

Decrease of fluorescence intensity after the K step in chlorophyll *a* fluorescence induction is suppressed by electron acceptors and donors to photosystem 2

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Abstract

Chlorophyll *a* fluorescence induction measured by a fluorometer with a high temperature stressed plant material shows a new K step which is a clear peak due to fast fluorescence rise and subsequent decrease of fluorescence intensity. We focused on an explanation of the decrease of fluorescence after the K step using artificial electron acceptors and donors to photosystem 2 (PS2). Addition of the artificial electron acceptors or donors suppressed the decrease of fluorescence after the K step. We suggest that the decrease mainly reflects (by more than 81 %) an energy loss process in the reaction centre of PS2 which is most probably a nonradiative charge recombination between $P680^+$ (oxidised primary electron donor in PS2) and a negative charge stored on either $Pheo^-$ or Q_A^- (reduced primary electron acceptor of PS2 and reduced primary quinone electron acceptor of PS2, respectively). We suggest that the energy loss process is only possible when the inhibition of both the donor and the acceptor sides of PS2 occurs.

Additional key words: charge recombination; fluorescence quenching; high temperature stress; *Pisum sativum*.

Introduction

In 1991-2, results of measurements of fluorescence induction using fluorometer PEA (Plant Efficiency Analyser, Hansatech, Norfolk, England) were introduced by

Received 19 April 1999, accepted 16 August 1999.

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Abbreviations: catechol - 1,2-benzenediol; DPC - 1,5-diphenylcarbazine; DQ - tetramethyl-*p*-benzoquinone (duroquinone); FI - fluorescence induction; HNQ - 2-hydroxy-1,4-naphthoquinone; OEC - oxygen evolving complex; P680 - the primary electron donor in photosystem 2; PEA - Plant Efficiency Analyser; Pheo - primary electron acceptor of photosystem 2 (pheophytin); PpBQ - phenyl-*p*-benzoquinone; PS - photosystem; Q_A - primary (bound) quinone electron acceptor of PS2; Q_B - secondary (mobile) quinone electron acceptor of PS2.

Acknowledgement: This work was supported by grant of Ministry of Education of Czech Republic number CEZ: J14/98: N70000010.

Strasser and Govindjee (1991, 1992). Using red LED diodes for excitation, PEA allows measurements of fluorescence induction (FI) from 10 μ s after the onset of excitation radiation which is characterised by the O-J-I-P pattern being clearly discriminated only when a logarithm time-axis is used (Strasser and Govindjee 1991, 1992, Strasser *et al.* 1995, see Fig. 1).

As fluorescence is mainly emitted by PS2 at room temperature (see Krause and Weis 1991, Dau 1994), the O-J-I-P pattern of FI also reflects the state of PS2. The O step of FI corresponds to the minimal fluorescence F_0 (also called dead fluorescence) which is obtained when all functional reaction centres of PS2 are open (Krause and Weis 1991). There is an experimental and theoretical evidence that the J step is the photochemical phase (Delosme 1967, Neubauer and Schreiber 1987, Strasser *et al.* 1995) connected with primary photochemistry, *i.e.*, reduction of the primary electron acceptor in PS2, pheophytin (Pheo), and Q_A (the primary quinone electron acceptor in PS2) by charge separation and charge stabilisation, respectively (Stirbet *et al.* 1995, 1998, Lazár *et al.* 1997a, 1998). On the other hand, the J-I-P rise is the thermal phase (Delosme 1967, Neubauer and Schreiber 1987), reflecting subsequent accumulation of single or double reduced Q_B (the secondary quinone electron acceptor in PS2) (Stirbet *et al.* 1995, 1998, Strasser *et al.* 1995, Lazár *et al.* 1997a) but also a heterogeneity in reduction of plastoquinone pool (Strasser *et al.* 1995, Barthélemy *et al.* 1997). As there is an equivalence of the J step with I_1 step and the I step with I_2 step (Strasser *et al.* 1995), the I_1 and I_2 being intermediate steps of FI curve measured under extreme high irradiance (Neubauer and Schreiber 1987), the J to I rise is affected by the state of the oxygen evolving complex (OEC) as was found for the I_1 to I_2 rise by Schreiber and Neubauer (1987). The I step bears on structure of the light-harvesting complexes (Barthélemy *et al.* 1997). Sometimes, there is a local fluorescence minimum (dip), labelled as D, between the I and P steps. Mathematical simulation suggests that D reflects a dynamic equilibrium between PS2 and photosystem 1 (PS1) (Stirbet *et al.* 1995, 1998) which agrees with the same interpretation of a dip measured under low irradiance (Munday and Govindjee 1969, Schreiber *et al.* 1972). For the latest review on FI see Lazár (1999).

