PHOTOSYNTHETICA 46 (4): 537-546, 2008

Irreversible changes in barley leaf chlorophyll fluorescence detected by the fluorescence temperature curve in a linear heating/cooling regime

J. FROLEC[*,**], P. ILÍK[*], P. KRCHŇÁK[*], P. SUŠILA[*], and J. NAUŠ[*]

Laboratory of Biophysics, Faculty of Science, Palacký University, tř. Svobody 26, CZ-771 46 Olomouc, Czech Republic[*]

Abstract

The chlorophyll fluorescence (F) temperature curves in a linear time-temperature heating/cooling regime were used to study heat-induced irreversible F changes in primary green leaves of spring barley (*Hordeum vulgare* L. cv. Akcent). The leaf segments were heated in a stirred water bath at heating rates of 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1} from room temperature up to maximal temperature T_m and then linearly cooled to 35 °C at the same rate. The F intensity was measured by a pulse-modulated technique. The results support the existence of the two critical temperatures of irreversible F changes postulated earlier, at 45−48 and 53−55 °C. The critical temperatures are slightly dependent on the heating rate. Two types of parameters were used to characterize the irreversibility of the F changes: the coefficient of irreversibility μ defined as the ratio of F intensity at 35 °C at the starting/ending parts of the cycle and the slopes of tangents of linear parts of the F temperature curve. The dependence of μ on T_m revealed a maximum, which moved from 54 to 61 °C with the increasing heating/cooling rate v from 0.0083 to 0.0500 ºC s^{-1}, showing two basic phases of the irreversible changes. The Arrhenius and Eyring approaches were applied to calculate the activation energies of the initial increase in μ. The values varied between 30 and 50 kJ mol^{-1} and decreased slightly with the increasing heating rate.

Additional key words: heat stress; *Hordeum*.

Introduction

As global warming becomes a recognized phenomenon, the need to know the reaction of cultural plants to varying temperature in more detail becomes more and more important along with developing reliable screening methods for detection of their high temperature resistance. In this work we have used the fluorescence temperature curve (FTC) for studying heat tolerance of barley leaves.

FTC is usually defined as the dependence of the chlorophyll (Chl) fluorescence (F) intensity on linearly increasing temperature. Although the shape of an individual FTC depends on plant material (Schreiber and Berry 1977), excitation wavelength and intensity (Schreiber and Berry 1977, Armond *et al*. 1978, Kuropatwa *et al*. 1992, Kouřil *et al*. 2004), detection wavelength, and the rate of heating (Kuropatwa *et al*. 1992), general characteristic phases of FTC can usually be distinguished (see Fig. 1). The heating medium bathing the sample is usually distilled water (in some cases another liquid or air). The excitation irradiance is either at the analytic (F_0 conditions) or actinic level.

The first part of FTC in a temperature range from 25 °C to about 40 °C is usually approximately constant. In the temperature interval from 35 to 45 °C an initiation of some adverse processes as a damage to the oxygen evolving complex (Havaux and Gruszecki 1993), dissociation of the oligomeric form of the light-harvesting complex 2 (LHC2) into monomeric ones and their separation from photosystem 2 (PS2) have been postulated

Received 14 April 2008, *accepted* 9 August 2008.
**Corresponding author; fax: +420-58-522-5737, e-mail: frolec.j@seznam.cz
Abbreviations: Chl – chlorophyll; E_a – activation energy; F – fluorescence; F(35) − fluorescence intensity after heating to 35 °C; F′(35) − fluorescence intensity after cooling to 35 °C; F_0 (F_m) − minimal (maximal) fluorescence intensity; F_v – variable fluorescence defined as $F_m - F_0$; FTC(s) − fluorescence temperature curve(s); LHC2 – light-harvesting complex of photosystem 2; M_1 (M_2) − the first (second) maximum of fluorescence intensity during heating; M_1' − the first maximum of fluorescence intensity during cooling; OEC – oxygen evolving complex; PS – photosystem; RC – reaction centre; S1 (to S5) − tangent of first (to fifth) linear part of FTC; t − heating time; T_c – critical temperature; T_{C1} (T_{C2}) − first (second) critical temperature interval of irreversible fluorescence changes; T_f – final temperature of heating/cooling regime; T_m – maximal temperature of linear heating; μ − coefficient of fluorescence irreversibility at 35 °C; v – heating rate.
Acknowledgements: This work was supported by grant No. MSM 6198959215 from the Ministry of Education of the Czech Republic and 522/08/H003 from the Grant Agency of the Czech Republic.

(Havaux and Tardy 1997). At temperatures of about 40 °C the F intensity begins to increase up to the first maximum, designated M_1 (Nauš *et al*. 1992b). The M_1 maximum appears around 50 °C. This heat-induced enhancement of F intensity seems to be caused by blocking of electron transport in RC2 (*e.g*. Schreiber and Armond 1978, Kouřil *et al*. 2004). This view has been supported by a mathematical simulation (Pospíšil and Nauš 1998). The tangent of this increase is used to determine the starting temperature of the initial F rise (T_c). The T_c temperature, also designated as the critical temperature, has often been used as an indicator of the thermal stability of the thylakoid membrane (*e.g*. Havaux *et al*. 1988). Upon high temperature hardening of plants, the T_c shifts by 2–3 °C to higher temperatures (Smilie 1979, Smilie and Nott 1979, Havaux 1993b, Lazár and Ilík 1997). The position of T_c also depends on the intensity of excitation (Kouřil *et al*. 2004). For higher intensities of exciting radiation a shift of T_c to higher temperatures has been detected.

In the following phase of FTC the F intensity gradually decreases, but within a temperature range of about 53−63 °C, a second F rise (to the maximum M_2) can be detected (see *e.g*. Kuropatwa *et al*. 1992, Ilík *et al*. 1995). This F rise probably originates from Chl *a* molecules released from the Chl-containing protein complexes denaturing at 55–60 °C (Ilík *et al*. 2003). The exact positions of both the M_1 and M_2 maxima and their ratio M_2/M_1 are dependent on the heating rate (Kuropatwa *et al*. 1992). For temperatures above that of M_2, the F intensity decreases or remains approximately constant (Ilík *et al*. 1995).

