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Peculiar properties of chlorophyll thermoluminescence emission of autotrophically or mixotrophically grown *Chlamydomonas reinhardtii*

Jean-Marc Ducruet[1,*], Aurelio Serrano[2], Mercedes Roncel[2], José M. Ortega[2]

[1] Groupe de Biospectroscopie Végétale, Ecophysiologie Végétale, ESE, Bât. 362, Université Paris-Sud-Orsay, 91400 Orsay. France.

[2] Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Avda. Américo Vespucio 49, 41092 Sevilla, Spain.

* Corresponding author. *Tel*: 33 6 87 70 36 72; *fax*: 33 1 69 15 73 53, *E-mail address*: jean-marc.ducruet@u-psud.fr

ABSTRACT

The microalgae *Chlamydomonas reinhardtii* and *Chlorella* sp. CCAP 211/84 were grown autotrophically and mixotrophically and their thermoluminescence emissions were recorded after excitation by 1, 2 or 3 xenon flashes or by continuous far-red light. An oscillation of the B band intensity according to the number of flashes was always observed, with a maximum after 2 flashes, accompanied by a downshift of the B band temperature maximum in mixotrophic compared to autotrophic grown cells, indicative of a dark stable pH gradient. Moreover, new flash-induced bands emerged in mixotrophic *Chlamydomonas* grown cells, at temperatures higher than that of the B band. In contrast to the afterglow band observed in higher plants, in *Chlamydomonas* these bands were not inducible by far-red light, were fully suppressed by 2 μM antimycin A, and peaked at different temperatures depending on the flash

number and growth stage, with higher temperature maxima in cells at a stationary compared to an exponential growth stage. These differences are discussed according to the particular properties of cyclic electron transfer pathways in *Chlamydomonas reinhardtii*.

Keywords

Thermoluminescence

Microalga

Cyclic electron flow

Photosystem II

Chlororespiration

Abbreviations

AG band	afterglow thermoluminescence band
B band	thermoluminescence band due to $S_{2/3}$ Q_B^- recombination
CCCP/FCCP	chloro/fluoro carbonyl cyanide phenylhydrazone
FQR	Ferredoxine-plastoQuinone-Reductase cyclic/Chlororespiratory pathway
NDH	NAD(P)H DeHydrogenase cyclic/Chlororespiratory pathway
TL	Thermoluminescence
Tm	Temperature of the maximum of a TL band; Tm(B) for B band..

1. Introduction

Photosynthesis relies on the conversion of a quantum of light energy into a charge separation within photosystem I (PSI) and photosystem II (PSII) reaction centers, creating charge pairs stabilized on electron carriers by activation energy barriers that limit charge recombination, i.e. the wasteful back reaction of the forward charge separation [1]. In PSII centers, charge recombination, limited to a slow rate at physiological temperatures, proceeds following different pathways [2], one of these leading to the recreation at a low yield of an exciton in the chlorophyll antenna, with a probability to deactivate as fluorescence. This delayed fluorescence emitted in darkness following an illumination is also called PSII luminescence. Thermoluminescence (TL) is a technique to study luminescence emission that consists in illuminating the sample at a temperature sufficiently low to make negligibly small the recombination rate of the charge pairs under investigation, then to reveal them successively as TL bands by a progressive warming. Due to the strong temperature dependency of charge recombination, TL has a greater resolving power than luminescence multiphasic decays recorded at a constant temperature immediately after an illumination. In unstressed dark-adapted photosynthetic material, one or few flashes give rise essentially to a so-called B band, located between 25 and 40 °C depending on species and conditions, which results from a recombination of an electron stored on the secondary quinonic acceptor Q_B and a positive charge stored on the S_2 or S_3 states of the oxygen evolving complex of PSII (OEC).

Analysis of luminescence signals becomes more complex for intact photosynthetic systems, in which a delayed luminescence bounce appears superimposed to the exponential decay phases of the different types of charge pairs initially stabilized on PSII electron carriers [3]. This delayed emission called "afterglow" can be related to a dark electron back-transfer from stroma to the acceptor side of PSII [4]. The afterglow (AG) emission, highly dependent on temperature, can be optimally recorded as a sharp TL band peaking at about 45 °C at a 0.5

°C/s warming rate in plant leaves [5]. Complex luminescence decays at constant temperatures, sometimes exhibiting one or two successive delayed luminescence bursts, have been reported in some algal species [6,7,8]. The kinetics observed were strongly dependent on the growth conditions, e.g. low or high CO_2, phosphate deficiency and ionic composition.

Green microalgae are considered by plant biologists as valuable unicellular model systems for photosynthesis studies. In particular, *Chlamydomonas reinhardtii* (*C. reinhardtii*), a flagellated photosynthetic protist that belongs to the class Chlorophyceae, has been widely used for biochemical, physiological and genetic studies. This cosmopolite microalga is able to growth in many different environments, normally deriving energy from oxygenic photosynthesis. *Chlamydomonas* exhibits a remarkable metabolic flexibility and can also thrive in total darkness with acetate as an alternative carbon source. Its adaptability and quick generation time have made it an important model organism for biological research and studies on this microalga have provided major research contributions in diverse areas of cell and molecular biology, like photosynthesis, phototaxis, cell motility, inorganic nutrient assimilation, abiotic stress, etc. [9]. Moreover, its recently sequenced nuclear genome has advanced the understanding of the ancestral eukaryotic cell of animals and plants [10].

Here we show that TL emission properties in *C. reinhardtii* vary widely depending on growth stage and nutritive medium and are quite different from those observed in *Chlorella* CCAP 211/84, another well known chlorophycean microalga, or in higher plants. Additional TL bands peaking at temperatures above the B band that appear in mixotrophic grown cells, can be ascribed to an “afterglow” mechanism of electron transfer with, however, noticeable discrepancies compared to what is observed in higher plants: namely, occurrence of several bands peaking at variable temperatures depending on culture growth stage, sensitivity to antimycine A, an inhibitor of the Ferredoxine-plastoQuinone-Reductase (FQR) and failure to induce these bands by a far-red illumination.