After heating plant material to about 47 °C (for 5 min or by linear heating to this temperature), a new step, K, at about 300 μ s appears in FI (Guissé *et al.* 1995a,b, Lazár and Ilík 1997, Lazár *et al.* 1997b, Srivastava *et al.* 1997, Strasser 1997). The appearance of the K step may be caused by an inhibition of OEC (Guissé *et al.* 1995a,b, Srivastava *et al.* 1997, Strasser 1997, Lazár and Pospíšil 1999) and by an inhibition of electron transport from Pheo to Q_A (Guissé *et al.* 1995b). The appearance of the K step may also reflect changes in the structure of the light-harvesting complex of PS2 (Srivastava *et al.* 1997). The assumption that at the time at which the K step appears in FI an accumulation of Q_A^- occurs, is a basis for suggesting that a decrease of fluorescence intensity after the K step reflects an oxidation of Q_A^- via subsequent electron acceptors of PS2 (Srivastava *et al.* 1997, Strasser 1997). All results indicate that the appearance of the K step reflects an irreversible high-temperature induced change of PS2 (Guissé *et al.* 1995b, Srivastava *et al.* 1997, Lazár and Ilík 1997, Lazár *et al.* 1997b).

In this work, we focus on the decrease of fluorescence after the K step. We found that the decrease is suppressed by the addition of artificial electron acceptors and donors to PS2 and we suggest that the decrease mainly reflects a nonradiative charge recombination between $P680^+$ and a negative charge stored on either $Pheo^-$ or Q_A^- .

Materials and methods

All measurements were done with primary leaves of two weeks old pea (*Pisum sativum*). The plants were grown at 25 °C in a growth chamber (85 % humidity) on artificial soil composed of perlite and supplied with Knop solution. The light/dark regime was 16 h light/8 h dark with continuous "white" irradiation of 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of Philips SL-Prismatic bulbs (25 W). The concentration of chlorophyll (*a+b*) in the leaves was determined according to Lichtenthaler (1987) to be about 140 mg m^{-2} .

As artificial PS2 electron acceptors we used phenyl-*p*-benzoquinone (PpBQ) and tetramethyl-*p*-benzoquinone (DQ) which accept electrons next to Q_A^- (Henrysson and Sundby 1990) and 2-hydroxy-1,4-naphthoquinone (HNQ) which accepts electrons from $Pheo^-$ (Renger and Kaye 1987, Nedbal *et al.* 1992). As artificial electron donors to PS2 we used 1,5-diphenylcarbazine (DPC) and 1,2-benzenediol (catechol - Yamashita and Butler 1968, Vernon and Shaw 1969). With the exception of HNQ which was from Lancaster (Mühlheim, Germany) all other chemicals were obtained from Sigma (Deisenhofen, Germany). The chemicals were dissolved in ethanol and then added to distilled water in a way that the ethanol formed only 1/100 of final solution volume of particular concentration. The low concentration of ethanol was chosen to avoid possible inhibition of electron transport by ethanol (Masamoto and Nishimura 1978). A concentration of a chemical which is sufficient enough to provoke an expected change in the course of FI was checked by measurements of FI with a high temperature stressed plant material upon different concentration (100, 200, 500, and 1000 μM) of the chemical (not shown). In this way we found for all artificial electron acceptors that the course of FI did not differ when the concentration was 200 μM and higher, and 500 μM and higher for the artificial electron donors. Thus we used the concentration of 200 μM for all electron acceptors and 500 μM for all electron donors.

