids at low pH (<6.5) the B band, induced by two flashes, splits into two bands B1 (S2Qb±) and B2 (S2Qb±), because S1 is more destabilized by protonation than S2 (Joliot and Joliot, 1980; Rutherford et al., 1984b; Demeter and Sallai, 1986; Miranda and Ducruet, 1995b). Upon treatment by a PSII-inhibiting herbicide (diuron, atrazine) which blocks the QA to Qb electron transfer, the electron is stored as QA±, a less stabilized state (i.e. the activation barrier is smaller) than Qb± and produces a Q band peaking at a lower temperature (around 5 °C at neutral pH) than the B band upon recombination with S2/S3. This band is associated with a C band at about 55 °C due to D*QA± (Johnson et al., 1994), D* being the oxidized form of Tyrosine D on the inactive branch of PSII. The functional electron donor to the PSII P680 centre is Tyrosine Z: Z±P680 → Z±P680−.

A fast reduction of Z+ by S states occurs, except when the oxygen-evolving manganese complex is damaged, which leads to the emergence of A band Z+Qb± at about −15 °C. Other TL bands exist at lower temperatures which
are not fully explained and will not be considered here since they have been of little interest in plant stress physiology, at least up to now.

Twenty years ago, thermoluminescence brought a confirmation of the charge stabilization model of PSII. It remains a valuable technique that gives a global view complementing more analytical tools and it is particularly well adapted to the study of PSII mutants. Furthermore, like chlorophyll fluorescence, (thermo)luminescence is relevant to various levels of integration, from subchloroplast particles to algal cells or leaves (Farineau, 1993; Homann, 1999). However, applications to leaf photosynthesis have been relatively few until now, due to instrumental constraints and to a lack of understanding of in vivo signals. Several reviews already exist on photosynthesis TL (Sane and Rutherford, 1986; Vass and Inoue, 1992; Misra et al., 2001; Tyysjärvi and Vass, 2003). The present review will be focused on TL emission by leaves and will address the instrumental, theoretical and practical issues of measuring TL in leaf discs. Taking advantage of new and recently available components, simple instruments designed for leaf studies can now be built. The properties of TL bands already characterized in thylakoids or subchloroplast particles are modified in vivo by the cellular environment, particularly when leaves are kept unfrozen during the cooling step. Different types of stresses also modify the shape and intensity of the existing bands or enhance minor bands. Furthermore, a TL ‘afterglow’ band observable only in intact systems (intact chloroplasts, algae, leaf fragments) reflects the flow of electrons from reducing compounds present in the stroma to the plastoquinone pool and the quinonic acceptors of PSII. This back-transfer follows one of the pathways involved in the cyclic/chlororespiratory electron flow and appears to be a sensitive indicator of photosynthetic metabolism.

Although PSII is totally destroyed at about 60 °C, huge chlorophyll TL bands can be observed at higher temperatures. They correspond to a heat-enhanced chemiluminescence from molecular species generated by radical forms of oxygen, such as lipid peroxides, which accumulate in stress situations. Despite the fact that the mechanisms of high-temperature TL emission (HTL) is completely different from photosynthetic TL, recording through a single temperature scan from 0 °C to 160 °C on the same leaf disc both the photosynthesis TL bands and the oxidative stress HTL bands proves to be of practical interest in environmental physiology.

Instrument

Measuring thermoluminescence requires both the detection of a low far-red emission with the same maximum as prompt fluorescence at about 730 nm in leaves (another maximum at 685 nm is strongly reduced by chlorophyll reabsorption) and a flexible regulation of sample temperature allowing a fast cooling and a linear warming. This is used to be achieved with costly, complex, cumbersome, and immovable apparatus, using liquid nitrogen for cooling (for example, see Ducruet and Miranda, 1992). New mass-produced (hence cheap) components, which appeared during the last ten years, have paved the way to affordable and easy-to-use instruments, easily transportable (as luggage) close to the experimental field. A truly portable instrument, powered by a 12 V battery and driven by a laptop computer is theoretically feasible, but TL experiments are done on dark-incubated material subsequently preilluminated under controlled conditions, so the interest in catching on-the-spot TL records from leaves submitted to natural sunlight has still to be documented. Leaf discs can be punched out in the field and dark-relaxed in a cooled box while being carried back to the TL instrument. Several TL instruments have been built in the Bioénergétique laboratory at CEA-Saclay as initially described in Miranda and Ducruet (1995a) and more completely in Ducruet et al. (1998). It might be and has indeed been reproduced or adapted in laboratories endowed with a mechanical workshop. (More information on instrument building and computer programs can be found in Journal of Experimental Botany online: see Supplementary data available in the online version of this article.)