2. Material and Methods

2.1. Algal strains and growth conditions

The unicellular green algae (Chlorophyceae) *C. reinhardtii* 21gr and *Chlorella* sp. CCAP 211/84 were grown in Sueoka liquid mineral medium (pH 7.1) under photoautotrophic conditions (continuous white light, 100 μmoles m^{-2} s^{-1}) as previously described [11]. When indicated *Chlamydomonas* and *Chlorella* cultures were supplemented with 12 mM sodium acetate or 25 mM glucose, respectively, and maintained under continuous illumination (mixotrophic conditions). Growth of the algal batch cultures was monitored by measuring the chlorophyll cell content after methanol extraction. Exponential growth phase and plateau growth phase cells were collected after two-day and five-days culturing, respectively.

2.2. Thermoluminescence

TL emission was recorded as previously described [5,12,13,14]. Briefly, temperature regulation, signal recording and flash sequences were driven by a computer through a National Instrument DAQ-Pad1200 interface, using dedicated software. Temperature regulation was performed by means of a Marlow thermoelectric "Peltier" element powered by a variable (0 to 5 A) computer-driven power supply. Luminescence emission was detected by a H5701-50 Hamamatsu photomultiplier module. Illumination was performed through a light guide parallel to the photomultiplier, both of them being attached to the same stand sliding horizontally from the illumination to the measuring position. Single turn-over flashes were provided by a xenon white light (Walz XST-103). Far-red was provided by an Epitex LED

emitting at 735 nm with negligibly small intensity below 700 nm. TL signals were analyzed with a dedicated numerical simulation software described in Ducruet [12].

Algal suspensions were maintained in the darkness or under dim green light in Erlenmeyer vessels (about 5 mm layer thickness) that were all shaken horizontally before each TL recording. Samples were then kept at 25 °C 2 minutes in complete darkness in the TL measuring cell, then cooled to 1 °C and submitted to flash or far-red illumination; the TL recording was started immediately.

3. Results

3.1. Variations of TL emission depending on autotrophic or mixotrophic growth conditions (Figure 1)

In dark-adapted autotrophically or mixotrophically grown *Chlorella* cells at stationary phase, single turn-over flash sequences induced a single TL band (Fig. 1) reaching a maximum intensity after 2 flashes and oscillating with a period 4, as classically observed also in plant leaves for the B band [15,16]. The maximal temperature Tm(B) of the B band after 2 or 3 flashes occurred at a lower temperature in mixotrophic compared to autotrophic grown cells.

A similar unique TL band was generally observed in *Chlamydomonas* cells from autotrophic cultures in the exponential growth phase (Fig. 2A). However, when cells from a culture that reached the stationary growth phase were analyzed, 2 or 3 flashes induced a noticeable shoulder at >30 °C that is suppressed by antimycin A, in addition to the B band centered near 25 °C (see Fig.5, Ctrl auto).

(Figure 2)

A clearly different emission pattern was observed in mixotrophically grown cells at a stationary stage (Fig 2B). The B band peaking at 25 °C after 1 flash was downshifted to lower temperatures and broadened after 2 or 3 flashes. In addition, new bands emerged above 30 °C. A band arised at 50 °C after 1 flash (luminescence emitting centers are 100% $S_2Q_B^-$) and another one at about 40 °C overlapping the smaller 50 °C band after 2 flashes (mixture of $S_2Q_B^-$, $S_3Q_B^-$). Interestingly, 3 flashes (mostly $S_3Q_B^-$) induced a band at 32 °C, not at 40 °C as observed after 2 flashes, with a shoulder towards high temperatures suggesting that the 50 °C band observed after 1 flash is still present after 3 flashes, despite the low amount of $S_2Q_B^-$; this was further confirmed by its sensitivity to antimycin A (Fig. 3C). As a consequence the bands observed at 50 °C, 40 °C and 32 °C cannot be simply ascribed to the proportion of S_2 and S_3 states in luminescence-emitting centers. A brief 5 s far-red illumination produced a sharp band at about 25 °C both in autotrophic (Fig. 2A) and mixotrophic (Fig. 2B) grown cells, similar to a B band, without any of the >30 °C non-B bands induced by flashes. It is worth noticing that the Tm of this band is about 3 °C lower in mixotrophic compared to autotrophic grown cells.

The strongly downshifted B band (Fig. 2B) due to an acidic dark-stable lumen pH is also broadened and flattened increasingly with the S_3/S_2 ratio, from 1 to 3 flashes. In these conditions, the apparent B band is not a genuine TL band but becomes an envelop of elementary B bands dependent on varied local pHs [17], making a simulation impossible. Nevertheless, discarding the B band, the main band peaking at 42°C after 2 flashes can be tentatively decomposed into 2 components, including a 48°C component similar to that found after 1 flash. (Fig. 2B, dots). This confirms that analyzing the main band after 2 flashes as the sum of 2 elementary TL band is possible, although this does not exclude a higher number of components that also provide a satisfactory fit.

In mixotrophically grown *Chlamydomonas* at an exponential growth stage (Fig. 2C), the same general pattern for flash-induced TL bands was observed with, however, two differences compared to mixotrophic grown cells at a stationary phase (Fig. 2B):

- the downshift of the B bands (arrows in Fig. 2) was not so pronounced at exponential stage (Fig. 2C) compared to stationary stage (Fig. 2B), the "relaxed" position of the B band being at 25 °C as observed in autotrophic exponential samples (Fig. 2A).
- the 50 °C, 40 °C and 32 °C bands found in mixotrophic grown cells at stationary stage (Fig. 2B) were had their peak temperature Tm dercreased to 44 °C, 33 °C and 25 °C, respectively, in cells at exponential growth stage (Fig. 2C).

The TL signal after 3 flashes was tentatively decomposed into 3 elementary components, including the B band (Fig. 2C, dots). Unexpectedly, the intensity of the highest temperature component at 42°C did not decrease with flash number, as it should if it was due to S_2 centers.

Preillumination during 20 min with blue light (480 nm) did not change significantly the Tm of the 30-35 °C band induced by 2 or 3 flashes (data not shown).

3.2. Effects of inhibitors of photosynthetic cyclic pathways

(Figure 3)

The origins of the non-B flash-induced bands was further investigated by adding antimycin A, a specific inhibitor of the FQR cyclic pathway at sub-micromolar concentrations [18,19]. It should be noticed that antimycin A had a slight inhibitory effect on the B band in autrophic (Fig. 3A) as well as in mixotrophic conditions (Fig. 3B).