The measurements of control FI were done at 25 °C after 30 min of adaptation of the leaves to darkness. When FI with the K step without any chemicals was measured, the leaves were kept for 30 min in darkness and then immersed for 5 min in a distilled water bath of 47 °C (45 °C, 49 °C) in darkness. While measuring fluorescence induction with the artificial chemical, the leaves were immersed into the solution of the chemical and kept there for 40 min in darkness before an incubation for 5 min in darkness at 47 °C. Immediately (couple of seconds) after pulling out the leaf from the water bath, FI was measured by Plant Efficiency Analyser (PEA, Hansatech, Norfolk, England). The irradiance was about 3 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of red radiation with the maximum at wavelength of 650 nm. All particular kinds of fluorescence induction curves (room temperature, with K step, with particular artificial electron acceptor or donor) were measured five times and a typical curve

was chosen for presentation. The irradiance was measured by a quantum radiometer LI-189 (LI-COR, Lincoln, USA).

Results

A typical FI measured by PEA fluorometer with the control (25 °C) and high temperature stressed (47 °C, 5 min) pea leaves (Fig. 1) shows the O, K, J, I, and P steps. If draining of electrons from Q_A^- via subsequent electron acceptors of PS2 is only responsible for the decrease of fluorescence after the K step as suggested by Srivastava *et al.* (1997) and Strasser (1997) then, according to their idea, addition of an artificial electron acceptor to the acceptor side of PS2 should cause a more

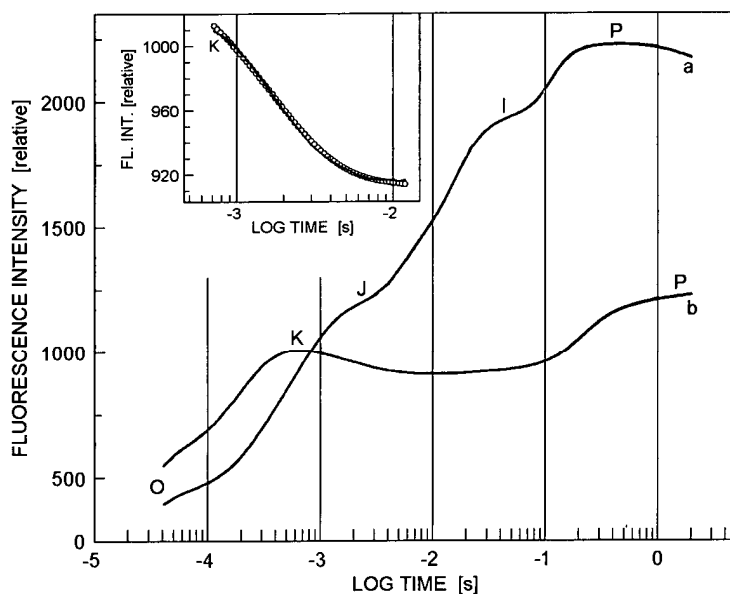


Fig. 1. A time course of FI measured by a fluorometer, labelled as PEA with the control (25 °C, curve *a*) and high-temperature stressed (47 °C for 5 min, curve *b*) pea leaves. Particular O, K, J, I, and P steps are labelled. The result of fitting (open circles) of the decrease of the experimental fluorescence intensity after the K step (full line) by the two-exponential decay is shown in the inset of Fig. 1.

pronounced decrease of fluorescence after the K step. Another effect of the addition of artificial electron acceptor should be a decrease of the amplitude of the K step due to this additional path for electron transport. However, we did not find any of these effects as can be seen in Fig. 2A which shows an action of the artificial PS2 electron acceptors PpBQ, DQ, and HNQ on FI measured at 47 °C. There was an increase of the minimal fluorescence F_0 by about 25 % and only a very small decrease of fluorescence after the K step. The fluorescence intensity kept an almost constant value. These results show that the decrease of fluorescence after the K step need not be caused only by draining of electrons from Q_A^- via subsequent electron acceptors

of PS2 as it has been suggested by Srivastava *et al.* (1997) and Strasser (1997). But because there was an increase of fluorescence to the P step (Fig. 2, curves *a*) indicating that there still exists the electron transport behind Q_A (see Introduction), the suggestion of Srivastava *et al.* (1997) and Strasser (1997) can not be completely ruled out. Similar results as with the artificial electron acceptors of PS2 were obtained with the artificial electron donors to $P680^+$, DPC and catechol, whose action on FI measured at 47 °C is shown in Fig. 2B.

