ISSN 1021-4437, Russian Journal of Plant Physiology, 2016, Vol. 63, No. 6, pp. 869–893. © Pleiades Publishing, Ltd., 2016.
Original Russian Text © V.N. Goltsev, H.M. Kalaji, M. Paunov, W. Bąba, T. Horaczek, J. Mojski, H. Kociel, S.I. Allakhverdiev, 2016, published in Fiziologiya Rastenii, 2016, Vol. 63, No. 6, pp. 881–907.

METHODS

Variable Chlorophyll Fluorescence and Its Use for Assessing Physiological Condition of Plant Photosynthetic Apparatus

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Received February 24, 2016

Abstract—Analysis of plant behavior under diverse environmental conditions would be impossible without the methods for adequate assessment of the processes occurring in plants. The photosynthetic apparatus and its reaction to stress factors provide a reliable source of information on plant condition. One of the most informative methods based on monitoring the plant biophysical characteristics consists in detection and analysis of chlorophyll *a* fluorescence. Fluorescence is mainly emitted by chlorophyll *a* from the antenna complexes of photosystem II (PSII). However, fluorescence depends not only on the processes in the pigment matrix or PSII reaction centers but also on the redox reactions at the PSII donor and acceptor sides and even in the entire electron transport chain. Presently, a large variety of fluorometers from various manufacturers are available. Although application of such fluorometers does not require specialized training, the correct interpretation of the results would need sufficient knowledge for converting the instrumental data into the information on the condition of analyzed plants. This review is intended for a wide range of specialists employing fluorescence techniques for monitoring the physiological plant condition. It describes in a comprehensible way the theoretical basis of light emission by chlorophyll molecules, the origin of variable fluorescence, as well as relations between the fluorescence parameters, the redox state of electron carriers, and the light reactions of photosynthesis. Approaches to processing and analyzing the fluorescence induction curves are considered in detail on the basis of energy flux theory in the photosynthetic apparatus developed by Prof. Reto J. Strasser and known as a "JIP-test." The physical meaning and relation of each calculated parameter to certain photosynthetic characteristics are presented, and examples of using these parameters for the assessment of plant physiological condition are outlined.

Keywords: chlorophyll *a* fluorescence, JIP-test, photosystem II, stress
DOI: 10.1134/S1021443716050058

INTRODUCTION

The plant organisms exist in permanent interaction with their environment. Environmental conditions of plant habitats are often adverse for plant functioning. The productivity of cultivated crops or survival of plant species under natural conditions depends strongly on environmental factors. When the crop plants are grown ***under optimal growth conditions***, the plant productivity is determined by the efficiency of photosynthetic apparatus.

Under natural conditions, the survival of each organism in an ecosystem depends on its ability of adapting to environmental conditions. The effective management of field crops, orchards, forests, and natural areas would depend on the invention of various

Abbreviations: AL—actinic light; Chl—chlorophyll; CS—cross section, the unit surface area excited by light in photosynthesizing samples; ETR—electron transport rate; FL—fluorescence; LHC—light-harvesting complex; PAM—pulse amplitude modulation (fluorometry); PAR—photosynthetically active radiation; PSI—photosystem I; PSII—photosystem II, OEC—oxygen-evolving complex; P680—reaction center of PSII; PPFD—photosynthetic photon flux density; PSA—photosynthetic apparatus; RC—reaction center.

means for assessing the physiological conditions of plants, their viability, and the occurrence of stress of variable strength. One important aspect in breeding the plants resistant to environmental stresses consists in experimental monitoring of plant physiological condition in vivo, detection of plant responses to stress factors, and the assessment of plant capacity to survive in adverse environmental conditions.