In a mixotrophic conditions, at a stationary phase, the band induced by 2 flashes peaking near 40 °C (Fig. 3B, 2F) was sharper in presence of 1% ethanol than that shown in

Fig. 2B without ethanol. This 40 °C band was clearly suppressed by antimycin A with a 0.4 µM half-inhibitory concentration (Fig. 3B). In mixotrophic grown cells at an exponential stage (Fig. 3C), the band at 45 °C after 1 flash was also present as a shoulder after 3 flashes, and was in both cases suppressed by 2 µM antimycin A. This suggests an involvement of the FQR pathway in all the non-B bands induced by flashes in mixotrophically grown *Chlamydomonas*. The main band after 3 flashes appeared unique and was located at about 25 °C (Fig. 2C, 3C), but antimycin A revealed a smaller B band at 18 °C, confirming the 25 °C band was for more than a half of non-B origin and related to FQR pathway.

Myxothiazol and HQNO, other inhibitors of the FQR pathway [19], also suppressed the >30 °C TL bands (data not shown).

3.3. Effect of phosphorylation uncouplers

(Figure 4)

Noticeably, nigericine was active at least in presence 3% ethanol (Fig. 4). Ethanol in the 0.5% to 3% range modified the 32-40 °C band(s) induced by 2 or 3 flashes, with a maximum sharpening and enhancing effect at 1% (Fig. 3B, Fig. 4), followed by a slight downshift at higher ethanol concentrations. This ethanol enhancing effect was less evident when the non-B bands induced by 2 or 3 flashes were peaking at lower temperatures (mixotrophic *Chlamydomonas* cells at exponential growth stage, control shown in Fig. 2C, Fig. 3C). 6 µM nigericine in 3% ethanol caused a fusion of the two bands of control into one single band at an intermediate position, with a Tm upshift of the B band and a downshift of the 40 °C band, as also observed in leaves of higher plants infiltrated by nigericine [5].

(Figure 5)

CCCP, another uncoupler and also an ADRY reagent that impairs the oxygen evolving complex [20], caused a complete suppression of the TL signal in autotrophic cultures, due to

its ADRY activity (Fig. 5). It should be noticed that autotrophic cells in Fig. 5 were at a stationnary stage and thereby exhibited a small TL shoulder at 35 °C, of non-B origin. However, in mixotrophic grown cells at stationary stage, CCCP decreased only by 40% the B band, which slightly upshifted from 12 °C to 15 °C without resuming its fully relaxed position at 25 °C as observed for nigericine; in contrast with the 35 °C band that was almost suppressed at the same concentration. This confirms that uncoupling suppressed the non-B emission while the ADRY effect of CCCP on the B band was alleviated in mixotrophic compared to autotrophic grown cells.

3.4 Effect of phosphate deficiency

(Figure 6)

A hypothetical cause of the enhancement and temperature upshift of non-B bands in mixotrophic conditions at stationary growth stage could be the depletion of some nutriment from the medium during batch culture growth. For example, assuming that phosphate might be the limiting element [6], a depletion of phosphate in a mixotrophic culture at exponential phase should produce a strongly upshifted antimycin-sensitive non-B bands similar to those observed in cells at mixotrophic stationary stage. A result opposite to that expected was obtained after 3 days of phosphate depletion, which suppressed the antimycin-sensitive emission instead of enhancing it (Fig. 6). The lack of phosphate is likely to prevent the building up of the assimilatory potential (NADPH *plus* ATP) in equilibrium with the pool of triose-phosphate, necessary to the back transfer of electrons towards Q_B via the FQR pathway.

4. Discussion

C. reinhardtii grown in mixotrophic conditions exhibits peculiar TL signals very different from those found in higher plant leaves or other microalgae such as *Chlorella*.

In a dark-adapted undamaged photosynthetic material, illumination by 1 or few flashes always produces a so-called B band due to a recombination of positive charges stored on S_2 and S_3 with an electron stabilized as Q_B^- on the secondary quinonic acceptor of PSII. Starting a 25% S_0 / 75% S_1 initial distribution and assuming a partly reduced Q_B (~40% Q_B^-) in an intact organism [16], the luminescence emitting states S_2 and S_3 are theoretically (neglecting the misses and double hits) 75% S_2 after 1 single turn-over flash, 25% S_2 75% S_3 after 2 flashes (the maximum of TL signal), 25% S_3 after 3 flashes, although this may differ *in vivo*. Due to a period 2 oscillation, the Q_B^-/Q_B ratio should be the same after 1 and 3 flashes, so that a higher luminescence yield for the S_3 Q_B^- than the S_2 Q_B^- pair has to be assumed to account for a stronger emission after 3 flashes than after 1 flash [16].

In *Chlorella* (Fig. 1) and more pronouncedly in *Chlamydomonas* (Fig. 2), the lower maximum temperature Tm(B) observed after 2 or 3 flashes in mixotrophic compared to autotrophic grown cells indicates an acidification of lumen pH that makes recombination faster [17,21]. In mixotrophic grown *Chlamydomonas* cells, at stationary stage, the B band was so much downshifted and broadened that is became hardly detectable (Fig. 2B). This larger downshift of the B band after 2 or 3 flashes generating mainly S_3 compared to 1 flash generating S_2 can be explained by a stronger proton uptake from lumen during the $S_3 \rightarrow S_2$ than the $S_2 \rightarrow S_1$ reverse transition [22,23] when the lumen pH decreases. This dark-stable acidic lumen in mixotrophic grown cells can be explained either by a chlororespiratory activity [24,25] or by a stronger respiratory activity producing more ATP that is hydrolyzed by chloroplastic ATP-ases, both activities being able to maintain a dark proton pumping into the lumen.

In addition to the B bands, an afterglow band AG is observed in higher plants after FR preillumination or sometimes after 2 or 3 flashes, peaking at about 45 °C at a 0.5 °C/s warming rate. It corresponds to the fraction of PSII centers in the $S_{2/3}Q_B$ non radiative state immediately after preillumination, in which the arrival of an electron transferred from stroma along cyclic/chlororespiratory pathway(s) produces the $S_{2/3}Q_B^-$ radiative state that emits luminescence [4]. This back electron transfer can be induced by warming above 35 °C that activates the cyclic pathway(s), unless these pathways are already activated prior to TL recording, which leads to a fusion of the AG with the B band as Q_B becomes reduced by stroma electrons before warming. Hence, it should be stressed upon the fact that a strong AG band indicates that the cyclic pathway(s) are constituted but are not activated before TL warming.

