2.4. Principle of the chlorophyll fluorescence

Photosynthesis is a main physiological function of the most plant organisms (higher plants and algae), cyanobacteria and other prokaryotes. Photoautotrophic organisms are separated in this group by their ability to photosynthesise. This separate groups of photosynthesising organisms have differences in their mechanisms of photosynthesis, however, its main action is related to the act of capturing the light energy and its conversion to the chemical energy of the glucose molecule and other chemical compounds. This process occurs in two phases, a light and a dark phase. During the light phase a sequence of oxidative-reduction reactions are carried out by the photochemical pathway including one or two photosystems. They create a transmembrane proton gradient, utilized for the synthesis of ATP. During the dark phase organic molecules are synthesized from the fixation of atmospheric CO_2, as well as from ATP and NADPH produced during the light phase. This occurs by the means of a sequence of metabolic pathways, the main of which is the so-called Calvin-Benson cycle (Taiz and Zeiger, 2006).

During the light reactions in higher plants two photosystems take place, PS II and PSI. Currently large number of methods for analysis of the functional condition of reactions from the light phase of photosynthesis exist, based on the method of the fluorescence of chlorophyll. In this current work, the methods of prompt chlorophyll fluorescence (PF) at 685 nm, giving information for the structural and functional condition of PS II *in vivo*, as well as the modulated 820 nm light reflection (MR820), characterizing PS I *in vivo* were used. Data was obtained with the M-PEA (multifunctional plant efficiency analyser, Hansatech) that can perform measurements by both methods, simultaneously.

Prompt chlorophyll fluorescence (PF)

The method of the prompt chlorophyll fluorescence is based on the specificity of the PS II to emit in the far-red range of the light spectrum (>700 nm) after illumination by light with wavelength of 650 nm (Goltsev et al., 2010, Goltsev et al., 2014). If the illuminated light is registered by a device with a high time-resolution (in the range of micro- and milliseconds) a characteristic curve of the intensity of the emitted fluorescence, described first by Kautsky and Hirsch (1931) as the transient curve of Kautsky can be observed. In the first several milliseconds, up to 1 sec. the intensity of the fluorescence quickly increases, and then starts to decrease more and more slowly almost to the initial levels of illumination. Due to the specifics of the speed of increase, curves usually are represented in a logarithmic scale. The curves, obtained from the measurement of most plants, growing in normal physiological condition in absence of stress are characterized with three clearly distinguishable phases (or steps) of increase, followed by steps of momentary maximum (relatively constant levels of fluorescence), denoted with the

letters J, I and P. They could be seen as a kind of “bottlenecks” of the electron-transport chain, where the charge accumulates for a certain period of time, until it is released to the next components (Gao et al., 2013). The slower phase after them, characterizing with a decrease in the intensity of the fluorescence can also be divided into stages, denoted as P, S and M, but, their analysis is limited in practice (Stirbert et al., 2013). The initial phases give the name JIP-test of the method of prompt chlorophyll fluorescence, which is widely used and can often be found in literature (Strasser and Govindjee, 1992; Strasser et al., 2004).

Induction OJIP-curves (Fig. 6, I) give detailed information about the redox states of PS II. According to the theory of Duysens and Sweers (1963), two states of the reaction centre of PS II exist: open, when the primary quinone acceptor of the reaction centre (Q_A) is oxidized and closed, when this acceptor is reduced (oxidized means deficit of electrons or positively charged, and in the case of quinone this is its neutral form Q_A; reduced means having excess of electrons or negatively charged, Q_A^-). The type of the moment state of the reaction centre of PS II has a direct impact upon the intensity of the fluorescence. When the reaction centre is “open”, the light energy turns it into excited state and donates an electron, reducing the primal quinone acceptor of electrons in PS II (Q_A). Thus, light energy will be spent for activation of the photochemical reactions and the fluorescence of the current reaction centre in open state will be minimal. When the reaction centre is “closed”, (according to Duysens and Sweers), Q_A^- is in a reduced state and cannot accept an electron from the reaction centre, therefore the energy will be emitted in the form of fluorescence, which will be maximal for this current reaction centre (in “closed” state). Other models for description the states of the reaction centre exist (with the phaeophytin, as the primal acceptor), but the model of Duysens and Sweers is commonly accepted and results from the current work will be analysed according to it.