A reversibility of Chl F changes after heating can provide information about the heat tolerance of the photosynthetic apparatus. The F reversibility has been examined in several works (Nauš *et al*. 1986, 1992a, Yamane *et al*. 1997) and mainly depends on the highest temperature reached during the sample warming and with the heating regime.

In our previous work we investigated the F response and F spectrum of linearly heated (from 20 to 60 °C with heating rate 0.0833 °C s^{-1}) barley leaves (heated in the air) and during the spontaneous cooling (Nauš *et al*. 1986). The measured FTCs were partially or fully reversible during the cooling for a maximal temperature of heating near the position of M_1. For the maximal temperatures above the first maximum of the curve, the F response has been mostly irreversible. The reversibility of FTC (barley leaves at a low actinic excitation) under a heating regime consisting of the linear increase of temperature (0.0833 °C s^{-1}), followed by a period of constant temperature (linear-constant heating regime), has been studied (Nauš *et al*. 1992a). Two starting temperatures of irreversible F changes, designed T_{C1} and T_{C2}, have been found within FTC. These temperatures have been postulated at 45–48 °C (T_{C1}) and 53–55 °C (T_{C2}) intervals. Lowering the temperature of the sample after reaching a temperature below T_{C1} or T_{C2} regions has led to a partially reversible transition of F intensity to the preceding level [to F(30) or M_1, respectively]. The temperature of the samples can be also changed in a temperature-jump regime (Schreiber and Berry 1977, Klinkovsky and Naus 1994, Yamane *et al*. 1997).

The weakness of the temperature-jump protocols resides in the fact that the conclusions are made from results usually obtained with one regime, a constant time of incubation at different temperatures or at a constant temperature and different times of incubation. The results might be different if the regime is changed. Both the duration of exposition and the incubation temperature are important. Moreover, in natural conditions the jump regime is usually not achieved. Therefore, we have exposed the leaves to a linearly increasing and decreasing temperature with different heating/cooling rates and with a wide range of temperatures to achieve a more natural but well-defined and reproducible regime. The slowly changing leaf temperature, simulated by our linear heating/cooling regime, may occur in natural conditions more commonly than a sudden change, *e.g*. during the diurnal cycle or sun-shade alternation (for plant species in the forest understorey).

In this article, we introduced new parameters for a description of irreversible heat-induced Chl F changes in barley leaves. We also propose this study as a protocol for determination of heat effects and thermotolerance of various plant species.

Materials and methods

Plants: Barley plants (*Hordeum vulgare* L. cv. Akcent) were cultivated in a home-made growth chamber for 9 d in a 16/8 h light/dark regime [85±20 μmol(photon) m^{-2} s^{-1} of "white" fluorescent light, relative humidity 50±10 %] in artificial soil composed of perlit and Knop solution at 22±3 °C. The plants used for measurements were in the growth stage 12 according to Zadox *et al*. (1974); the central segments of primary barley leaf blades were measured.

Heating device and linear heating: FTCs were measured using a laboratory-made heating device and a fluorimeter *PAM 2000* (*Walz*, Effeltrich, Germany). This heating device allowed for the realizing of linear temperature-time dependence during heating and subsequent cooling at different rates. The device is composed of a power supply and temperature-control unit. The segments placed on a holder were immersed in a stirred distilled water bath. The heating/cooling of the water bath used Peltier cells. The temperature of the bath was determined by a thermocouple and the regime was controlled by an application written in *LabView 3.1* (*National Instruments*,

Austin, TX, USA). The signal from the thermocouple was used by this application to regulate the temperature of the water bath with respect to the given regime. The linear heating/cooling device can work with a precision of 0.5 °C. The leaf segments were immersed in distilled water and heated at 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1} from room temperature up to the maximal temperature T_m and then cooled at the same rate to the final temperature T_f = 35 °C.

Chl F measurements and E_a calculation: Chl F intensity was measured with a fluorimeter *PAM 2000* (*Walz*, Effeltrich, Germany) excited by weak red radiation [655 nm, 0.3 μmol(photon) m^{-2} s^{-1}] and detected at wavelengths higher than 700 nm. The leaf segment placed on the holder was excited from the adaxial side at a distance of approximately 5 mm from the end of the light pipe. The slopes of tangents of the linear FTCs parts were estimated for the given temperature range (see Results) by a linear regression procedure using *Microsoft Excel 2000*.

Integrals from Eqs. (9) or (10) were numerically evaluated using a trapezoidal rule with an increment of 0.5 °C. In order to find E_a for each heating rate, the fitting procedure of Eqs. (9) or (10) was performed using *Microsoft Excel solver 2000* and *MatLab* software (*Mathworks 7.0*, Natick, MA, USA) to find the global minimum of the sum of the square deviations between the experimental and model data.

Results

Coefficient of irreversibility and critical temperatures: An up/down linear FTC course of a spring barley leaf segment under analytic radiation depends on the heating rate v and T_m. Typical curves for heating/cooling rates 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1} and for T_m = 60 °C are shown in Fig. 1 (starting temperature 25 °C, final temperature 35 °C). The curves have two parts designated by arrows. The right-hand arrow indicates initial heating, the left-hand arrow subsequent cooling. Although the maximal temperature T_m = 60 °C was the same for each curve, a clearly distinct kinetic behaviour depending on the heating rate is shown.

In order to characterize the changes in F intensity after the heating/cooling cycle we established a new parameter – the coefficient of irreversibility

$$\mu = F'(35)/F(35) \quad (1)$$

where F′(35) is the F intensity at 35 ºC after cooling and F(35) is the F intensity at the same temperature during heating of the sample. Thus μ represents the degree of F irreversibility at 35 ºC.