In order to attain this goal, it is necessary to apply a nondestructive experimental method that, on the one hand, preserves natural conditions of intact plants and, on the other hand, is the most informative for the multiparametric evaluation of the stress response on the background of a wide diversity of processes occurring in plant cells [1]. To enable the routine use of such a test, it is necessary that the method would satisfy additional important characteristics: the simplicity of use, a short measurement time, easy and unambiguous interpretation of the data, the feasibility of automated measurements, and the autonomously operated equipment (for field measurements). It is also desirable that information signals from the analyzed sample are obtained without the need for expensive consumable materials.

Chlorophyll fluorescence provides a very sensitive method to evaluate overall changes in the status of plant bioenergetics [2, 3]. The fluorescence changes are related directly or indirectly to all stages of photosynthetic light reactions: water photolysis, electron transport, generation of the pH gradient across thylakoid membranes, and ATP synthesis.

Chlorophyll fluorescence measurements were applied in agricultural practice [4, 5], fruit gardening [6], forestry [7], seed quality assessment [8], environmental studies [9], crop growing, food storage, and in processing of fruits and vegetables [10].

Chlorophyll fluorescence was also used to predict crop yields under various environmental conditions [4]. In recent years, chlorophyll fluorescence parameters were used as the criteria for selecting plants in the breeding programs [11]. In this review, we will consider the experimental approach that is based on in vivo measurements and analysis of variable fluorescence of chlorophyll *a*. We first analyze the current notions on the mechanisms of chlorophyll fluorescence emission in plants and examine relations between the kinetic characteristics of light emission with the reactions and processes in the photosynthetic apparatus (PSA) at the membrane and molecular levels. The basic experimental methods and instruments for measurements of light emission will be considered, as well as procedures for analysis of fluorescence signals, parameter calculations, and the overall assessment of plant physiological condition.

VARIABLE CHLOROPHYLL *a* FLUORESCENCE: SENSITIVE INDICATOR OF THE PRIMARY EVENTS IN PHOTOSYNTHESIS

Mechanisms of Fluorescence Emission by Chlorophyll Molecules

Substances can emit light in the optical spectral region upon heating [12] or due to luminescence. Following the definition in the Glossary of Terms recommended by IUPAC [13], luminescence is the spontaneous emission of radiation from an electronically excited species or from a vibrationally excited species not in thermal equilibrium with their environment. According to S.I. Vavilov, *luminescence of the radiating body in the given spectral region is the radiation arising in excess to thermally determined radiation, provided that this excess radiation has a finite duration exceeding the period of light wave oscillations.*

The first part of Vavilov's statement separates luminescence from thermal radiation, and its second part delineates distinctions between luminescence and scattered light. Luminescence is emitted by the excited molecules, although the source of excitation energy can vary. Depending on the type of energy used for excitation, different types of luminescence can be distinguished. Excitation of molecules during some chemical reactions can result in chemiluminescence. Specifically, if such reactions proceed in cells of some organisms and involve the enzyme systems, the light emission is called bioluminescence. In the case of electric current flowing in gas discharge tubes, the arising glow is called electroluminescence. If the energy for excitation of molecules comes from ionizing radiation, sound waves (ultrasound), and mechanical impacts on solid bodies (e.g., friction of surfaces), these luminescence types are termed radioluminescence, sonoluminescence, and triboluminescence, respectively. Upon heating of physical or biological systems containing long-lived metastable states, thermoluminescence can be emitted [14]. The most common type of luminescence is photoluminescence. In this case, the excitation energy comes from the absorption of infrared, visible, or ultraviolet light [13].

When a photon is absorbed, the molecule passes into the excited state (Fig. 1). Depending on the amount of energy absorbed, the molecule acquires one of several energy levels. During the time scale of picoseconds, a part of excitation energy is spent for vibrational motions of nuclei and for translational motions of solvent molecules. This energy portion dissipates as heat, and the molecule relaxes to the lowest vibrational energy level of the electronically excited state. Usually, this level corresponds to the first singlet excited state of the molecule.