Temperature regulation is performed with a 40×40 mm thermoelectric Peltier plate (Duratec from Marlrow, or Thermatec from Melcor for measuring HTL up to 160 °C). A thin thermocouple is placed in the centre of the plate with thermal grease and covered by a copper or aluminium thin plate (1 mm) or adhesive sheet, which forms the bottom of a 25 mm hole bored in a heat-resistant plastic pressed against the plate through an O-ring. A drop of water (100 μl) is placed on the centre and the leaf disc is pressed on the bottom by a washer with a 20 mm inner diameter. A circular Pyrex window may be placed between the leaf and washer to reduce water loss from the sample during warming (except when recording HTL).

Luminescence emission from a leaf disc is sufficiently strong to be recorded with an analogue photomultiplier tube (PMT). Much more expensive photon counting systems are not necessary, their only advantage here being to allow the quantum noise to be known as the square root of counts, hence to assess rigorously the quality of fit (χ²) of a simulated TL signal. A compact red-sensitive PMT (Hamamatsu H5701-50 or H7711-50, with built-in amplifier and +15/–15 V power supply) is positioned 1.5 cm above the sample by a light-proof holder that can slide laterally to an illumination position where a light guide comes in front of the sample whilst the PMT is protected from strong actinic light (Ducruet et al., 1998; Fig. 1A). Alternatively, luminescence can be conveyed to the detector by a light guide, which prevents heating of the PMT windows during HTL measurements. In this new
configuration, the common part of a 4-arm or 5-arm PAM-Walz light guide is maintained on top of the sample by a holding ring (Fig. 1B). Luminescence is conveyed to the PMT via one arm of the guide whilst the four other arms are used for illumination (flashes, continuous visible or far-red light or weak blue LED for $F_0$ excitation) and, if needed, for recording fluorescence kinetics. The PMT is protected by a shutter during illumination periods. A loss of luminescence in the light guide is partly compensated by a higher luminescence collection at the guide entrance placed very close to the leaf disc. With an electric shutter, a whole preillumination/acquisition cycle, lasting about 10/15 min, can be programmed.

The instrument is driven by a PC computer, with a specially developed acquisition program, through an interface which should provide at least two analogue-to-digital (A/D) ports for temperature and TL signal entrance, respectively, a digital-to-analogue 0 to +5 V (A/D) port for proportional current regulation in the Peltier plate through a power amplifier, and five TTL ports providing +5 V to trigger various light sources and shutters. Either Advantech PCL718/818 cards plugged into a computer slot, or a National Instrument DAQ-Pad 1200 (Fig. 1A) on the computer parallel port are used. Before TL recording, it is possible to define up to 10 preincubation periods at different temperatures combined with a maximum of 10 flash sequences and/or 10 preillumination periods. An ultra-weak blue LED (480 nm) can be pulsed once or periodically to measure $F_0$ emission, subsequently separated from TL emission by TL interpolation.

Ultimately, TL measurement can be fully integrated in fluorescence and absorbance (e.g. P700) instruments such as PAM101, requiring basically a thermostated sample holder at the common part of a light guide, a PMT with shutter on one of the multiple arms and optionally a weak blue LED for $F_0$ excitation on another arm.

**Signal analysis**

Many minerals heated at hundreds of degrees emit luminescence, so that thermoluminescence has been used initially in geology, archeological dating and radiation dosimetry. The theory of charge recombina-
tion had first been worked out for minerals by Randall and Wilkins (1945). They considered that the recombination of fixed +/- charge pairs obeys a first order kinetics. This has been applied to the analysis of photosynthetic TL by Vass et al. (1981) using an analytical method. From the same theoretical background, a numerical method is also feasible (Ducruet and Miranda, 1992), the advantages of which are (i) to require no approximation, (ii) to take into account the actual temperature as measured under the leaf sample (hence the small irregularities on the simulation curve, reflecting those of the temperature gradient). It consists in calculating iteratively, from low to high temperature, the expected proportion of recombination \( L(T) \) for each TL band at every measured temperature, using the Arrhenius–Eyring equation:

\[
L(T)=N^R P T^s \exp \left(-E_a/k_BT\right)
\]

(1)

\(N\) represents the number of charge pairs still able to recombine at temperature \(T\), and is decreased at each sampling step by \(L(T)\). The initial concentration \(N_0\) of identical charge pairs detected through the measuring system is the area of the resulting TL band. It can be known exactly with photon counting detection, but only as an arbitrary quantity with analogic detection (i.e. amplitude of integrated area of a TL band in arbitrary units). \(R\) is the order of reaction (generally \(R=1\), as would be expected for charge pairs not exchangeable between PSII centres), \(P\) is the pre-exponential factor which can be related to the Arrhenius frequency factor \(s\) as \(P=K(T)\times s\), \(K(T)\) being an unknown factor; \(\Delta t\) is the duration of sampling step (generally 1 s); \(E_a\) is the activation energy, and \(k_B\) is the Boltzmann constant.