A main characteristics of mixotrophically grown *Chlamydomonas* cells is the presence of at least two non-B bands above 30 °C: (i) a 45-50 °C band induced by 1 flash; (ii) a stronger band induced by 2 or 3 flashes, with a higher Tm for 2 flashes (40 °C at plateau stage, 32 °C at exponential stage) than for 3 flashes (32 °C at plateau stage, 25 °C at exponential stage). In autotrophic cells reaching a stationary stage, these non-B bands would correspond only to a small shoulder on the high temperature edge of the B band (Fig. 5, Ctrl auto). Furthermore, far-red light that generally induces a strong AG band near 45 °C in leaves produces a band at 25 °C in *Chlamydomonas* cells, at a position similar to that of the B band in autotrophic conditions. A possible explanation for the absence of a specific effect of far-red in this microalgal species is the lack of far-red absorbance bands in its PSI antenna [26].

Another noticeable feature is that these non-B bands above 30 °C are suppressed by antimycin A (Fig. 3), that specifically inhibits the FQR cyclic pathway at sub-micromolar concentrations [18,19,27]. FQR is generally considered as active under light, not in darkness,

and consistently the AG emission in leaves of *Arabidopsis* is strongly inhibited in NDH-mutants while being almost insensitive to antimycin A. However, the opposite situation occurs in tobacco leaves, in which the AG band is strongly lowered by antimycin [28], as here observed with *Chlamydomonas*. The involvement of the antimycin-sensitive FQR pathway in the TL bands above 30 °C in *Chlamydomonas* supports their identification as afterglow bands, even though their properties are quite different of those observed in leaves. This role of FQR in the AG emission in *Chlamydomonas* is also consistent with the absence of NDH pathway in this microalgal species, although an NDH2 non-electrogenic pathway is present [29,30,31]. The full suppression by antimycin A of non-B (AG) bands seen by TL does not exclude that other pathways may be involved in dark plastoquinone reduction [25] and cyclic electron flow around PSI [32,33] without generating TL emission.

The non-B bands are fully inhibited by the other inhibitors of the FQR pathway myxothiazol and HQNO, which confirms that FQR is involved in these TL emissions.

The uncoupler nigericine suppressed these non-B bands and relaxed the B band to its unshifted position at 25 °C (Fig. 4). The ΔpH causes the downshift of the $S_3Q_B^-$ B band and is also necessary to the AG emission**,** both effects being cancelled by uncoupling. The same effect was observed in pea leaves uncoupled either by nigericine infiltration or by freezing below -5 °C, the nucleation temperature under which ice crystals collapse the proton gradient by disrupting the membrane [5].

FCCP [34] and CCCP induce similar effects, i.e. a suppression of the AG band and a partial, albeit not as complete as observed with nigericine, relaxation of the B band downshift. Interestingly, these ADRY reagents [20] that destroy the oxygen evolving complex, fully suppressed the B band in autotrophic grown cells, but only partly in mixotrophic grown cells. A more acidic lumen pH in the later may tentatively explain the weaker ADRY activity of FCCP and CCCP.

A blue illumination downshifts by about 10 °C the AG band in *Arabidopsis* where NDH is prominent and has little effect in tobacco where FQR is prominent [27]. Consistently with the FQR pathway in *Chlamydomonas* demonstrated by antimycin A inhibition of AG emission, 20 min blue illumination has almost no effect on the Tm of the AG band.

In mixotrophically grown *Chlamydomonas* cells, acetate provides additional NADPH in the stroma through the respiratory pathway [33], thus stimulates the cyclic versus linear electron transfer to maintain the ATP/NADPH ratio required by the Benson-Calvin cycle, thereby increasing the capacity of cyclic pathway(s). However, that does not explain why the AG-like bands produced by 2 or 3 flashes peak at variable temperatures, from 32 ºC to 40 °C after 2 flashes, from 23 °C to 32 °C after 3 flashes, depending on the growth stage of the culture, with their Tm increasing from the exponential phase to the stationary phase cells. A possible explanation would be the strong absorption of some nutriments during culture growth, resulting in a depletion of the medium when reaching the stationary phase. An experiment of phosphate starvation (Fig. 6) resulted in a decrease of the non-B antimycin-sensitive bands instead of an expected increase suggested by delayed luminescence bounces induced by phosphate starvation in *Scenedesmus obtusiusculus* [6]. However the depletion of other nutrients has also to be considered in *Chlamydomonas*, for example nitrogen deficiency [35], or iron deficiency that in this microalga increases RNA and protein levels of PGRL1, a component of the FQR pathway required for efficient cyclic electron flow under this stressing condition [36].

5. Conclusion

TL after various flash numbers of white light or far-red continuous illumination reveals a complex emission pattern in mixotrophic *C. reinhartii* grown cells exhibiting new

bands with properties of afterglow (AG) emission, i.e. electron transfer from stroma reductants to Q_B via the cyclic FQR. Such a distorted TL emission exhibiting a strongly downshifted B band and an increased intensity of AG band (Fig.2B) can also occur in higher plants, for example in *Arabidopsis thaliana* submitted to drought stress [37] These bands, however, display peculiar characteristics in *C. reinhardtii*, clearly different from those of the AG band observed in higher plants and more pronounced in stationary phase than in exponential growth phase cells.

Further work is needed to relate these special properties of *Chlamydomonas* TL emission to those of the cyclic/chlororespiratory pathways, for example the lack of NDH pathway joint to the existence of a non-electrogenic NDH2. Their strong dependence on the medium trophic conditions and culture growth stage suggests the importance of some nutriments, the depletion of which would favor the role of cyclic pathways. Therefore, TL can bring useful information for probing the energetic metabolism of *C. reinhardtii*, a microalga widely used both as a model photosynthetic organism and as a biotechnological source of hydrogen.

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Legends

Fig. 1 - Thermoluminescence emission curves of *Chlorella* sp. CCAP 211/84 cells grown autotrophically (line) or mixotrophically (dots) at the stationary phase. Cells were excited by 1, 2, or 3 flashes at 1 °C.