The backward transition of the molecule from the singlet excited state (S_1) to the ground state (S_0) is accompanied by dissipation of the excitation energy that is either wasted on vibrational motions of nuclei

and translational movements of solvent molecules (i.e., converted into thermal energy with a rate constant k_d) or is emitted as a light quantum, known as *fluorescence* (k_f). The radiative transition can be also observed in molecules existing in the triplet excited state. This second type of photoluminescence is called *phosphorescence* [15]. At sufficiently high temperatures (~20°C), the molecules can pass from the triplet excited state back to the S_1 level, which is followed by *delayed fluorescence* [16]. The return of the molecule to the S_1 state depends on the absorption of excess energy, which may come from heat energy of surrounding solvent molecules (*delayed fluorescence of the E-type*). The excited singlet molecules can be also formed as a secondary event during recombination of the two triplet states (*delayed fluorescence of the P-type*).

The main pigment in the majority of oxygenic photosynthetic organisms is a molecule of chlorophyll *a* [17]. The chlorophyll *a* molecules associated with the antenna complexes perform mostly the function of light harvesting. When the microenvironment contains appropriate molecules capable of interacting with an electron shell of the excited pigment, excitation is transferred at a high speed between neighboring pigment molecules. Although individual energy transfer events occur as a stochastic process, the energy migration proceeds on the whole from the peripheral molecules toward the photochemically active molecule of the reaction center.

The excited pigment molecule in the antenna complexes can participate in several excitation-deactivating processes that compete with the radiative transition. The fluorescence quantum yield φ_f depends on the ratio of rate constants for all radiative and nonradiative deactivation processes (Equation 1):

$$\varphi_f = \frac{k_f}{k_f + k_d + k_p + k_t + k_{ic} + k_q[Q]}, \quad (1)$$

where k_f, k_d, k_p, k_t, k_{ic}, and k_q represent, respectively, the rate constants of fluorescence, internal conversion, photochemical reaction, energy transfer to neighboring molecules, intersystem crossing, and quenching of excitations by quencher molecular species. The lifetime of the excited state of the molecule, which is equal to the experimentally determined time of the fluorescence decay (τ), is determined by the overall rate of the processes that deactivate the excitation energy:

$$\tau = \frac{1}{k_f + k_d + k_p + k_t + k_{ic} + k_q[Q]}. \quad (2)$$

The intensity of emitted fluorescence is proportional to the rate of photon absorption by pigment molecules (I_a) and to the quantum yield of fluorescence (φ_f):

$$F = I_a\varphi_f. \quad (3)$$

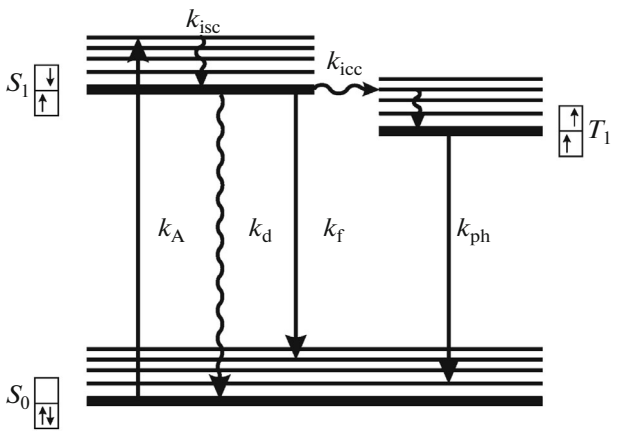

Fig. 1. Jablonski diagram illustrating the electronic and vibrational energy levels of molecules and the transitions between them. Bold horizontal lines represent energy levels of the ground (index 0) and excited (index 1) states of the molecule. Letters S and T designate the singlet and triplet levels and the arrows in the paired rectangles show orientations of the electron spins on the ground and excited orbitals. Symbols k_A, k_d, k_f, k_{ph}, k_{isc}, and k_{icc} stand for the rate constants of photophysical processes of light absorption, thermal dissipation, fluorescence, phosphorescence, intrasystem conversion, and intercombination conversion (intersystem crossing) associated with the spin reversal of the excited electron, respectively.