To what extent can an experimental TL signal be simulated using the theoretical equation (1)? Recombination of charge pairs in PSII is not exactly the same phenomenon as in minerals, since those charges are transferred downhill on carriers which are in temperature-dependent equilibrium. DeVault et al. (1983) and DeVault and Govindjee (1990) carried out a theoretical study of photosynthetic TL, showing that an apparent activation energy can be calculated even though several electron transfer steps have to be passed through from the stabilized state to the recombination step (for a recent review on the theory of photosynthetic TL, see Tyysjärvı and Vass, 2003). Practically, a first order theoretical simulation fits well to the central part of a B band (Table 1) induced by a single turn-over flash which only forms the pair \(S_2Q_b\), except at the low and high temperature edges due to the presence of minor bands. This shows that \(P\) and hence \(K(T)\) are constant throughout a B band. By contrast, a \(S_3Q_a\) Q band obtained in the presence of diuron is poorly fitted, because the recombination of the \(S_3Q_a\) pair proceeds from a closed state \(Q_a\) of all PSII centres to an open state \(Q_a\) (Ducruet and Miranda, 1992). Indeed the \(F_0\) fluorescence measured simultaneously drops, reflecting the progressive opening of centres. This raises the problem of retrapping: a quantum of excitation formed in the antenna by charge recombination migrates between chlorophylls almost as if it were coming from an absorbed photon, with a probability to be (re)trapped either by the same centre or by neighbouring centres. The retrapping is integrated in the \(K(T)=P/s\) factor. In a B band, \(S_2Q_b\) or \(S_3Q_b\), all centres are open (\(Q_a\)) throughout the recombination process, so \(K(T)\) remains constant, so that a good fit can be obtained. In a Q band, a closed \(S_2Q_a\) centre opens as soon as charges recombine, but neighbouring centres change progressively from a closed to an open state as recombination proceeds, which increase the retrapping probability due to the connectivity of antennae between PSII units. Hence, \(K(T)\) decreases as temperature increases. Attempts have been made to extract entropy value \(\Delta S\) from the pre-exponential factor \(P\) and hence deduce the free energy of activation \(\Delta E\) from the activation enthalpy \(\Delta H=\Delta Ea\). However the frequency factor \(s\) is not experimentally accessible but only the pre-exponential factor \(P=K(T)s\), where \(K(T)\) is unknown.

Those theoretical considerations are important to clarify what can be reliably deduced from simulation. First simulation is necessary for the decomposition (it is not a deconvolution) of an experimental TL signal into elementary bands. This is done by adjusting the three parameters \(E_a\), \(P\) and \(N_0\) in equation (1) for each elementary TL band so that their sum fits the actual signal, by minimizing the sum of square of differences. A graphical fitting is first done interactively on the computer screen, with the amplified fitting residue optionally displayed, then refined by loop minimization. Simulation is theoretically justified, as explained above, for B bands, which are prominent in leaves. It provides an overall activation energy \(E_a\) (enthalpy) which reflects the band width (the higher \(Ea\), the sharper the band) and allows quantitative comparisons to be done between the shape of similar bands in different samples. It also gives the relative intensity of each band. Finally, it allows the maximum temperature \(T_m\) of elementary bands to be known, which cannot be determined directly on the TL signal when bands strongly overlap, even less when they appear as shoulders.

**Experimental**

In the presently available TL instruments, pieces of leaves, usually discs, have to be excited just before the experiment and placed in the measuring chamber. In order to study the stabilization of charge pairs within PSII, it is possible to perform the dark-adaptation and the preillumination of the leaf sample in the same chamber. However, as TL becomes a tool in plant ecophysiology, it may become necessary to
submit the attached leaf to more elaborate conditioning outside the TL chamber, for example, in a gas exchange clip, then quickly to punch out a disc for TL recording.