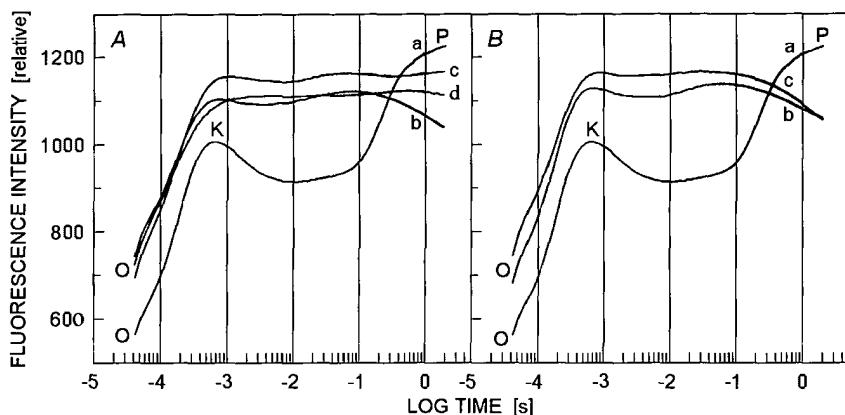


Fig. 2. A time course of FI measured by PEA fluorometer with pea leaves incubated for 5 min at 47 °C in darkness (curves *a*), and with pea leaves which were before the high temperature incubation (A) immersed for 40 min in darkness in 200 μ M solution of the artificial electron acceptor of PS2, PpBQ (curve *b*), DQ (curve *c*), and HNQ (curve *d*), or (B) immersed for 40 min in darkness in 500 μ M solution of the artificial electron donor to $P680^+$, DPC (curve *b*), and catechol (curve *c*). The O, K, and P steps are labelled.

One of the most general explanations of the non-zero value of F_0 is that it is a consequence of the transfer equilibrium of excitons in PS2 antenna and reaction centre of PS2 (Owens 1996) as derived by Laible *et al.* (1994). Hence the addition of the artificial electron acceptors and donors to PS2 can somehow change the transfer equilibrium leading to the increase of F_0 .

Considering the suggestion by Srivastava *et al.* (1997) and Strasser (1997) that the decrease of fluorescence after the K step is caused by the draining of electrons from Q_A^- via subsequent electron acceptors of PS2 and our Fig. 2, there are probably two processes responsible for the decrease of fluorescence after the K step in FI. Thus, the decrease should be well fitted by a two-exponential decay, the first decay representing an overall rate of a process(es) which is suppressed by the addition of artificial electron acceptors and donors to PS2 (Fig. 2) and the second decay representing an overall rate of electron transport from Q_A^- to the subsequent electron acceptors of PS2. Results of such fitting performed for the decrease of the fluorescence intensity after the K step measured for 47 °C (see inset in Fig. 1) and also for other temperatures where the K step still appears as a clear peak (45 or 49 °C) are presented in Table 1. The best fit of the decrease of fluorescence after the K

step was obtained by the two-exponential decay for 45 and 47 °C, whereas by only one-exponential decay for 49 °C. Even if FIs with the K step were measured five times for each temperature (45, 47, and 49 °C), the absolute values of fluorescence intensity and the shapes of FI curves at a position of the P step were different at given high temperature (not shown), indicating that different inhibition of the acceptor side of PS2 occurs in different sample. Hence, we propose that this different inhibition of the acceptor side of PS2 (inhibition of electron transport from Q_A to Q_B) is expressed by the process described by τ_2 because of a high scatter found for τ_2 . Also a decrease of the mean percentage amplitude A_2 with increasing temperature found for the process characterised by τ_2 (Table 1) and an increased value of τ_2 with increasing temperature indicate that the process describes an electron transport from Q_A^- to subsequent electron acceptors of PS2 which is more inhibited by increasing temperature (Havaux 1993). On the other hand, the time constant τ_1^* must somehow reflect a process which is suppressed by the action of artificial electron acceptors and donors to PS2 (Fig. 2). A value of the mean percentage amplitude of this process, A_1 , and its rise with increasing temperature (Table 1) indicates that the decrease of fluorescence after the K step mainly reflects this process (by more than 81 %) and that its contribution to the decrease of fluorescence after the K step increases with increasing temperature.