Chlorophyll *a* fluorescence in solutions is characterized by the quantum yield of 20–35% and decays with the lifetime of 6–20 ns. By contrast, for chlorophyll *a* molecules associated with proteins in the antenna complexes of thylakoid membranes, the quantum yield φ_f is ≈ 2–10% [18] and the fluorescence decay time is in the order of 1–2 ns [19]. At room temperature, fluorescence is mainly emitted by the antenna complexes of photosystem II (PSII), whereas the contribution of photosystem I (PSI) equals 5–30% in C_3 plants [18]. However, this contribution may increase significantly at low temperatures or under recording the long-wavelength light emission. The major part of fluorescence quanta are emitted by the antenna complex, i.e., by the proteins CP43 and CP47 [20].

At a constant rate of photon absorption, the intensity of fluorescence emitted by the photosynthesizing object is determined by the rates of fluorescence and the concurrent processes, including thermal dissipation (all processes except for photochemical reaction cumulatively termed as "nonphotochemical dissipation" or "nonphotochemical quenching" with the rate constant k_N) and the photochemical reaction in the PSII reaction center characterized by the rate constant k_P:

$$F = I_a \frac{k_f}{k_N + k_P}. \quad (4)$$

The rate constant of nonphotochemical quenching of excitation energy results from the superposition of rate constants for fluorescence, internal conversion, dissipation of excitations by quencher molecules, and migration of excitation energy to PSI (k_T):

$$k_N = \sum k_i = k_f + k_d + k_T + [Q]k_q. \quad (5)$$

Although fluorescence is predominantly emitted by chlorophyll *a* molecules in PSII antenna, it should be clear from equations 4 and 5 that the emission intensity depends on the proportions between the rates of excitation energy utilization in each molecule in the antenna complex, the rate of its redistribution between photosystems, and on the rate of photochemical processes. Furthermore, in the last few decades, an experimental approach was developed that revealed the dependence of fluorescence intensity on the electron transport between PSII and PSI [1, 21–23]. This approach based on the analysis of photoinduced fluorescence changes in dark-adapted photosynthetic samples will be considered in the next section.

Dynamics of Variable Fluorescence: Induction Curves

Chlorophyll *a* fluorescence is the secondary radiation of light energy absorbed by chlorophyll molecule. The portion of absorbed energy that is not used for photosynthesis and is lost as fluorescence is small, constituting only 3–5%; nevertheless, it represents a source of important information. Chlorophyll *a* fluorescence (FL) emitted in photosynthesis is a measure of the energy of absorbed light quanta that escaped from the use in photosynthesis. Under normal and efficient operation of photosynthetic reactions, the intensity of chlorophyll *a* fluorescence remains low, while any disturbance of photosynthesis that reduces its effectiveness results in a significant increase in FL [24].

When a dark-adapted photosynthesizing object is illuminated, the fluorescence intensity undergoes changes with time, exhibiting characteristic transients known as variable fluorescence induction [25] or the Kautsky curve [26]. The history of studying fluorescence and fluorescence induction curves (FIC) was considered in detail in [20, 27] and on the web site www.fluoromatics.com/kautsky_effect.php.

The induction curve can be divided into the rapid and slow phases. The rapid increase in fluorescence starts on the moment of switching on the light (level O) and terminates within 1 s at the peak level (P). During slow changes, the dominant phase is the fluorescence decline from the maximum P to the stationary level T (Fig. 2b). Both the increase in FL to the maximum and the subsequent decay to the steady state are usually multiphasic [20, 22, 23, 28–30]; i.e., they comprise several ascending and descending stages with different characteristic times. The shape of photoinduced transients in fluorescence induction curves reflects the kinetics of photosynthetic processes affecting the fluorescence quantum yield. Therefore, the most informative points of the induction curve were sequentially designated as O, J, I, P, M, S, and T. This modern nomenclature was developed by Strasser and Govindjee for the fast transients [31, 32] and by Papageorgiou and Govindjee for the slow transients [33, 34].