Historically, charge separation before TL had to be induced by continuous illumination at low temperatures or even throughout a slow (for instrumental reasons) cooling process. This had allowed some major TL bands to be detected in the 1970s, but failed to reach mechanistic assignments because complex signals thus generated were resulting in undefined charge patterns, with possible artefacts due to photoinhibition. Short sequences of single turn-over xenon flashes, each of them inducing one and only one charge separation per PSII centre, provided a unique tool to create well-defined charge patterns on PSII electron carriers and therefore played a major role in the identification of TL bands in the early 1980s (Rutherford et al., 1982, 1984). Excitation by short flash sequence (at least one and two flashes and a dark control) should always be done to characterize control and treated leaf samples, notwithstanding further experiments using continuous illumination.

Freezing or not freezing? In conventional TL protocols, the sample is deep-frozen possibly down to liquid nitrogen temperature to be sure that no recombination occurs before TL recording. A complete study on the effect of freezing on TL emission has been carried out by Homann (1999). As a consequence, a comparison should be done for every new leaf material between TL emission in frozen and unfrozen samples, to determine if freezing distorts the signal. If not, it remains, as further discussed below, that freezing acts as an uncoupler and can be used as a tool to relax the dark-stable proton gradients. It should be noticed that the TL signal, even undistorted, has a lower intensity after freezing (non-radiative recombination may occur even at low temperatures by tunneling, i.e. temperature independent non-radiative recombination).

This carries instrumental consequences: Peltier plates provide both a rapid cooling (20 °C to 0 °C in a few seconds using 5 A current) and a better control of the lower temperature than by dipping the holder in liquid nitrogen. Furthermore, a single-stage Peltier plate with a water flow at 0 °C can reach −30 °C. This allows the resolution of the A band at −15 °C. When freezing is avoided, the TL gradient should be started at 0 °C immediately after preillumination in order not to let the Q band, peaking near 5 °C, to be partly discharged. This Q band appears as a shoulder at the beginning of the thermogram whereas the A band is not seen and the major bands B1, B2, AG, and C can be fully resolved (Table 1).

Various treatments or stress factors may affect TL emission, compared to a control, by inducing new bands, stored in the vacuole such as phenolics, in lesser amounts in spinach or pea leaves. A complete study on the effect of freezing on TL emission has been carried out by Homann (1999). As a consequence, a comparison should be done for every new leaf material between TL emission in frozen and unfrozen samples, to determine if freezing distorts the signal. If not, it remains, as further discussed below, that freezing acts as an uncoupler and can be used as a tool to relax the dark-stable proton gradients. It should be noticed that the TL signal, even undistorted, has a lower intensity after freezing (non-radiative recombination may occur even at low temperatures by tunneling, i.e. temperature independent non-radiative recombination).

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### Table 1. Thermoluminescence bands observable in leaves

<table>
<thead>
<tr>
<th>Name</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; standard (~)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; shift</th>
<th>Origin</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosystem II bands (after preillumination)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>−15 °C</td>
<td></td>
<td>Z’Qb&lt;sup&gt;−&lt;/sup&gt; (S&lt;sub&gt;3&lt;/sub&gt;Qa&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Damage to Mn oxygen-evolving complex</td>
</tr>
<tr>
<td>Q</td>
<td>+5 °C</td>
<td></td>
<td>S&lt;sub&gt;2&lt;/sub&gt;Q&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Damage to secondary Qb quinonic acceptor (induced by diuron or atrazine-like herbicides)</td>
</tr>
<tr>
<td>B</td>
<td>+35 °C</td>
<td>+28 °C</td>
<td>S&lt;sub&gt;2&lt;/sub&gt;Qb&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Typically observed after one flash (Fig. 3A, B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+22 °C</td>
<td>S&lt;sub&gt;1&lt;/sub&gt;Qb&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Typically observed after three flashes (Fig. 3B)</td>
</tr>
<tr>
<td>AG</td>
<td>+45 °C</td>
<td>+35 °C</td>
<td>S&lt;sub&gt;2&lt;/sub&gt;/S&lt;sub&gt;3&lt;/sub&gt;Q&lt;sub&gt;b&lt;/sub&gt;+e&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Typically induced by 720 nm light (sometimes by flashes)</td>
</tr>
<tr>
<td>C</td>
<td>+55 °C</td>
<td></td>
<td>D&lt;sup&gt;−&lt;/sup&gt;Qa&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Appears when Qb is blocked by diuron or damaged (D is the non-functional donor to PSII, Z is the functional one)</td>
</tr>
<tr>
<td>Oxidative bands (not dependent on preillumination)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTL1</td>
<td>65–85 °C</td>
<td></td>
<td>Aldehydes+H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;?</td>
<td>Also pseudo-HTL2 in wet samples (Fig. 4)</td>
</tr>
<tr>
<td>HTL2</td>
<td>120–140 °C</td>
<td></td>
<td>Lipid peroxides</td>
<td>Correlated with content in lipid peroxides in dry samples (Fig. 4)</td>
</tr>
<tr>
<td>HTL3</td>
<td>&gt; 160 °C</td>
<td></td>
<td>Induce by warming</td>
<td>Increased by O&lt;sub&gt;2&lt;/sub&gt;, reduced by N&lt;sub&gt;2&lt;/sub&gt; flushed during TL warming</td>
</tr>
</tbody>
</table>