Discussion

As already mentioned in the Introduction, there is sometimes a decrease of fluorescence to a local minimum (D) in FI measured without high-temperature treatment which may reflect a dynamic equilibrium between PS2 and PS1. Thus, according to the assumptions used for the successful simulation of D (Stirbet *et al.* 1998), a faster oxidation of the reduced PS2 electron acceptors by PS1 is necessary. In other words, the oxidation of Q_A^- by subsequent electron acceptors in some conditions results in the dip D. However, the same process, *i.e.*, the oxidation of Q_A^- by the subsequent electron acceptors may cause the decrease of fluorescence after the K step (Srivastava *et al.* 1997, Strasser 1997). But it implies from Table 1 that the oxidation of Q_A^- by the subsequent electron acceptors contributes by maximally 19 % (for 45 °C) to the overall decrease of fluorescence after the K step. This small extent of a contribution of the Q_A^- oxidation to the overall decrease of fluorescence after the K step seems to be a reasonable estimate because a rather strong inhibition of the electron transport from Q_A^- to Q_B occurs at the temperature when the K step appears (Ducruet and Lemoine 1985, Bukhov *et al.* 1990, Cao and Govindjee 1990, Havaux 1993, Goltsev *et al.* 1994). Thus, we conclude that the origin of the decrease

*The value of τ_1 is not the real (intrinsic) time constant of the process. The intrinsic time constant of the process could be obtained by a mathematical deconvolution of the decrease of fluorescence after the K step using an appropriate model describing all reactions occurring in the reaction centre of PS2 at high temperatures.

of fluorescence after the K step in only the oxidation of Q_A^- by the subsequent electron acceptors as suggested by Srivastava *et al.* (1997) and Strasser (1997), *i.e.*, in a dynamic equilibrium between PS2 and PS1, is improbable.

Table 1. Results of the best fit of the decrease of fluorescence intensity after the K step by exponential decay(s) in relation to temperature of the measurement. A_1 and A_2 are the values of average percentage amplitudes for the first and second exponential decay, respectively, whereas τ_1 and τ_2 are the average time constants for the first and second exponential decay, respectively. The average values of A_i and τ_i and SD (standard deviation) were computed from five measurements at given temperature.

Temperature [°C]	A_1 [%]	$\tau_1 \pm \text{SD}$ [ms]	A_2 [%]	$\tau_2 \pm \text{SD}$ [ms]
45	81	1.54 ± 0.43	19	19.3 ± 18.1
47	84	1.64 ± 0.27	16	41.9 ± 24.7
49	100	1.41 ± 0.22	-	-

In addition to the inhibition of the acceptor side of PS2 mentioned above, there is an inhibition of OEC (Cramer *et al.* 1981, Thompson *et al.* 1986, Havaux 1993) at the temperature when the K step appears, both leading to an accumulation of $P680^+$, $Pheo^-$, and Q_A^- . But note, that the accumulation of these forms occurs after an absorption of minimally two photons by PS2. The first photon results in a formation of $P680^+$ and Q_A^- , $P680^+$ being immediately reduced by an electron stored on the secondary electron donor in PS2, Y_Z (tyrosine 161). $P680$ then absorbs the second photon leading to the second charge separation resulted in a formation of $P680^+$, $Pheo^-$, and Q_A^- . If OEC is absolutely destroyed by high temperature, it cannot donate electron to Y_Z which in turn cannot reduce $P680^+$ now. However, if OEC is not absolutely destroyed by the high temperature, another photon(s) can be utilised for photochemistry. As these reactions result in a closure of the reaction centre of PS2, there is an initial increase of fluorescence from the O to the K step in FI. At the position of the K step, there should be a maximal accumulation of $P680^+$, $Pheo^-$, and Q_A^- . That is why one can suggest that the decrease of fluorescence after the K step is somehow driven by the accumulation of $P680^+$, $Pheo^-$, and Q_A^- .

$P680^+$ is a quencher of fluorescence (Butler 1972, Mauzerall 1972, Sonneveld *et al.* 1979, Deprez *et al.* 1983, Shinkarev and Govindjee 1993). In the case of action of the artificial electron acceptors which oxidise $Pheo^-$ and/or Q_A^- , due to a malfunction of OEC only $P680^+$ accumulates. If the quenching of fluorescence by $P680^+$ molecule itself was the case of the decrease of fluorescence after the K step, then identical FI curves should be obtained without and with the artificial electron acceptors because the quenching of fluorescence by $P680^+$ molecule itself should not be affected by the state of the acceptor side of PS2. But we have not observed such behaviour (Fig. 2A). Thus, we conclude that the quenching of fluorescence by $P680^+$ molecule itself is probably not the origin of the process which is suppressed by the addition of artificial electron acceptors and donors to PS2, *i.e.*, the quenching of fluorescence by $P680^+$ molecule itself probably does not cause the main process

responsible for the decrease of fluorescence after the K step in FI as presented in Table 1. A possibility that $P680^+$ molecule can quench fluorescence signal during FI has also been excluded by Lavergne and Trissl (1995).