The temperature 35 °C was chosen as a representative temperature for initiation of the irreversible changes. This choice is rather arbitrary and may be changed, *e.g.* using lower final temperatures in the range of 30−35 °C. To

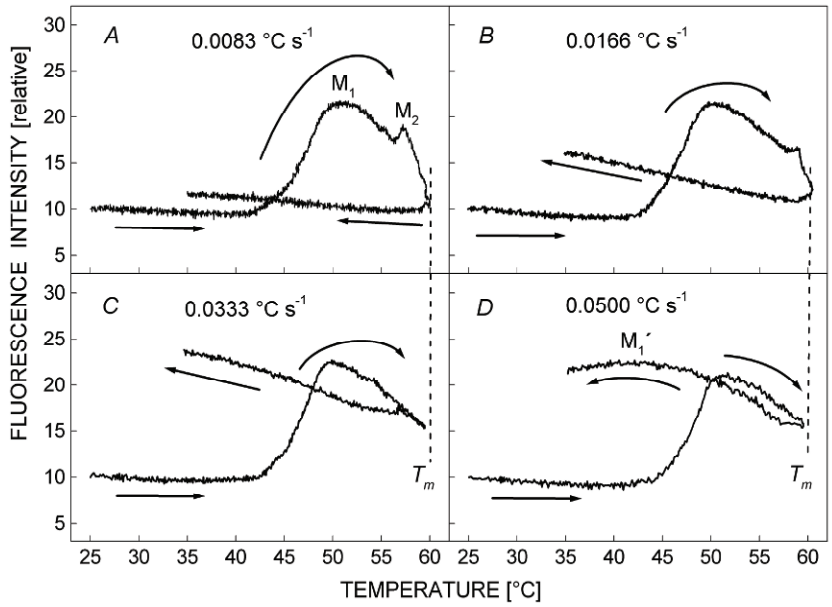

Fig. 1. Typical courses of the up-down linear FTC representing the changes in the measured F_0 level of a spring barley leaf segment for the heating/cooling rate [°C s^{-1}] of 0.0083 (*A*), 0.0166 (*B*), 0.0333 (*C*), and 0.0500 (*D*). The maximal temperature of heating (T_m) was 60 °C. The curves are normalized to the fluorescence intensity at 25 °C. *The arrows* indicate heating (*to the right*) and cooling (*to the left*) temperature-courses. The positions of the first and second FTC maxima reached during heating (M_1 and M_2) and the maximum reached during cooling (M_1′) are marked.

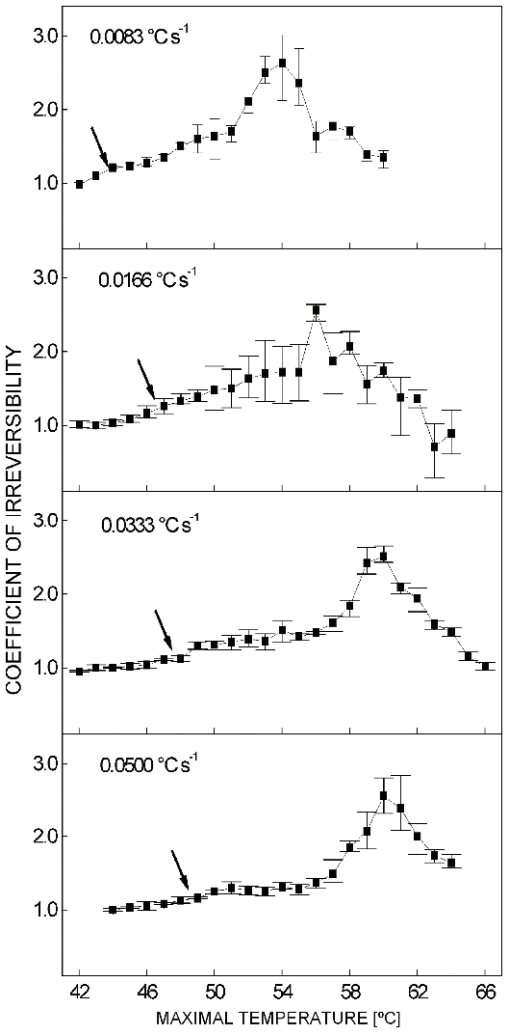

Fig. 2. The dependence of the fluorescence coefficient of irreversibility at 35 ºC (μ, see Eq. 1) on the maximal temperature of heating (T_m) for different heating/cooling rates (0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1}). *The arrows* indicate an approximate starting point of a steeper increase. The means of 3 experiments and the variation interval are shown.

support our choice, several findings may be cited. Gounaris *et al*. (1983) have found that a normal morphology of the bean chloroplast membranes is preserved up to 35 °C. Temperatures above 35 °C cause a lateral migration of spinach thylakoids' PS2 core from the grana out to the stroma region (Sundby and Andersson 1985), and at similar temperatures (about 38 °C) a partial decrease of oxygen evolution (mostly reversible) has been observed for spinach chloroplasts (Yamane *et al*. 1998) and barley leaves (Čajánek e*t al*. 1998). Another reason for choosing the final temperature of 35 °C is a matter of rationality. Cooling to lower temperatures than 35 °C would demand a somewhat longer measurement time.

Fig. 2 depicts the dependence of μ on T_m for different heating/cooling rates *v*. An increase in this rate led to a shift of the maximum μ to higher temperatures. This clearly corresponds with the shift of the temperature of the M_2 maximum in FTC. In contrast, no maximum corresponding to the M_1 maximum of FTC (see Fig. 1) appeared in the μ dependencies.

Tangents: We also evaluated the slopes of tangents of linear parts of the measured FTC to characterize the changes in the shape of this type of FTC (up/down linear FTC). The lines were estimated by a regression procedure. The temperature range for these regression lines was established so as to reach a minimal deviation between F intensity and the corresponding regression line (Fig. 3). The up/down linear FTC (Fig. 3) is one of the most complex curves (with 5 lines, S1 to S5), for some of the other curves some of these linear parts do not exist. The first (designated S1) and second (S2) tangent is defined in the temperature ranges of 46−48 °C and 51 ºC to T_m, respectively. They describe the shape of FTC during the linear heating (*i.e*. the F rise to the first maximum M_1 and subsequent F decrease). The S3, S4, and S5 tangents, however, describe the F course during the linear cooling from T_m to 35 °C. The S3 line was discernible only for T_m above 54 °C (see Fig. 4). Since there appeared a maximum of F intensity at about 50 °C during cooling for curves measured at a higher heating rate and T_m below 60 °C, we established the temperature range for the S3 part and the heating rate of 0.0500 ºC s^{-1} between 54 and 50 °C. For the other heating rates (0.0083, 0.0166, and 0.0333 ºC s^{-1}) the intervals for S3 were from 54 to 48 °C. The temperature range for the S4 and S5 tangents was 48–42 °C and 42–35 °C, respectively.