Fig. 2 - Thermoluminescence emission curves of *C. reinhardtii cells.* **A**: autotrophic growth, exponential phase. **B**: mixotrophic growth, stationary phase. **C**: mixotrophic growth, exponential phase. Excitation by 1, 2, 3 flashes (lines) or 5 s far red-light (dashes). The arrows point at the maximum of the B band. Symbol ? indicates the estimated position of B band. Simulated signal for 2 flashes in thicks dots, components in thin dots (B: 2 components, excluding B band. C: 3 components, including B band).

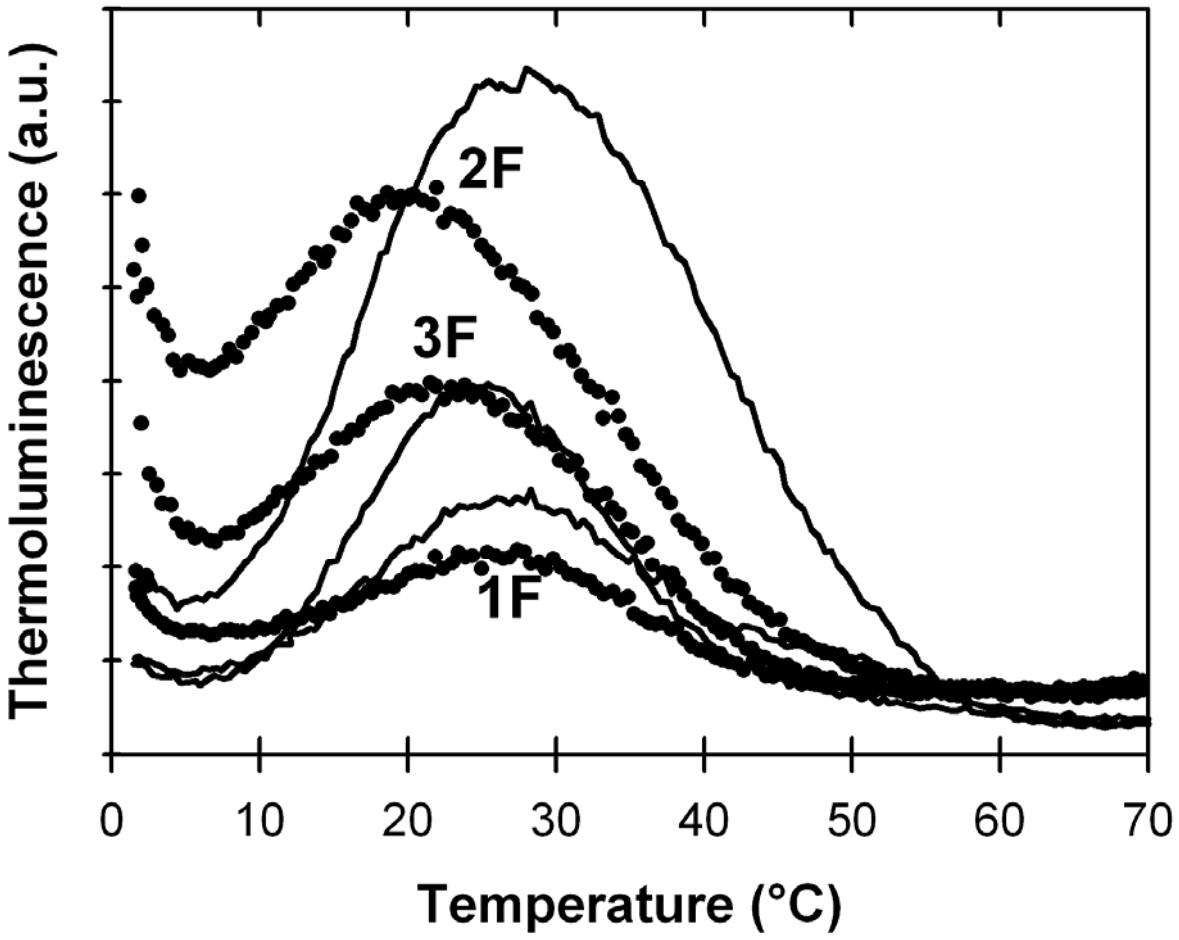
Fig. 3 - Effect of antimycin A on thermoluminescence of *C. reinhardtii* cells. **A**: autotrophic growth, after 3 flashes in samples supplemented with 1% ethanol (line, Ctrl); after 3 flashes without ethanol (dots) and with 2 μM antimycin A and 1% ethanol (dashed). **B**: mixotrophic growth, stationary phase, after 2 flashes in samples with 1% ethanol (2F), 0.4 μM, 1 μM or 2 μM antimycin A and 1% ethanol. **C:** mixotrophic growth, exponential phase, after 1 or 3 flashes in samples with 1% ethanol (lines) or 2 μM antimycin A and 1% ethanol (dashed and dot lines, respectively).

Fig. 4 - Effect of nigericine on thermoluminescence of *C. reinhardtii*. Cells grown mixotrophically at stationary phase. After 2 flashes without addition (thin line), with 1% ethanol (dots), 3% ethanol (thick line, Ctrl) or 3% ethanol and 6 μM nigericine (dashed line, Nig.).

Fig. 5 - Effect of CCCP on the thermoluminescence of *C. reinhardtii*. Autotrophically (thin and dashed lines, auto) and mixotrophically (thick and dashed lines, mixo) cells grown, at stationary stage. Where indicated 1 μM CCCP was added.

Fig. 6 - Effect of phosphate deficiency on thermoluminescence of *C. reinhardtii.* Cells growth mixotrophically, at exponential phase. Previous to TL experiments cells were incubated three days with (**A**) and without phosphate (**B**). For the experiment without phosphate, cells were grown in Sueoka liquid mineral medium in which phosphate salts were substituted by potassium nitrate (see Materials and Methods). TL emission curves were recorded after 3 flashes. Control without addition (dots); with 1% ethanol (lines); with 1% ethanol and 2 μM antimycin A (dashed lines).