Duysens and Sweers were the first to undertake interpretation of the variable fluorescence kinetics [35]. According to their hypothesis, supported later by Butler [36], the reaction centers of PSII exist in two alternative states (open and closed ones) that differ in the quantum yield of fluorescence. The reaction center is considered to be open if its excitation leads to the photochemical reaction. In this case, the energy of absorbed light quantum is used for photosynthesis, whereas the fluorescence quantum yield is low. The "closing" of the reaction center results from the reduction of the quinone acceptor Q_A in PSII.

According to the *reversible radical pair model (RRP)* suggested by Schatz [18, 37, 38], the primary photochemical reaction in the PSII reaction center is reversible (Scheme 1):

$$\begin{aligned} &Chl_{II} - P_{680}PheoQ_A \xleftrightarrow{h\nu} Chl_{II}^* - P_{680}PheoQ_A \\ &\leftrightarrow Chl_{II} - P_{680}^* PheoQ_A \leftrightarrow Chl_{II} - P_{680}^+ Pheo^- Q_A \\ &\leftrightarrow Chl_{II} - P_{680}^* PheoQ_A \leftrightarrow Chl_{II}^* - P_{680}PheoQ_A \\ &\xleftrightarrow{h\nu} Chl_{II} - P_{680}PheoQ_A \end{aligned}$$

Scheme 1. Sequence of events in PSII and the reaction center (RC) underlying the emission of variable fluorescence.

On the other hand, the transfer of excitation energy from the antenna to the RC chlorophyll is also in equilibrium with the backward process. Thus, according to the hypothesis originally proposed by Duysens and Sweers [35] and subsequently developed by Butler [39], the fluorescence is at its minimum when the primary quinone acceptor Q_A is oxidized. The PSII reaction centers where the quinone acceptor is in the oxi-