We evaluated the slopes of the tangents for each heating rate in dependence on T_m (Fig. 4). There was no evident trend in the dependences of S1 and S2. They

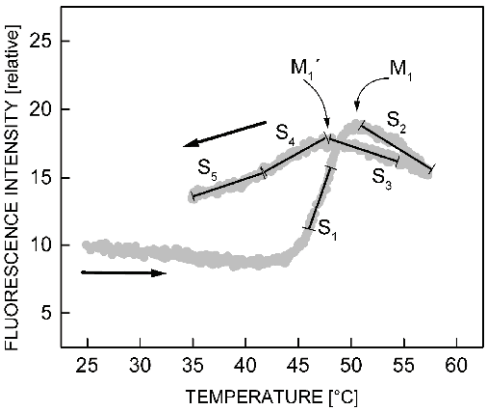

Fig. 3. A schematic illustration of tangents (S1−S5) of the linear parts of the up/down linear FTC. The temperature ranges of the tangents are 46–48 °C (S1), 51 °C – T_m (S2) during heating, and 54–48 °C (S3), 48–42 °C (S4), and 42–35 °C (S5) during subsequent cooling. *The arrows* indicate heating (*to the right*) and cooling (*to the left*) time-course. The position of the first FTC maximum reached during heating (M_1) and cooling (M_1') are marked. Barley leaf segment, heating/cooling rate of 0.0333 ºC s^{-1}, T_m = 57 °C.

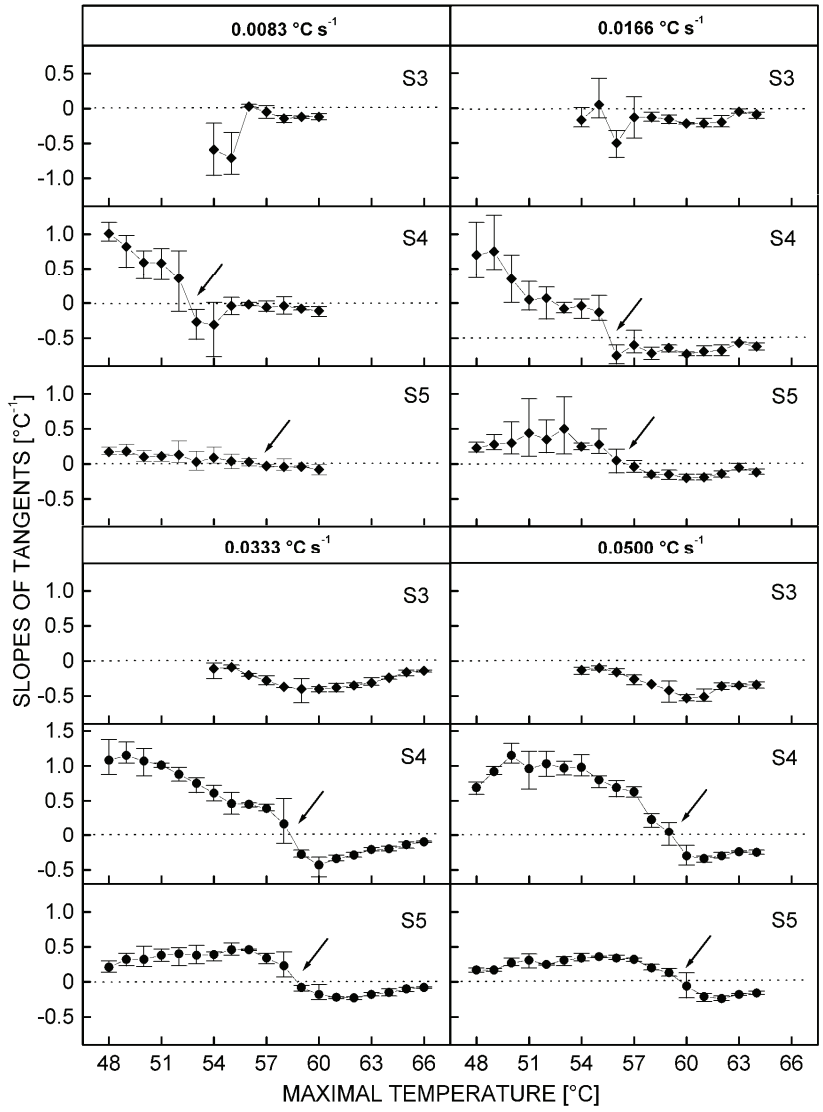

Fig. 4. The dependence of slopes of tangents (S3, S4, S5) on the maximal temperature of heating (T_m) for heating/cooling rates of 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1}. The means of 3 experiments and the variation intervals are shown. *The arrows* indicate a change of sign of a given slope from positive to negative. Evaluated from the up/down linear FTCs of spring barley leaf segment.

conserved almost identical values (on an average about 2.25 and −0.71 for the S1 and S2, respectively), consequently these data are not shown. On the contrary, the slope of the S3 tangent clearly reached a minimum at T_m = 55, 56, 59–60, and 60 °C for the heating rates of 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1}, respectively (Fig. 4). Thus, an increase in the heating rate led to a shift of this minimum to higher temperatures. The behaviour of the S4 tangent was similar to that of the S3. The minima of S4 occurred at very similar temperatures (T_m = 54, 56, 60, and 61 °C for the heating rates of 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1}, respectively) reaching negative values. During the third (last) phase of cooling (characterized by S5), the dependences had a similar course to those of S4, only the amplitudes were lower (the FTC course was rather horizontal).

Activation energies: In order to better characterize the irreversible processes during our heating regimes, we tried to calculate the activation energies necessary for the F irreversible increase. The initial increasing part (see below the description of Eq. 9) of the μ(T_m) curve was used for such an evaluation. We used the protein-denaturation model described by the scheme (see *e.g.* Bischof and He 2005):

$$n \xrightarrow{k} d \tag{2}$$

In this model, the proteins irreversibly transform from a native (n) to a denatured (d) state with a rate constant k. The first order of chemical kinetics is expected:

$$\frac{\mathrm{d}N}{N} = -k\ \mathrm{d}t \tag{3}$$

where N is the number of native proteins. A temperature dependence of k is generally assumed to be governed by an Arrhenius dependence

$$k = A \exp\left(-\frac{E_a}{RT}\right) \tag{4}$$

where A is the frequency factor, E_a is the activation energy, and R is the molar gas constant. According to Eqs. (3) and (4) and after integration we get a fraction of native proteins

$$\frac{N}{N_0} = \exp\left(-\int_{t_0}^{t_m} A \exp\left(-\frac{E_a}{RT}\right) dt\right) \tag{5}$$

where N_0 is the total number of proteins. The fraction of denatured proteins (F_d) is

$$F_d = 1 - \frac{N}{N_0} = \frac{N_0 - N}{N_0} = 1 - \exp\left(-\int_{t_0}^{t_m} A \exp\left(-\frac{E_a}{RT}\right) dt\right) \tag{6}$$

In our experiment the actual temperature [K] during heating was a linear function of time $T = T_0 + vt$, where T_0 is the starting temperature, v the heating rate, and t the time.