Ducruet
by modifying the shape and/or the intensity of existing bands (essentially the B band). Comparing the absolute intensity of a TL band between different samples raises the same problem as using the absolute values of fluorescence parameters such as $F_0$ or $F_m$, i.e. the variability of these measurements due to natural heterogeneity between leaf samples. This variability can be reduced by comparing leaves of the same age and development. However, it is also advisable to relate those values to an internal standard, either another TL band when possible or the $F_0$ level measured simultaneously by a pulse of ultra-weak blue light.

Figure 2 show an example of TL and fluorescence $F_0$ recorded at two heating rates after a far-red excitation. The TL signal obtained at 0.5 °C s$^{-1}$ show a peak at 42 °C and a shoulder near 20 °C, which correspond to an afterglow (AG) band and a downshifted B band, respectively, as explained below. A slower heating rate (0.25 °C s$^{-1}$) results in a downshift of the bands, since more recombination events occur in the same temperature interval (all $T_m$ in this review are at 0.5 °C s$^{-1}$). The $F_0$ is constant up to 30 °C and can be used as an internal standard to calibrate the intensities of TL bands. It starts increasing at about 40 °C, sometimes with a shoulder in the 30–40 °C range, and peaks above 50 °C (Schreiber and Berry, 1977), reflecting heat damage to PSII.

Classical TL bands in vivo

The B band at about 35 °C is prominent in a healthy dark-adapted leaf submitted to one or several single turn-over flashes, although minor bands can also be detected: the Q band on the lower edge, AG or C bands on the upper edge. Increase of the Q band ($T_m$ ~5 °C), generally associated with a C band (~55 °C), reflects damage to the secondary quinonic acceptor Qb of PSII, induced for example by photoinhibition (Janda et al., 1992). The A band (~−15 °C) appears in the case of damage to the oxygen evolving complex. However, continuous illumination at freezing temperatures can induce these Q and A bands as artefacts, another reason for preferring flash excitation. For routine work, two flashes can be used since they generate S$_2$ and S$_3$ states from the dark-stable S$_0$ and S$_1$, hence the strongest B band.

Besides the emergence of new stress-induced TL bands, the characterization of the B band(s) itself can be very informative. In frozen spinach leaves, the B band exhibits the classical period 4 oscillation pattern (Rutherford et al., 1984a). During photoinhibitory treatments, the intensity of the B band decreases in parallel to the $F_s/F_m$ fluorescence ratio, reflecting the destruction of PSII centres. After cold adaptation, the activation energy is decreased in spinach leaves (Briantais et al. 1992) and in some chilling-tolerant inbred lines of maize (unpublished results). A slightly lower activation energy $E_a$ could be observed in crop plant species adapted to cool climates compared to thermophilic species, and their $E_a$ was further decreased upon cold hardening (Glémin et al., 1992). Such experiments were done after freezing the leaf fragments to ~40 °C, which breaks the thylakoid membrane so that PSII centres are in a fully relaxed state, without an influence of a dark-stable transthyllakoidal proton gradient.

Compared with frozen leaves in which the B band peaks at about 35 °C, whatever the flash number, unfrozen leaves show a B$_2$ S$_2$Qb$^-$ band after one flash near 32 °C and a B$_1$ S$_3$Qb$^-$ band after three flashes near 28 °C. After two flashes, B$_2$ is prominent with B$_1$ appearing as a shoulder on its upper edge (this B$_1$ B$_2$ nomenclature can be confusing, and is indeed confused in the literature, since historically numbering has been done from low to high temperature, and it was only known later that B$_1$ is induced by two flashes and B$_2$ by one flash). These downshifts can be explained by an acidic pH of the lumen, whereas the stroma pH remains fairly constant (Heldt et al., 1973), which destabilizes S$_3$ strongly and S$_2$ to a lesser extent (Joliot and Joliot, 1980; Rutherford et al., 1984b; Demeter and Sallai, 1986; Miranda and Ducruet, 1995b), as later explained by the pH-dependent protonation pattern of S states (Lavergne and Jünge, 1993).