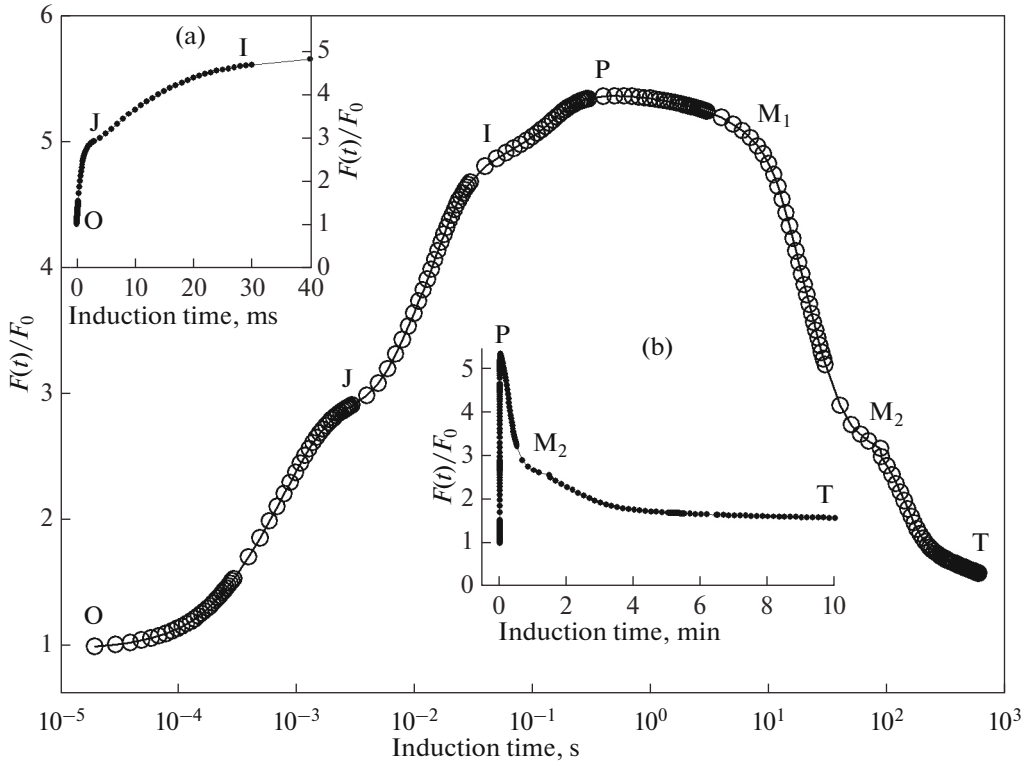

Fig. 2. Induction curves of variable fluorescence in dark-adapted bean leaves (30 min dark adaptation) presented on different time scales. The main panel represents the induction curve as a semilogarithmic plot. The insets show the induction curves in a linear time scale: (a) fluorescence changes in the first 40 ms of measurement; (b) changes in variable fluorescence over 10-min illumination. Fluorescence was measured with an M-PEA fluorometer (Hansatech Instruments, United Kingdom) at a photon flux density of 4000 μmol/(m^2 s).

dized form are called "open" and those with the reduced Q_A are termed "closed." The efficiency of fluorescence emission by closed RC is approximately five times higher than the fluorescence quantum yield of open centers [30]. The excess fluorescence emitted by the photosynthetic objects and exceeding the level of minimum fluorescence is called *variable fluorescence*. Fluorescence of PSI is independent of the redox state of the RC and electron acceptors. It does not participate in the variable fluorescence but contributes slightly to the constant background fluorescence (F_0) and the maximal quantum yield of fluorescence F_v/F_M.

This is due to the relative longevity of $P700^+$, whereas $P680^+$ rapidly obtains an electron from Y_Z. In the closed RC of PSI, the long-lived $P700^+$ captures the energy of excitation of chlorophyll antenna complexes and turns it into heat, so that fluorescence remains low [40]. Franck et al. [41] evaluated the contribution of PSI fluorescence by comparing the fluorescence emission spectra of the open and closed RC. Their data suggest that the contribution of PSI to the overall emission of open RC is 40%.

Variable fluorescence can be described within the framework of the reversible radical pair model [37, 42] that assumes the equilibrium between the excited states of antenna chlorophylls and P680 chlorophyll and, on the other hand, assumes the partial equilibrium between the states P680*I and $P680^+I^-$. The earlier hypothesis proposed by Klimov and Allakhverdiev [43, 44] considered variable fluorescence as a result of radiative recombination of the pair $P680^+ I^-$ in closed RC. According to the notion of Schatz et al. [37], shared also by other authors [45], the charge separation in RC with the reduced quinone acceptor (Q_A^-) is highly constrained because of the strong electrostatic repulsion. In this case, the contribution of charge recombination in variable fluorescence would be insignificant.

Fluorescence changes are related to the processes occurring within and around the PSII reaction centers, which alter the redox state of the acceptors and the quantum yield of light emission (Fig. 3). In darkened samples, all photosynthetic reaction centers are open and can be excited, because the PSII electron carriers are in the oxidized state. When photochemically active actinic light (AL) is switched on (the AL irradiance is sufficiently high to elicit photochemical changes), the fast phase of fluorescence induction begins. The AL intensity usually ranges from 200 to 500 μmol/(m^2 s). Measurements of chlorophyll FL at