Inhibition of the donor side of PS2, leading to the accumulation of $P680^+$, is accompanied by a stimulation of a 50- μ s recombination fluorescence (Schreiber and Neubauer 1989, 1990) which induces a correlated increase of fluorescence quenching (both photochemical and nonphotochemical - Schreiber and Neubauer 1989). The 50- μ s recombination fluorescence has even its maximum at 46 °C (Schreiber and Neubauer 1990) which is very near the temperature of the K step appearance in our case. Thus, it is highly probable that some kind of a charge recombination process involving $P680^+$ is responsible for the decrease of fluorescence after the K step.

As mentioned above, in addition to the inhibition of the donor side of PS2, there is an inhibition of the electron transport from Q_A^- to Q_B at high temperatures leading to the accumulation of $P680^+$ and Q_A^- . Thus, one can suggest that a recombination between these two forms can occur. This agrees to results of Johnson *et al.* (1995) who have suggested that a direct charge recombination between $P680^+$ and Q_A^- is favoured rather than the recombination involving Pheo $^-$ in the case of PS2 with inactivated donor side. On the other hand, Briantais *et al.* (1996) found an increase of the rate constant of the charge recombination between $P680^+$ and Pheo $^-$ with increasing temperature. Furthermore, as both the electron acceptor accepting electrons from Pheo $^-$ (HNQ) and the electron acceptors draining electrons from Q_A^- (PpBQ, DQ), used in our experiments, induce a transfer of $P680^+Pheo(-)Q_A(-)$ to $P680^+PheoQ_A$, it is not possible to exactly discriminate on the basis of our acceptor experiments if a carrier of negative charge for the charge recombination is Pheo $^-$ or Q_A^- .

No matter if $P680^+$ recombines with Pheo $^-$ or Q_A^- , the recombination results either in a direct formation of P680 or in a formation of $P680$ via $^3P680^*$ (see, *e.g.*, Pospíšil 1997). The former case occurs by a nonradiative recombination where the excess energy is changed to heat. On the other hand, $^3P680^*$ can also be nonradiatively quenched to P680 by triplet oxygen or singlet β -carotene (see Pospíšil 1997 for review). The formation of P680 enables a utilisation of radiant energy for photochemistry and not for fluorescence emission thus causing the decrease of fluorescence.

In conclusion, we think that the decrease of fluorescence after the K step does not mainly result from the oxidation of Q_A^- by the subsequent electron acceptors as suggested by Srivastava *et al.* (1997) and Strasser (1997). We also exclude the possibility that the decrease of fluorescence after the K step is caused by the quenching of fluorescence by $P680^+$ molecule itself. On the other hand, we suggest that the given decrease is caused by some kind of the recombination between $P680^+$ and a negative charge. But based on our experimental results and also on the literature, we are not able to exactly determine if the negative charge is stored on Pheo $^-$ or Q_A^- . We can also only speculate if fluorescence is consequently quenched due to formation of P680 directly or via $^3P680^*$.

Finally, we point out that no matter to an exact mechanism of the recombination process leading to the decrease of fluorescence after the K step, the mechanism can only occur when inhibition of both the donor and acceptor sides of PS2 happen

because both inhibitions are necessary for the accumulation of $P680^+$, $Pheo^-$, and Q_A^- . The inhibition of both sides of PS2 at this temperature is reported in the literature. On the other hand, when only the inhibition of OEC occurs (it starts from about 32 °C - Havaux 1993) leading to an accumulation of $P680^+$, and the acceptor side of PS2 is not inhibited (up to 42 °C - Havaux 1993), no accumulation of $Pheo^-$ and Q_A^- occurs and the mechanism responsible for the fluorescence decrease after the K step cannot take place. This is also consistent with experimental FI curves lacking a decrease of the fluorescence intensity after the J step measured at temperature range where only inhibition of OEC could happen (see Fig. 1 in Lazár and Ilík 1997).

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