Figure 3 shows examples of B bands produced by flash excitation on healthy leaves. In Fig. 1A, the S$_2$Qb$^-$ charge pairs generated in a maize leaf by a single turn-over flash produce a TL band that can be satisfactorily fitted by a one-component numerical simulation using equation (1) as previously explained. The apparent activation energy $E_{act}=1.04$ eV corresponds to that of a thermophilic species, whereas $E_{act}$ found in cold-tolerant species are somewhat lower, between 0.75 and 0.9 eV (Briantais et al., 1992; Glémin et al., 1992). In most cases, the fit is not so good due to the presence of a small Q band on the lower edge.
and an AG band on the upper edge of the B band. Figure 3B shows a typical example of B bands generated by flashes in pea leaves. It should be noticed that TL intensities between different leaf samples (here pea leaflets) cannot be exactly compared. Nevertheless, the B band is clearly maximum after two flashes (all centres are in the S2 or S3 states) then decreases after three flashes. A B band much higher after three flashes than after one flash would indicate that the flash is not saturating, thus inducing ‘misses’ (i.e. a fraction of centres does not undergo charge separation, due to the scarcity of absorbed photons). The B band after one flash (S2Qb−) can be simulated with one component (another component a higher temperature was introduced to correct the baseline). The simulation is not so simple after three flashes (S2Qb−), since S2Qb− (due to misses) and AG (explained below) components had to be introduced. Finally, The B band after two flashes was fitted with the three components, with Ea and P for S2Qb− and S2Qb− blocked. Although a minimum number of three components is used here, fairly good fits (local minima) can be obtained with completely different sets of parameters: a decomposition led only by the search for an absolute minimum can be misleading, because the fit may be biased by the presence of minor bands. Therefore, decomposition of complex signals should be started with components determined on simpler signals. Simulation should be considered mainly as a practical tool for a signal decomposition, taking into account the theoretical charge patterns. It is not always necessary: in Fig. 3B, the B band after two flashes is complex, but the apparent Tm after one and three flashes provide a good estimate of the downshift due to S5 compared to S2, ascribable to an acidic lumen pH.

**Afterglow (AG)**

Bertsch and Azzi (1965) have described a luminescence bounce superimposed on luminescence decays following a far-red (>700 nm) illumination. Björn (1971) showed that it was related to cyclic electron flow and provided evidence of the enhancing effect of temperature on this ‘afterglow’. This emission reflects a back-flow of electrons from reductants present in the stroma to the quinonic acceptors of PSII, allowing their recombination with S2 and S3 states (Sundblad et al., 1986, 1988). Whilst S2Qb− and S3Qb− centres produce a B band, the S2Qb and S3Qb centres should not lead to luminescence emission, unless an electron is progressively fed back to Qb, resulting in an AG emission. It should be stressed that this emission, even though generally induced by far-red light which preferentially excites PSI, is originating from PSII, as showed by its period-4 oscillation and by luminescence emission spectroscopy (Hideg et al., 1991). The weak absorption of far-red light by PSII is sufficient to create luminescence active states S2 and S3. This AG emission initially reported in algae also occurs in higher plants (Nakamoto et al., 1988).

The afterglow emission is enhanced and accelerated by temperature elevation up to 40/45 °C (Björn, 1971), the threshold for heat damage to PSII (Schreiber and Berry, 1977). However, more subtle changes occur in the 25–40 °C temperature range. PSI centres are located mainly in grana and PSI centres in stroma lamellae. Moderate heating above 30 °C induces an unstacking (Weis, 1984; Sundby et al., 1986) accompanied by structural changes (Thomas et al., 1986). Photoacoustic spectroscopy confirmed a triggering of PSI cyclic electron flow in this temperature range, driving electrons from stroma reductants towards the acceptor side of PSI (Havaux, 1996), consistent with the role of state transitions in the induction of the cyclic pathway (Finazzi et al., 2002). In order to avoid triggering the AG process during the far-red excitation period, a temperature jump method can be used, which consists in illuminating the sample at 10 °C, then quickly raising the temperature to 25 °C, 30 °C or 35 °C. An alternative method is to warm the sample progressively after far-red excitation at 10 °C or 0 °C, in order to reveal the temperature-induced back-transfer of electrons towards PSII as a sharp TL band at about 45 °C (Miranda and Ducruet, 1995a; Fig. 2). The sharp band at 45 °C exhibits the properties of an afterglow emission, with a maximum AG/B band intensity ratio after three flashes and a similar sensitivity to characterizing chemicals. Addition of an uncoupler or freezing below −5 °C suppresses the AG bands and the downshift of the B band, resulting in a unique band at 35 °C. A basic property of the AG emission is its suppression by 5 μM antimycin A, as already shown by Björn (1971), a low concentration which also selectively inhibits the ferredoxin-plastoquinone-reductase or FQR pathway, but not the NAD(P)H-plastoquinone-oxidoreductase or NDH pathway (Ravenel et al., 1994; Bendall and Mannasse, 1995; Scheller, 1996). Consistently, the AG emission remains almost unchanged in NDH-deficient mutants of tobacco (L Cournac and J-M...
Ducruet, unpublished data; A Liszkay-Krieger, personal communication). The molecular background of the FQR activity remains unknown.