After differentiation we get

$$dT = v\, dt \tag{7}$$

The μ coefficient reached minimal and maximal values of 1.0 and 2.5, respectively (Fig. 2). We expect (see Discussion) that the excess of F (μ − 1) is proportional to the number of denatured centres ($N_0 - N$). Then

$$F_{d1} = \frac{2\,\mu - 2}{3} = \frac{N_0 - N}{N_0} \tag{8}$$

Thus, in a fully native system (*i.e.* $N = N_0$ or μ = 1) both of these fractions equal 0 ($F_{d1} = 0$) and for a fully denatured system (*i.e.* $N = 0$ or μ = 2.5) equal 1 ($F_{d1} = 1$). According to Eqs. (7) and (8) we can transform the time integral (Eq. 6) into the temperature integral and write

$$F_{d1} = \frac{2\,\mu - 2}{3} = 1 - \exp\left(-\int_{T_1}^{T_m} \frac{A}{v} \exp\left[-\frac{E_a}{RT}\right] dT\right) \tag{9}$$

where the initial T_1 temperature was 42, 42, 42, and 43 °C for heating rates of 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1}, respectively. At these temperatures μ equals 1 (Fig. 2). The highest T_m was placed at 52, 54, 58, and 58 ºC for the heating rates 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1}, respectively. Thus we do not include the two highest values from $\mu(T_m)$ curve for the given heating rate as around the maximum another process apparently occurs. This is shown in Fig. 2, where a steeper increase to a maximum of μ begins at these temperatures (for each heating rate).

Apart from Arrhenius (Eq. 4) we also tested the Eyring equation (both describe the temperature dependence of reaction rate constants), where in addition the right side of this equation is multiplied by the absolute temperature T. From Eq. (9) we have numerically evaluated the activation energies separately for each heating rate assuming the same frequency factor. Values of about 48 kJ mol^{-1} (Arrhenius dependence) or 46 kJ mol^{-1} (Eyring dependence) were found using a least-squares' analysis (see Table 1). These results were also supported by fitting Eq. (9) in *MatLab* software.

Table 1. Calculated activation energies of the fluorescence (F) irreversible increase. For each combination of the model and heating rate, the obtained activation energy [kJ mol^{-1}] (*left columns*) and appropriate sum of least squares (*right columns*) are shown. The quantities F_{d1} and F_{d2} represent the fraction of denatured proteins proportional to the measured excess of F irreversibility (see Eq. 9) and F irreversibility derived from F_v/F_m parameters (see Eq. 10), respectively.

Method	Heating rate [ºC s^{-1}] 0.0083		0.0166		0.0333		0.0500	
Arrhenius dependence (F_{d1})	49.8	0.032	48.7	0.008	48.2	0.044	47.7	0.075
Eyring dependence (F_{d1})	47.0	0.032	45.9	0.007	45.4	0.044	44.9	0.075
Arrhenius dependence (F_{d2})	31.2	0.005	30.2	0.001	29.5	0.006	29.1	0.011
Eyring dependence (F_{d2})	41.1	0.005	40.2	0.001	39.6	0.007	39.1	0.011

Björkman and Demmig (1987) found (see Discussion) that the F quantum yield of the closed PS2 (level of F_m) is about 5 times greater than that of the open PS2 (level of F_0). If we consider no other process, than μ reaches minimal and maximal values of 1 and 5, respectively, and quantity F_{d2} could be expressed analogically as F_{d1} in Eq. (9):

$$F_{d2} = \frac{\mu - 1}{4} = 1 - \exp\left(-\int_{T_1}^{T_m} \frac{A}{v} \exp\left[-\frac{E_a}{RT}\right] dT\right) \tag{10}$$

The least-squares analysis revealed that this definition (F_{d2}) leads to a more accurate fitting (see Table 1). The calculated values reached about 30 kJ mol^{-1} (Arrhenius) or 40 kJ mol^{-1} (Eyring).

Discussion

A survey of the interpretation of F_0 fluorescence has been done in several reviews (*e.g.* Lazár 1999, 2003). It is generally believed that F_0 fluorescence can be composed of several components. The most important are three components:

(*1*) Emission of PS2 (usually about 70 % or more). The sources of F emission are either excitations which did not reach a reaction centre, RC (Melis and Ow 1982) or which are a result of the transfer equilibrium between the antennae and RC (Owens 1996). The emission comes in all probability from the inner antenna of PS2, not from LHC2 (Kouřil *et al*. 2004).

(*2*) Some emission may come from Chls not connected to RCs (Lavorel and Joliot 1972, Ilík *et al*. 2003).

(*3*) Part of the F_0 emission has been also ascribed to emission of PS1 (Stahl *et al*. 1989, Trissl *et al*. 1993, Pfündel 1998, Gilmore *et al*. 2000). The contribution of this emission strongly depends on the spectral region of detection (Stahl *et al*. 1989) and may reach up to 50 % (Pfündel 1998).

Changes in F_0 can comprise a decrease of F_0 (*e.g.* F_0 quenching, *e.g.* Gilmore and Yamamoto 1993, Härtel and Lokstein 1995, Špunda *et al*. 1998, Kurasová *et al*. 2003) or an increase of F_0 or both. In our case, an increase of F_0 after the thermal cycle prevailed. Several main reasons for this increase can be listed:

(*1*) A transfer from State 2 to State 1. However, usually the opposite effect can be observed (Ruban and Trach 1991).

(*2*) A disconnection of electron transfer from Q_A^- to Q_B or a higher back transfer from Q_B^- to Q_A (Kouřil *et al*. 2004).

(*3*) Accumulation of inactive or Q_B-non-reducing RC2 with reduced Q_A (Cao and Govindjee 1990, Lazár *et al*. 1997).