The phases (stages) of the transient JIP-curve are defined on the basis of the model of Duysens and Sweers for “opening” and “closing” of the reaction centres. Most devices for measurement of prompt chlorophyll fluorescence calculate these stages at fixed intervals after the start of the illumination with 685 nm. Modern devices have very high time-resolution, and the first value of the fluorescence intensity could be determined after 0.01 ms. This value is denoted with the letter O on the curve, and the intensity of fluorescence for this time, as F_O. Usually, for the purposes of most studies before the illumination with actinic light, plant samples stay for a certain period of time in the dark, called “dark adaptation”. During this period the reaction centres of PS II relax to open state. This is the reason for the initial phase of fluorescence to be minimal. The last point from the transient JIP-curve (P),

located at about 300 ms from the start is the next important point from the transient curve. Normally, its intensity of fluorescence F_P is identical to the maximal intensity of fluorescence F_M (in plants at optimal physiological condition). Most of the reaction centres in PS II are closed at this point, which is the reason for the maximal intensity of the fluorescence at this position. In the period from O to P, the photochemical quenching is the cause for the lower intensity of fluorescence. The photochemical pathways from the light reactions of photosynthesis are gradually filled up with electrons, donated from the reaction centres of PS II. This leads to closing of the reaction centres and a larger part of the energy is redirected as fluorescence. After the point of maximal fluorescence F_M, a period of a gradual decrease in the fluorescence intensity follows. During this period activation of the later stages of photosynthesis such as the cycle of Calvin-Benson occurs, as well as other processes, differing from the photochemical reactions in the photooxidative chains. They were denoted already by Kautsky with the common name non-photochemical quenching. They include the thermal dissipation that increases gradually, as well as the re-grouping of the light-harvesting complexes and thylakoid membranes after the filling up of the plastoquinone pool. Between the points of minimal (initial) O and maximal fluorescence (P), clearly distinguishable stages of relatively constant intensity of fluorescence exist, denoted with J (at 2 ms) and I (at 30 ms), respectively. They reflect the reduction of the primary electron acceptor of PS II Q_A -> Q_A^- as well as the reduction of the plastoquinone pool PQ -> PQH_2. Between the O-J phase of prompt fluorescence kinetics, other two harder to distinguish stages L (150 μs) and K (300 μs) exist (Strasser et al., 2004; Gao et al., 2013). They reflect structural and functional specifics in PS II. The point L relates to the possibility for energy transfer between the components of PS II. The shape and position of K is dependent on the level of electron transfer at the donor and acceptor sites of PS II.

The differences, obtained from subtraction of the intensities F_O, F_J, F_I at different stages of the transient curve to the intensity of the maximal fluorescence F_P (F_M) are denoted as a variable fluorescence in the corresponding point of the time scale. They describe the so-called energy fluxes (Pallotin, 1976). When relating these fluxes to each other the so-called parameters of prompt chlorophyll fluorescence can be obtained. For example, the difference between the minimal and maximal fluorescence ($F_M – F_O = F_V$) reflects the ability of the reaction centres from PS II to capture light energy and is often denoted as TR_O (trapped energy flux). In a similar way the difference $F_M – F_J$ reflects the electron transport beyond Q_A. It could be written also as ET_O (electron transport flux). $F_M – F_I$ relates to the reduction of PS I by the electrons, coming from PS II, as well as the reduction of the next components (Fd, $NADP^+$). Therefore it is denoted as RE_O (reduction of end acceptors of PS I acceptor side). When the relations of the forementioned differences are calculated to F_M, the so-called quantum