In healthy leaf material a sharp AG band reveals the induction by heat of the corresponding cyclic pathway. A broadening and/or a downshift of the AG band suggest that this cyclic electron flow is already active at lower temperatures, so that the effect of heat induction is less pronounced. Cyclic electron flow seems to be triggered by stress situations in order to meet an increased demand in ATP (Manuel et al., 1999). Under illumination, the AG band is downshifted in some plant species (see Fig. 3) and it resumes its location at 45 °C within a few hours in the dark, reflecting the activation of a cyclic pathway by light and its progressive inactivation in the dark (M Roman et al., unpublished data). Reduction of plastoquinone in the dark is also part of the chlororespiratory pathway discovered in C. reinhardtii (Bennoun, 1982) and in higher plants (Garab et al., 1989), the physiological role of which is still unknown.

In some particular conditions, an AG emission appears after white light or xenon flash illumination. This occurs when the use of photosynthetic energy (ATP+NADPH) is slowed down by a lack of CO₂ or phosphorus (Mellvig and Tillberg, 1986; Palmqvist et al., 1986). It can be observed in young pea leaves (Miranda and Ducruet, 1995a). In a CAM-inducible species, an AG TL band appears when CAM metabolism is activated, in correlation with the increase in the dihydroacetone-P/phosphoglyceric acid ratio (Krieger et al., 1998), an indicator of the assimilatory potential [NADPH+ATP] (Heber et al., 1986). The AG band induced by two flashes also reflects the chloroplast energetic state in pea leaves (Roman and Ducruet, 2000).

The afterglow is both a time-dependent and a temperature-dependent process, and hence not relevant to the Randall–Wilkins model of pre-existing charge pairs. Indeed, in some cases, no TL simulation could be done. However, a satisfactory fit is generally possible, suggesting that the temperature and not the back-transfer time is the limiting factor in TL afterglow emission (e.g. TL signals in Fig. 2 can be decomposed in the two bands B and AG, which have not been shown for clarity).

In agreement with several authors who reported various stress effects on the far-red induced afterglow observed on luminescence decays (Mellvig and Tillberg, 1986; Schmidt and Senger, 1987), the far-red-induced AG band of TL appears as a sensitive stress indicator (Janda et al., 1999, 2000). Perhaps too sensitive? Indeed, it is modified by external factors such as previous light episodes, morning or evening sampling, sunny or cloudy day, temperature, and age of leaves (Roman, 2002), all factors which leave the fluorescence parameter $F_v/F_m$ unchanged (but may affect the $q_p$ and $q_N$ quenchings). More work is needed to harness the AG emission into a reliable tool for ecophysiology.

**High temperature bands**

When a sample is submitted to stress treatments, strong TL bands can be observed above 50/60 °C without prior illumination, in a temperature range fully destructive for PSII. They constitute the chemiluminescence high-temperature bands or HTL, unrelated to photosynthesis with the exception that dark-excited chlorophylls are luminescence emitters in the red.

Around 1990, scientists at the university of Moscow reported TL bands above 60 °C, with a main band at 130 °C in algae or leaves submitted to oxidative stress (Venediktov et al., 1989; Vavilin et al., 1991; Merzlyak et al., 1992). Independently, a band peaking at 75 °C induced by oxidative treatments was described by Hideg and Vass (1993). A similar band was found in tobacco leaves treated by a fungal elicitor (Stallaert et al., 1995) and in greening barley leaves (Marder et al., 1998), without bands at higher temperatures. Finally, the 130 °C band reappeared when the sample was pressed uncovered in the dry atmosphere of a ‘classical’ TL set-up where liquid nitrogen acted as a drying vapour trap, the intensity of this band being correlated with the content of lipid peroxides (Vavilin and Ducruet, 1998). By contrast, when samples (chloroplast, algae, leaf squares) were kept in water medium up to 100 °C to prevent desiccation, as usually done in TL photosynthetic studies, only the band at about 75 °C could be seen (Fig. 4). The 130 °C band in an uncovered leaf sample was not reduced by flushing the compartment with nitrogen gas, demonstrating that peroxides were not formed during warming, but disappeared when the sample was maintained wet by a window,