Fig. 1


Thermoluminescence (a.u.)
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3F
1F
0
10
20
30
40
50
60
70
Temperature (°C)

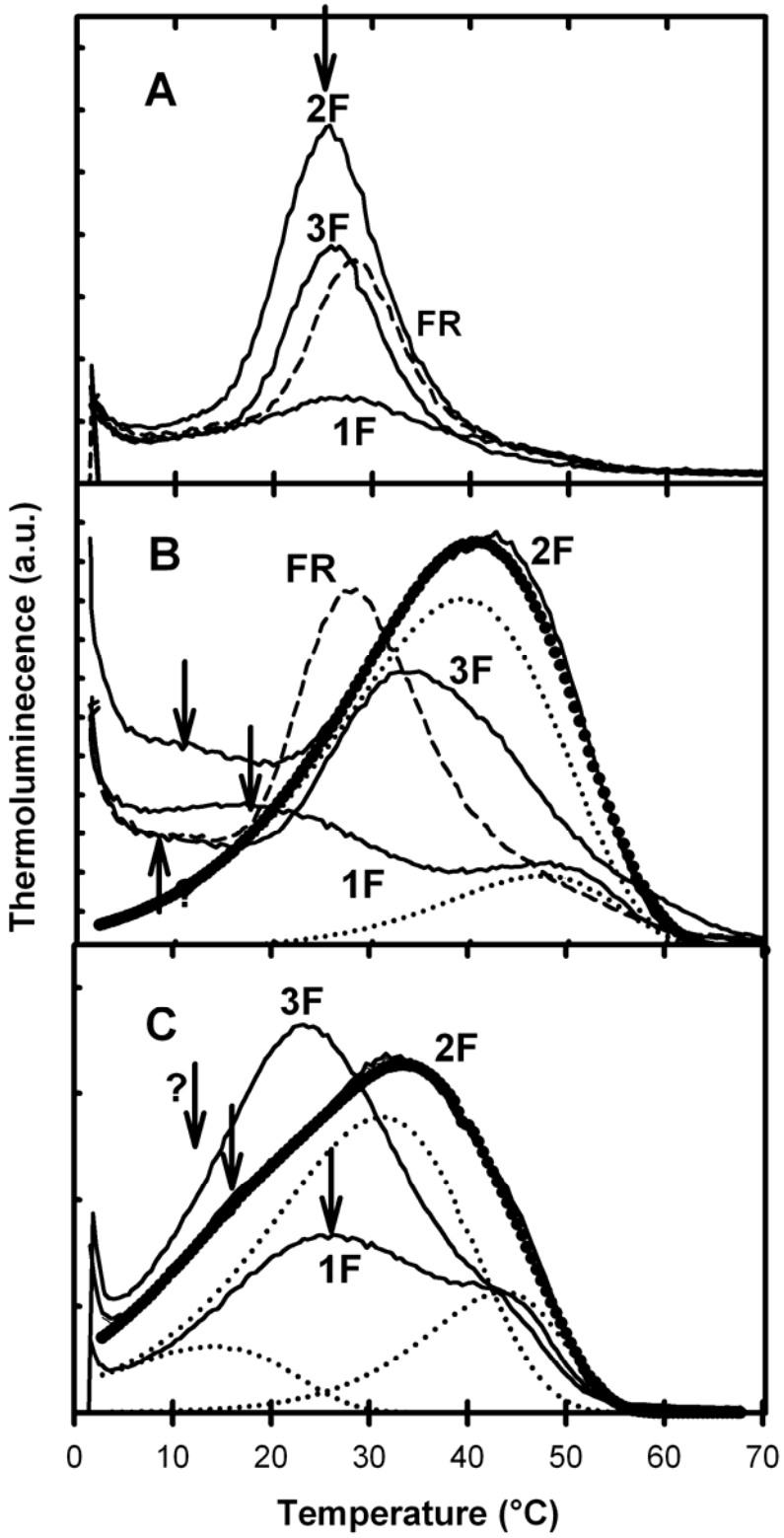

A
2F
3F
FR
1F
B
FR
2F
3F
1F
C
3F
2F
?
1F
Thermoluminecence (a.u.)
0
10
20
30
40
50
60
70
Temperature (°C)