(*4*) A decrease in the Q_A^- S2 recombination (Kouřil *et al.* 2004).

(*5*) Disconnection of the inner antennae (Havaux 1993a, Bartošková *et al.* 1999) or a minor component of LHC2 (Briantais *et al.* 1996) from the core of PS2.

(*6*) Formation of free chlorophylls having high fluorescence quantum yield (Ilík *et al.* 2003).

(*7*) Other minor effects like a decrease in quenching of different origins, changes in the cyclic electron transport around PS2, changes in the internal conversion, *etc*. can also play a role (see *e.g.* Briantais *et al.* 1996, Lazár 2003).

Our results do not make it possible at this point to distinguish between the mechanisms. Additional projects are under way to elucidate the nature of the F irreversible increase. However, all the possible mechanisms may be characterized as a denaturation of some PS2 turning them from the fully functional state to an adversely changed one.

Coefficient of irreversibility and critical temperatures: The μ coefficient reflects the relative changes in F intensity at 35 °C after the heating/cooling cycle. The μ *versus* T_m dependences (Fig. 2) are basically biphasic. These two distinct phases, an increasing and decreasing one, indicate the two main processes in the irreversible changes.

The initial phase with μ>1 is increasing and reaches a maximum at temperatures of 52−62 °C in dependence on *v*. This increase of μ in all probability reflects an increase in the amount of irreversibly closed (blocked) PS2 centres (see Introduction). The F quantum yield of the closed PS2 (level of F_m) is about 5 times greater than that of the open PS2 (level of F_0) as judged from the standard value of F_v/F_m (Björkman and Demmig 1987) and thus the increasing amount of closed PS2 increases the μ value.

The most striking feature of this first phase is the absence of a distinct resemblance to the original FTC (Fig. 1). The $\mu(T_m)$ curve is fluently increasing with no maximum corresponding to the M_1 maximum (around 50 °C). This shows that there is a complex reaction of the photosynthetic apparatus to the increasing temperatures up to 62 °C, which is, as a whole complex, partly reversible. The reverse part of FTC also has a maximum (designated here as M_1', *i.e.* it is composed of an increasing and decreasing part thus partly copying the original curve). The reversibility is higher, as can be expected, for higher heating/cooling rates *v* and lower T_m. The second phase of the $\mu(T_m)$ curve means that there is another complex of processes different from those of phase 1.

The critical temperatures T_{C1} and T_{C2} have been postulated earlier in the temperature regions 45−48 and 53−55 °C, respectively (Nauš *et al*. 1986, 1992a). Lowering the temperature of the sample after reaching a temperature below T_{C1} or T_{C2} regions leads to a partially reversible transition of F intensity to the preceding level [to F(30) or M_1, respectively] (Nauš et *al*. 1992b). It is rather difficult to identify the T_{C1} region by the $\mu(T_m)$ curve. However, if we look for a starting point of the steeper increase of μ, we find a temperature coinciding well with the postulated T_{C1} region. These points occurred at about T_m = 43, 46, 47, and 48 °C for the heating rates of 0.0083, 0.0166, 0.0333, and 0.0500 °C s^{-1}, respectively (see Fig. 2, *arrows*). A fully reversible response (μ = 1) was observed for T_m = 42–43 °C. Similar results were obtained for spinach leaves at a temperature-jump regime where the maximal temperature for the fully reversible response of F intensity (regarding the intensity at room temperature) was about 42 °C (Yamane *et al*. 1997). They used a regime consisting of 5 min treatment at high temperatures followed by incubation at room temperature for 5 min. For rice cv. Norin 8 and Chl *b*-less rice mutant leaves was the maximal stress temperature for a fully reversible response about 48 and 54 °C,

respectively. For higher temperatures, the F recovery was only partially reversible. The authors have explained this effect as an increase in the F_0 level.

The second critical temperature T_{C2} may be identified as the region of $\mu(T_m)$ maximum (54−59 °C in dependence on the heating/cooling rate). Reaching of these temperatures led to the appearance of M_2 or to a different FTC reverse course during the cooling.

The obtained results support the existence of critical temperatures of irreversible F changes, designated as T_{C1} and T_{C2} (Nauš *et al*. 1986, 1992b). These triggering temperatures were postulated at 45–48 °C (T_{C1}) and 53–55 °C (T_{C2}). We have obtained similar temperature ranges: T_{C1} = 43–48 °C, T_{C2} = 54–60 °C, whereas the particular critical temperature depends on the heating rate.

Tangents: A detailed characterization of the course of the up/down linear FTC can be made by a set of 5 linear parts (Fig. 3) and the slopes of their corresponding tangents (Fig. 4). The slopes of the S1 and S2 tangents describing the heating part (to the M_1 maximum) have only changed slightly in our measurements (not shown). Around M_1 the reversibility of some FTCs (especially for 0.0166 °C s^{-1}) was similar to the course of the earlier measured curves (Nauš *et al*. 1986).

The most interesting results have been obtained with the slopes of the S3, S4, and S5 tangents of the cooling part of the curve. The minimum of the S3 tangent slope represents the maximal temperature T_m which triggers a maximal F increase during the first phase of cooling. The slopes of the S4 and S5 tangents usually change their sign with increasing T_m (see Fig. 4). The region of T_m where the slope S3 is negative and S4 positive means that there is a maximum M_1' in the reverse part of the curve. This can be found between 54 and 59 °C for higher rates of heating/cooling (0.0333 and 0.0500 °C s^{-1}, Fig. 4). When using heating/cooling rates lower than 0.0166 °C s^{-1} no M_1' maxima were discernible.

The most relevant change for the character of the FTC curve seems to be the change of sign from positive to negative of the slope of the S4 tangent. It is consistent with the presence of the M_1' maximum. The change of the S4 sign appears between 52 and 59 °C depending on the heating/cooling rate. For T_m below these temperatures, the changes in the sample are at least partly reversible. After this point the signs of the S3 and S4 slopes are the same and the reverse part of FTC has only an increasing tendency with no maximum.

Activation energies: We applied the Arrhenius and Eyring approaches to calculate E_a of the initial increase in the F irreversibility. The activation energies ranged 30−50 kJ mol^{-1} (see Table 1). Most of the obtained values may correspond to energies of enzyme controlled metabolic processes including membrane transport (Bischof and He 2005).