![Fig. 4. HTL emission from spinach leaf discs stuck on a 24 mm diameter copper sheet, frozen for 20 h at −20 °C then thawed for 1 h at room temperature. Dry: disc was desiccated under vacuum before TL recording without glass window. Wet: undesiccated disc, TL recording with glass window above sample. Control: unfrozen, dry conditions.](https://academic.oup.com/jxb/article-abstract/54/392/2419/621936)
suggesting that the lipid peroxides were hydrolysed during the heating process before radiative thermolysis could occur (Ducruet and Vavilin, 1999). So, the 75 °C TL band recorded in wet conditions would be a pseudo-band resulting from the competition between radiative thermolysis (rising edge) and heat-enhanced non-radiative hydrolysis (falling edge).

Other bands have been reported in the 60–90 °C temperature range. In barley cultivated in low light, a band near 60 °C was observed without prior illumination (Skotnica et al., 1999). In crucifers such as Arabidopsis thaliana (Havaux and Niyogi, 1999) or rapeseed, a sharp band at 70 °C, also present in unstressed controls, might be tentatively ascribed to the chemiluminescence of compounds specific to crucifers. The addition of both aldehydes and H₂O₂ to chloroplast suspensions produced TL bands near 60/70 °C (Ducruet and Vavilin, 1999).

Practically, the 130 °C can be used as an indicator of oxidative stress (Havaux and Niyogi, 1999). Care should be taken not to cover the sample with a window, and to let it dry during TL warming. Flushing nitrogen gas in the holder during measurements can speed up drying and make sure that no sample oxidation occurs (although this has not been observed in air, except at temperatures above 140 °C). A problem is the wilting or popping up of leaf sample above 100 °C due to dryness. This can be avoided either by a metal grid pressing on the leaf or by sticking the leaf disc on a piece of aluminium or copper adhesive sheet for electronics. In this latter case, storing leaf discs stuck on a thin flat metal sheet in a cool (but not freezing) dry oxygen-free vessel and sending these for measurements could be considered. Another potential application of HTL emission would be the imaging of oxidative stress in whole leaves.

**Perspectives**

TL is clearly a destructive technique although to some degree non-invasive, since it preserves the leaf integrity until the measuring step, somewhat like a biochemical analysis done on a freshly excised sample. Warming a spot of an attached leaf would be feasible using a variable temperature air flow or an infrared heater, but lateral diffusion of daylight through the mesophyll would imply that it works in full darkness. Fresh leaf discs offer a level of integrity even better than intact chloroplasts or protoplasts, and are considered as intact systems for studying photosynthetic metabolism. Ultimately, an outdoor light-tight TL measuring head for punching out a disc from a leaf under sunlight and immediately starting the gradient up to 160 °C is technically feasible: this would open an entirely new field of research.

Luminescence is the informative phenomenon and TL is a particular technique providing a better resolution of emitting charge pairs at the expense of a thermal treatment applied to the sample. Among alternative methods, the so-called ‘delayed light emission’, not to be confused with delayed fluorescence or delayed luminescence (perhaps light-modulated luminescence would be a better term) consists of a pulsed illumination with recording of luminescence induction through the light pulses and of luminescence induction through the dark intervals. This luminescence kinetics reflects the energization of chloroplasts up to a stationary state, which cannot be done in one TL scan. DLE has produced intriguing results such as temperature breakpoints consistent with the thermal adaptation of various plant species or differences in the characteristic freezing temperatures which uncouples thylakoids in wheat varieties of contrasting frost hardiness (Fedoulov, 1998), although the intertwining of excitation and detection makes interpretation more complex (Malkin et al., 1994). DLE deserves to be reappreciated as a tool complementary to TL, with basically the same instrument, using LED pulses for excitation and a modulated shutter or attenuator protecting the PMT during the light pulses.

In conclusion, the progress in instrumentation and in understanding TL signals in vivo allows the use of (thermo)luminescence in plant ecophysiology in order to complement gas exchange, chlorophyll fluorescence, and leaf absorption/reflectance measurements. Several applications to investigate abiotic and even biotic (Rahouti et al., 1999) stress have already been published. The potential of photosynthetic TL and of HTL has now to be assessed within the frame of ecophysiological research.

**Supplementary data**

More information on instrument building and computer programs can be found at *Journal of Experimental Botany* online.

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