yields can be obtained. The first of these parameters is the maximal quantum yield of the primal photochemical reaction. It shows the amount of energy, trapped by the reaction centres of PS II in the start of the light reactions and could be found from the formula: $\varphi_{Po} = TR_O /ABS = F_V/F_M = (F_M-F_O)/F_M$. The next of the quantum yields reflects the amount of transported electrons from PS II to PS I. It is called the quantum yield of the electron transport: $\varphi_{Eo} = ET_O /ABS = (F_M-F_J)/F_M$. The last of the parameters, describing quantum yields is the so-called quantum yield of the reduction of end acceptors of PS I and, as its name proposes it shows the degree of reduction of end electron acceptors of PS I. It is expressed with the equation $\varphi_{Ro} = RE_O /ABS = (F_M-F_I)/F_M$. These parameters are amongst the most widely used in fluorescent analysis.

Other parameters, often measured in studies with prompt chlorophyll fluorescence are the quantum probability ψ_o for movement of electrons beyond Q_A and the performance indexes, as well as the parameters, reflecting the energy dissipation. The performance index on absorption basis (PI_{abs}) combines the forementioned quantum yield parameters (Goltsev et al., 2012) and reflects the condition of components from PS II and intersystem components (Strasser et al., 2004, 2010). Besides them, the total performance index (PI_{total}) includes the relative amount of chlorophyll in a reaction centre and is a parameter, describing the overall condition of every photooxidative component (Strasser et al., 2004, 2010). Energy dissipation could also be described by the parameters DIo/RC and φ_{Do}. They reflect the dissipation flux and the quantum yield of energy dissipation, respectively. Their application includes the measurement of dissipated or unused energy from the plant during light phase of photosynthesis and could be useful for describing the damages, caused by stress.

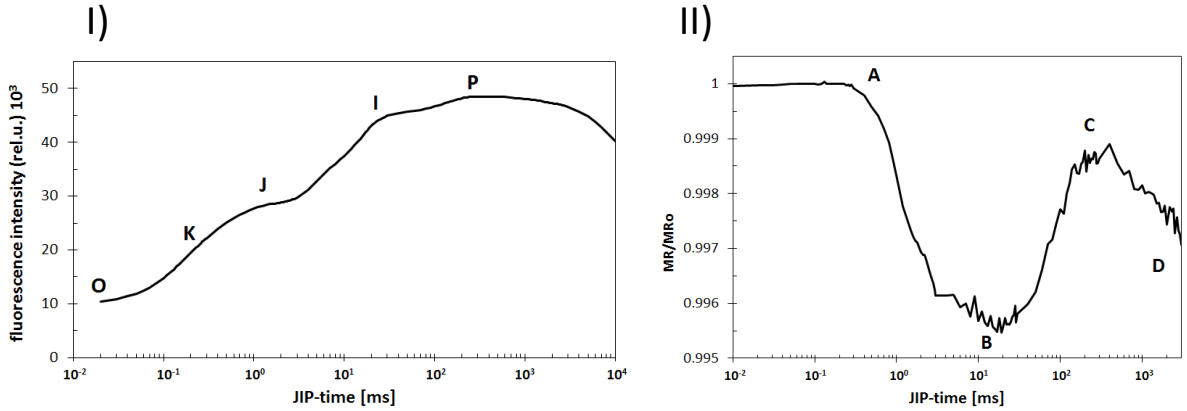

Fig. 6 I) A typical JIP-curve of the prompt fluorescence. The curve was displayed on a logarithmic scale from 0.01 μs to 10 s. The characteristic points O, K, J, I and P, could be seen, which are

taken in the calculations of parameters of the fluorescence. The curve represents the prompt chlorophyll fluorescence of A. alpina from Rila mountain, at temperature of 22°C.

II) A typical curve of the modulated 820 nm light reflection. The stages of oxidation (A-B) and re-reduction (B-C) could be seen, as well as the last phase of oxidation C-D, matching with the stages after the point P from the JIP-curve. The curve represents the prompt chlorophyll fluorescence of A. alpina from Rila mountain, at temperature of 22°C.