Fig. 2

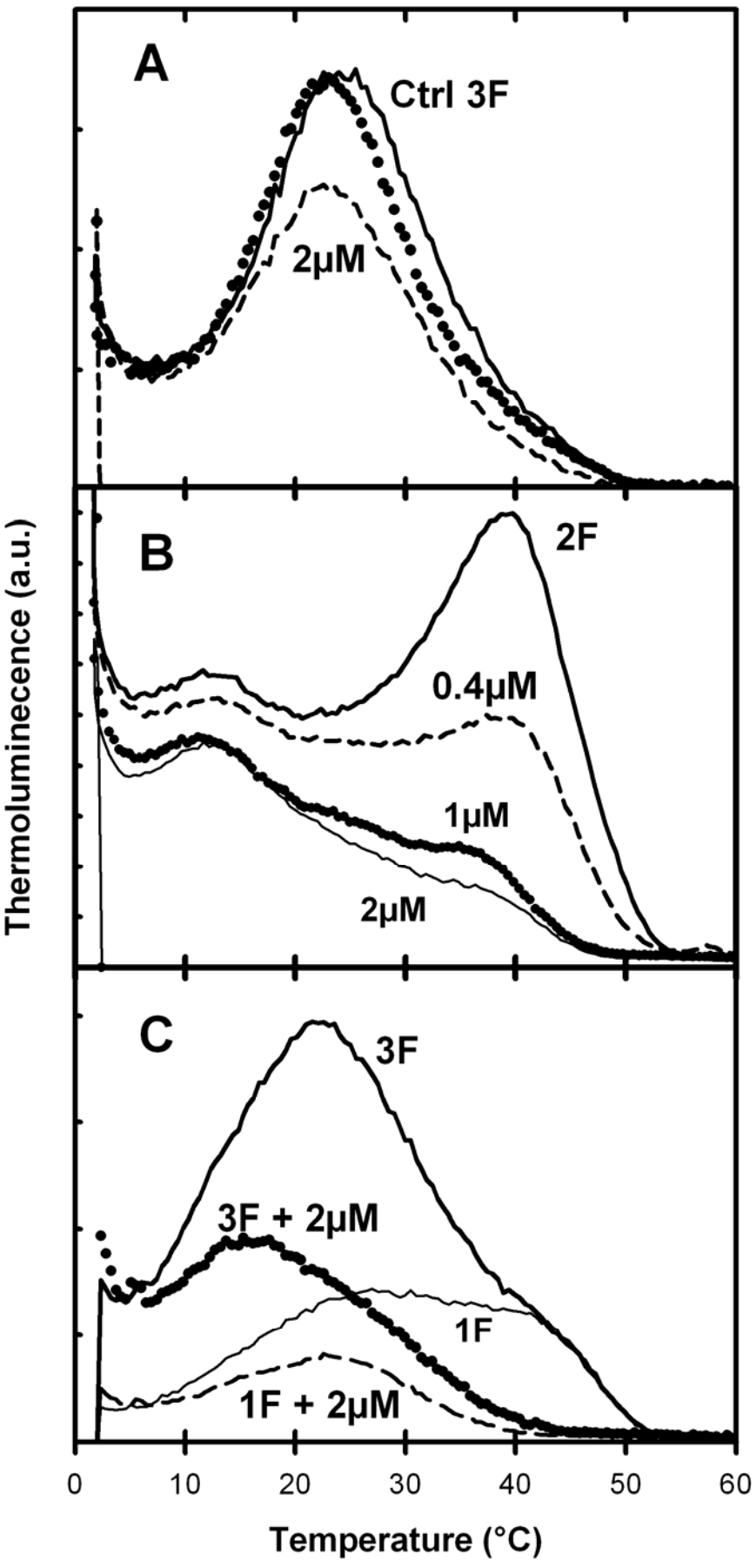

A
Ctrl 3F
2μM
B
2F
0.4μM
1μM
2μM
C
3F
3F + 2μM
1F
1F + 2μM
Thermoluminecence (a.u.)
0
10
20
30
40
50
60
Temperature (°C)

Fig.3

Fig.4

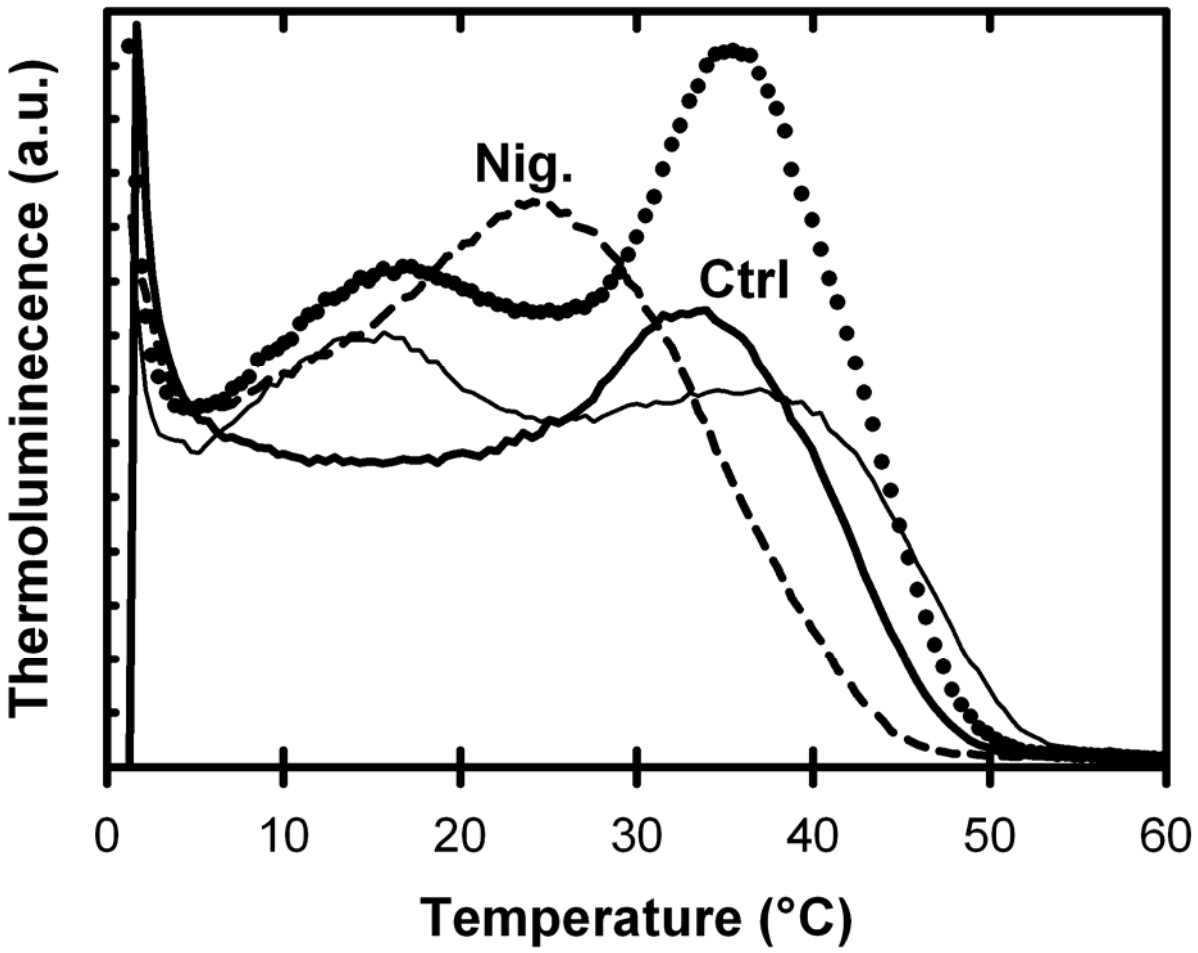

Thermoluminecence (a.u.)
Nig.
Ctrl
0
10
20
30
40
50
60
Temperature (°C)