The reasons for the irreversible F increase may be different and several mechanisms come into consideration. Our study concentrated on a formal description of the irreversible F increase and a more detailed study is necessary in the future to reveal the most probable mechanism. However, due to the fact that the best fit was obtained with an assumption of closing PS2 at the acceptor side characterized by the tendency of F to increase up to the F_m level (see Introduction), we suggest the closure of certain PS2 RCs, *i.e.* the accumulation of Q_A^- probably connected with OEC desintegration, as the most probable mechanism of the initial irreversible changes.

The Arrhenius or Eyring dependences only predicted a slight tendency to decrease with the increasing rate of heating. This decrease is consistent with the view that it is more acceptable for plants if the temperature increases slowly rather than rapidly (*e.g.* Ginzburg and Salomon 1986, Howarth 1991, Crafts-Brandner and Salvucci 2002), because under slow heating, plants have enough time to acquire some type of thermo-tolerance. The value of E_a is similar to those of enzymatic membrane processes and thus no drastic denaturation can be expected to be the reason for these changes. For denaturation, there is a need of a higher temperature (Smith and Low 1989) and higher activation energy (Bischof and He 2005). Lower activation energies in contrast indicate some minor structural changes in PS2. They do not reflect changes in the secondary structure of PS2 proteins. Apart from this interpretation, we suggest the methods presented in this article as a protocol for exact monitoring plant thermo-tolerance.

References

Armond, P.A., Schreiber, U., Björkman, O.: Photosynthetic acclimation to temperature in the desert shrub, *Larrea divaricata*. II. Light-harvesting efficiency and electron transport. – Plant Physiol. **61**: 411-415, 1978.

Bartošková, H., Komenda, J., Nauš, J.: Functional changes of photosystem II in the moss *Rhizomnium punctatum* (Hedw.) induced by different rates of dark desiccation. – J. Plant Physiol. **154**: 597-604, 1999.

Bischof, J.C., He, X.: Thermal stability of proteins. – Ann. New York Acad. Sci. **1066**: 12-33, 2005.

Björkman, O., Demmig, B.: Photon yield of O_2 evolution and chlorophyll fluorescence characteristic at 77 K among vascular plants of diverse origins. – Planta **170**: 489-504, 1987.

Briantais, J.-M., Dacosta, J., Goulas, Y., Ducruet, J.-M., Moya, I.: Heat stress induces in leaves an increase of the minimum level of chlorophyll fluorescence, F_0: A time-resolved analysis. – Photosynth. Res. **48**: 189-196, 1996.

Čajánek, M., Štroch, M., Lachetová, I., Kalina, J., Špunda, V.: Characterization of the photosystem II inactivation of heat-

stressed barley leaves as monitored by the various parameters of chlorophyll *a* fluorescence and delayed fluorescence. – J. Photochem. Photobiol. B **47**: 39-45, 1998.

Cao, J., Govindjee: Chlorophyll *a* fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes. – Biochim. biophys. Acta **1015**: 180-188, 1990.

Crafts-Brandner, S.J., Salvucci, E.M.: Sensitivity of photosynthesis in a C_4 plant, maize, to heat stress. – Plant Physiol. **129**: 1773-1780, 2002.

Gilmore, A.M., Itoh, S.S., Govindjee: Global spectral-kinetic analysis of room temperature chlorophyll *a* fluorescence from light-harvesting antenna mutants of barley. – Phil. Trans. roy. Soc. London B **335**: 1371-1384, 2000.

Gilmore, A.M., Yamamoto, H.Y.: Linear models relating xanthophylls and lumen acidity to non-photochemical fluorescence quenching. Evidence that antheraxanthin explains zeaxanthin-independent quenching. – Photosynth. Res. **35**: 67-78, 1993.

Ginzburg, C., Salomon, R.: The effect of dormancy on the heat shock response in *Gladiolus* cormels. – Plant Physiol. **81**: 259-267, 1986.

Gounaris, K., Brain, A.P.R., Quinn, P.J., Williams, W.P.: Structural and functional changes associated with heat-induced phase-separations of non-bilayer lipids in chloroplast thylakoid membranes. – FEBS Lett. **153**: 47-52, 1983.

Härtel, H., Lokstein, H.: Relationship between quenching of maximum and dark-level chlorophyll fluorescence in vivo. Dependence on photosystem II antenna size. – Biochim. biophys. Acta **1228**: 91-94, 1995.

Havaux, M.: Characterization of thermal damage to the photosynthetic electron transport system in potato leaves. – Plant Sci. **94**: 19-33, 1993a.

Havaux, M.: Rapid photosynthetic adaptation to heat stress triggered in potato leaves by moderately elevated temperatures. – Plant Cell Environ. **16**: 461-467, 1993b.

Havaux, M., Ernez, M., Lannoye, R.: Correlation between heat tolerance and drought tolerance in cereals demonstrated by rapid chlorophyll fluorescence tests. – J. Plant Physiol. **133**: 555-560, 1988.

Havaux, M., Gruszecki, W.I.: Heat-induced and light-induced chlorophyll *a* fluorescence changes in potato leaves containing high or low levels of the carotenoid zeaxanthin: Indications of a regulatory effect of zeaxanthin on thylakoid membrane fluidity. – Photochem. Photobiol. **58**: 607-614, 1993.

Havaux, M., Tardy, F.: Thermostability and photostability of photosystem II in leaves of the chlorina-f2 barley mutant deficient in light-harvesting chlorophyll *a/b* protein complexes. – Plant Physiol. **113**: 913-923, 1997.

Howarth, C.J.: Molecular responses of plants to an increased incidence of heat shock. – Plant Cell Environ. **14**: 831-841, 1991.

Ilík, P., Kouřil, R., Kruk, J., Myśliwa-Kurdziel, B., Popelková, H., Strzałka, K., Nauš, J.: Origin of chlorophyll fluorescence in plants at 55–75 °C. – Photochem. Photobiol. **77**: 68-76, 2003.

Ilík, P., Nauš, J., Cikánek, D., Novotný, R.: Chlorophyll fluorescence changes at high temperatures induced by linear heating of greening barley leaves. – Photosynth. Res. **44**: 271-275, 1995.

Klinkovsky, T., Naus, J.: Sensitivity of the relative F_{pl} level of chlorophyll fluorescence induction in leaves to the heat stress. – Photosynth. Res. **39**: 201-204, 1994.