Modulated 820 nm light reflection (MR_{820})

The next method, available with the M-PEA is used to perform measurement of the redox states of the reaction centre of PS I and plastocyanin. It is called the modulated 820 nm light reflection measurement, because an 820 nm light pulse is emitted and the reflected light is measured. In their oxidized state, $P700^+$ and PC^+ have a greater ability to absorb light in the 820 nm range, thus a lower amount of the emitted light by the device is reflected back, as compared with the reduced state of these two components of the photooxidative chain. The transient curve (Fig. 6, II), obtained with this method has a characteristic downwards course in the period about 1 ms after the start of illumination, related to the oxidation of P700 and PC. After about 15-20 ms the curve changes its course to upward direction, which corresponds to the secondary reduction (re-reduction) of $P700^+$ and PC^+. During the period around 300 ms after the start of illumination, corresponding to the maximal fluorescence of the reaction centre of PS II, the signal returns to its values before the oxidation and then starts decreasing again. It is worthy of note, that M-PEA can measure simultaneously both types of fluorescence (PF and MR). When both curves are compared (Goltsev et al., 2010), it can be seen that the downward part of the MR curve (A-B phase from Fig. 6 II the oxidation of PC and the reaction centre of PS I) matches with the J-phase of PF, whereas the rising part of the curve (the phase B-C from Fig. 6 II of the reduction of PS I) matches with the stage I-P in PF. The MR-curve in its upper part corresponds to the inclusion of the newly synthesised compounds (ATP, NADPH) from the light phase in the Calvin-Benson cycle (the phase of the carbon reactions) (Strasser et al., 2010). By reading the data, obtained from the measurement of the activity of both photosystems, the processes of oxidation and reduction taking place during the light reactions, could be followed. During the period of the first two milliseconds from the start of illumination with actinic light, a donation of electrons from both photosystems begins. The time, however, is not enough for the electrons to leave PS II and, as a consequence, reduced forms of Q_A

accumulate, which could be seen as an increase of the fluorescence until the J peak. Meanwhile, PS I oxidises as a result from absorption of actinic light, but at the same time it is reduced from the electrons, coming from the plastocyanin or even the plastoquinone pool. In fact, the degree of oxidation of the plastoquinone pool, changes under stress, as well as different conditions of the environment, and is related to the reduction of PS I and the oxidation of PS II. Depending on the speeds of the reactions of reduction and oxidation, the faster process predominates. The reaction of plastocyanin oxidation and reduction of P700 has a duration of about 200 µs, which is exactly the same, as the initial stationary phase of the MR-curve. After that the electrons deplete and P700 oxidises quickly about 2 ms after the start of the illumination. The speed of oxidation is dependent upon the functional state of PS I. The rate of the reactions after ferredoxin is speed-limiting in this case. After the reduction of Q_A by PS II, which is reflected on the J peak in the PF-transient curve, the slower reduction of PQ occurs. For a molecule of PQ to be reduces, two electrons are necessary, and a pool of about 7-10 molecules functions in the intersystem electron transport chain, so the process of pool reduction is slower, as well. The dynamic of this process is shown as increase of the fluorescence up to the point I. In the same time P700 remains in oxidized form. After the PQ-pool is reduced and enough electrons are accumulated, they pass to P700, reducing it. This reduction can be observed as increase in the MR-curve. The process is much slower than the oxidation, because it reflects the occurrence of two concurrent processes, namely the oxidation of P700 when it absorbs light quanta from PS II and the reduction of $P700^+$ by the reduced PQ. After electrons from PS II reach PS I, the reduction of end electron acceptors such as the NADPH reductase starts and the cycle of Calvin activates. This releases space for other electrons to enter, which is shown as a decrease in the PF and MR curves movement. A large part in this decrease in the fluorescent curves takes the re-organisation of PS II from state 1 (granal) to state 2 (lamellar) (Stirbert et al., 2013). It activates few seconds after illumination with saturating actinic light and is related to the phases of the transient PF curve after P.