Fig. 5

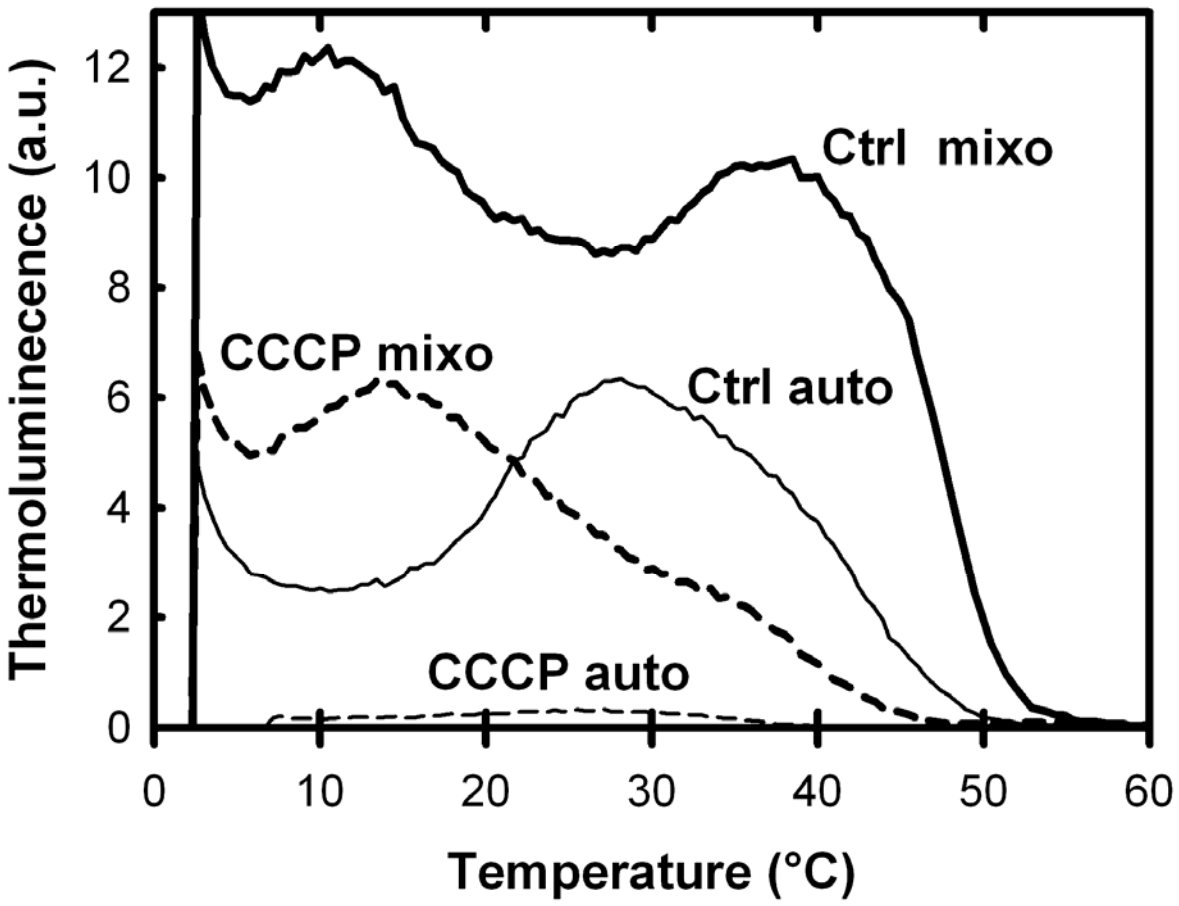
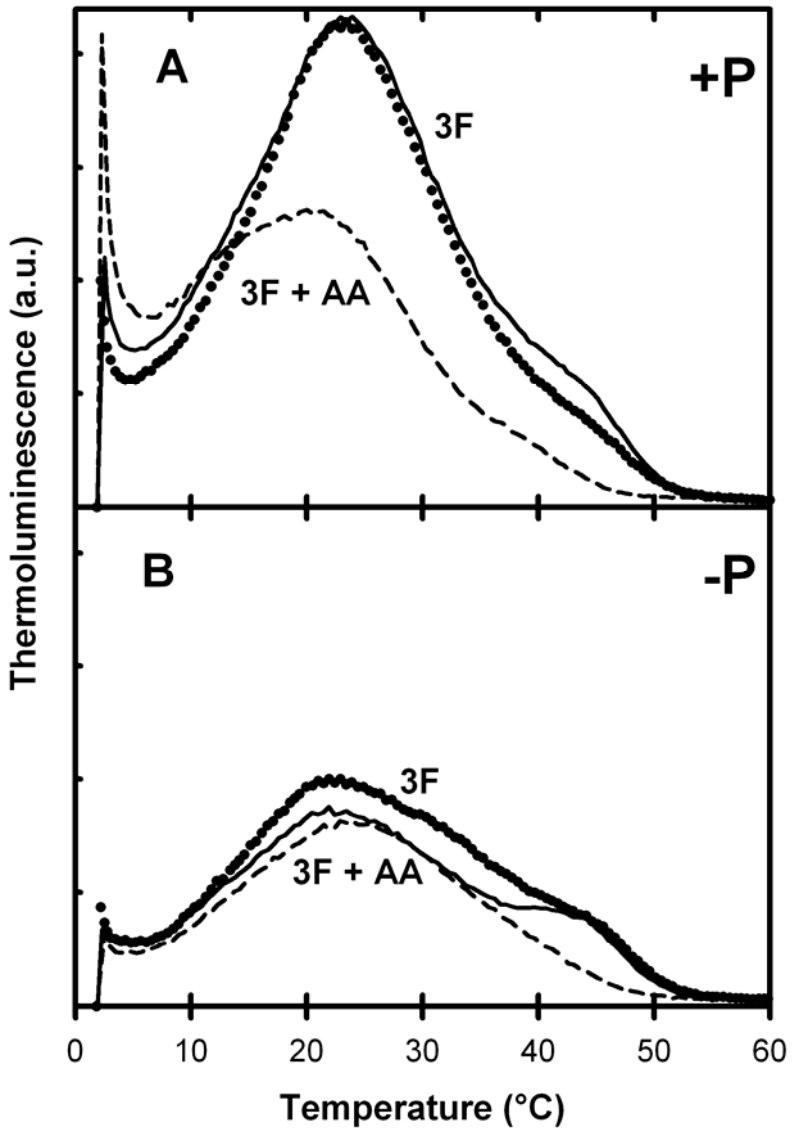

Thermoluminecence (a.u.)
12
10
8
6
4
2
0
0
10
20
30
40
50
60
Temperature (°C)
Ctrl mixo
CCCP mixo
Ctrl auto
CCCP auto

Fig.6


A
+P
3F
3F + AA
B
-P
3F
3F + AA
Thermoluminescence (a.u.)
0
10
20
30
40
50
60
Temperature (°C)