Kouřil, R., Lazár, D., Ilík, P., Skotnica, J., Krchňák, P., Nauš, J.: High-temperature induced chlorophyll fluorescence rise in plants at 40-50 °C: experimental and theoretical approach. – Photosynth. Res. **81**: 49-66, 2004.

Kurasová, I., Kalina, J., Štroch, M., Urban, O., Špunda, V.: Response of photosynthetic apparatus of spring barley (*Hordeum vulgare* L.) to combined effects of elevated CO_2 concentration and different growth irradiance. – Photosynthetica **41**: 209-219, 2003.

Kuropatwa, R., Nauš, J., Mašláň, M., Dvořák, L.: Basic properties of the chlorophyll fluorescence temperature curve in barley leaves. – Photosynthetica **27**: 129-138, 1992.

Lavorel, J., Joliot, P.: A connected model of the photosynthetic unit. – Biophys. J. **12**: 815-831, 1972.

Lazár, D.: Chlorophyll *a* fluorescence induction. – Biochim. biophys. Acta **1412**: 1-28, 1999.

Lazár, D.: Chlorophyll *a* fluorescence rise induced by high light illumination of dark-adapted plant tissue studied by means of a model of photosystem II and considering photosystem II heterogeneity. – J. theor. Biol. **220**: 469-503, 2003.

Lazár, D., Ilík, P.: High-temperature induced chlorophyll fluorescence changes in barley leaves. Comparison of the critical temperatures determined from fluorescence induction and from fluorescence temperature curve. – Plant Sci. **124**: 159-164, 1997.

Lazár, D., Nauš, J., Matoušková, M., Flašarová, M.: Mathematical modelling of changes in chlorophyll fluorescence induction caused by herbicides. – Pestic. Biochem. Physiol. **57**: 200-210, 1997.

Melis, A., Ow, R.A.: Photoconversion kinetics of chloroplast photosystems I and II. Effect of Mg^{2+}. – Biochim. biophys. Acta **682**: 1-10, 1982.

Nauš, J., Dvořák, L., Kuropatwa, R., Mašláň, M.: Transitions in thylakoid membranes of barley leaves studied by chlorophyll fluorescence temperature curve. – Photosynthetica **27**: 563-570, 1992a.

Nauš, J., Kuropatwa, R., Klinkovský, T., Ilík, P., Lattová, J., Pavlová, Z.: Heat injury of barley leaves detected by the chlorophyll fluorescence temperature curve. – Biochim. biophys. Acta **1101**: 359-362, 1992b.

Nauš, J., Šnáblová, M., Dvořák, L., Kupka, Z.: Temperature dependence of *in vivo* chlorophyll *a* fluorescence. – Acta Univ. palacki. olomouc. Fac. Rer. nat. Phys. **85**: 47-63, 1986.

Owens, T.G.: Processing of excitation energy by antennas pigments. – In: Baker, N.R. (ed.): Photosynthesis and the Environment. Pp. 1-23. Kluwer Acad. Publ., Dordrecht 1996.

Pfündel, E.: Estimating the contribution of Photosystem I to total leaf chlorophyll fluorescence. – Photosynth. Res. **56**: 185-195, 1998.

Pospíšil, P., Nauš, J.: Theoretical simulation of temperature induced increase of quantum yield of minimum chlorophyll fluorescence $\Phi_{F(0)}$. – J. theor. Biol. **193**: 125-130, 1998.

Ruban, A.V., Trach, V.V.: Heat-induced reversible changes in Photosystem 1 absorption cross-section of pea chloroplasts and sub-chloroplast preparations. – Photosynth. Res. **29**: 157-169, 1991.

Schreiber, U., Armond, P.A.: Heat-induced changes of chlorophyll fluorescence in isolated chloroplasts and related heat-damage at the pigment level. – Biochim. biophys. Acta **502**: 138-151, 1978.

Schreiber, U., Berry, J.A.: Heat-induced changes of chlorophyll fluorescence in intact leaves correlated with damage of the photosynthetic apparatus. – Planta **136**: 233-238, 1977.

Smilie, R.M.: Coloured components of chloroplast membranes

as intrinsic membrane probes for monitoring the development of heat injury in intact tissues. – Aust. J. Plant Physiol. **6**: 121-133, 1979.

Smilie, R.M., Nott, R.: Heat injury in leaves of alpine, temperate and tropical plants. – Aust. J. Plant Physiol. **6**: 135-141, 1979.

Smith, K.A., Low, P.S.: Identification and partial characterization of the denaturation transition of the photosystem II reaction center of spinach chloroplast membranes. – Plant Physiol. **90**: 575-581, 1989.

Špunda, V., Čajánek, M., Kalina, J., Lachetová, I., Šprtová, M., Marek, M.V.: Mechanistic differences in utilization of absorbed excitation energy within photosynthetic apparatus of Norway spruce induced by the vertical distribution of photosynthetically active radiation through the tree crown. – Plant Sci. **133**: 155-165, 1998.

Stahl, U., Tusov, V.B., Paschenko, V.Z., Voigt, J.: Spectroscopic investigations of fluorescence behaviour, role and function of the long-wavelength pigments of Photosystem I. – Biochim. biophys. Acta **973**: 198-204, 1989.

Sundby, C., Andersson, B.: Temperature-induced reversible migration along the thylakoid membrane of photosystem II regulates its association with LHC-II. – FEBS Lett. **191**: 24-28, 1985.

Trissl, H.-W., Gao, Y., Wulf, K.: Theoretical fluorescence induction curves derived from coupled differential equations describing the primary photochemistry of photosystem II by an exciton-radical pair equilibrium. – Biophys. J. **64**: 974-988, 1993.

Yamane, Y., Kashino, Y., Koike, H., Satoh, K.: Increases in the fluorescence F_0 level and reversible inhibition of Photosystem II reaction center by high-temperature treatments in higher plants. – Photosynth. Res. **52**: 57-64, 1997.

Yamane, Y., Kashino, Y., Koike, H., Satoh, K.: Effects of high temperatures on the photosynthetic systems in spinach: Oxygen-evolving activities, fluorescence characteristics and the denaturation process. – Photosynth. Res. **57**: 51-59, 1998.

Zadox, J.C., Chang, T.T., Konzak, C.F.: A decimal code for the growth stages of cereals. – Weed Res. **14**: 415-421, 1974.