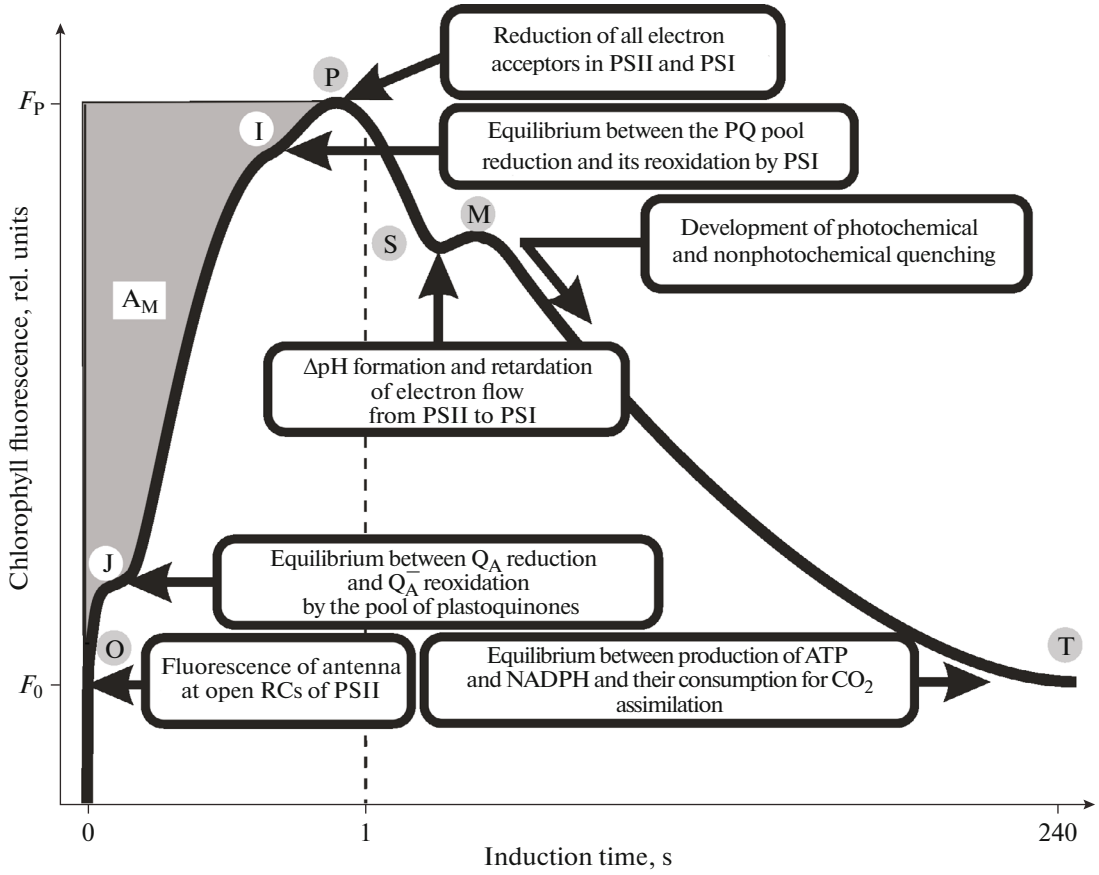

Fig. 3. Typical shape of the induction curve of chlorophyll fluorescence and description of processes determining fluorescence changes. Successive phases of the chlorophyll fluorescence induction curve are designated by letters O, J, I, P, S, M, and T. The shaded gray area above the O–P transition in the induction curve (A_M) represents the total electron capacity of electron transport chain.

the first point of the induction curve should be accomplished within at least 40 μs (the point O on the induction curve). This value, called the initial fluorescence (F_0 or F_O) is mainly related to energy losses in the pigments of PSII antenna [30]. The rise in chlorophyll FL yield from F_O to F_P undergoes several stages and depends on proper functioning of the PSII acceptor and donor sides of electron transport in PSII, as well as on the efficiency of interactions between photosynthetic units, which determines the possibility of excitation energy transfer between neighboring reaction centers.

As a result of charge separation in the excited reaction center of PSII, electrons are transferred from P680 to pheophytin (Pheo), thus reducing this primary acceptor of PSII. Next, the electron is transferred from $Pheo^-$ to Q_A, a plastoquinone associated with D2 protein at the Q_A binding site, which is the primary stable electron acceptor in PSII. The reduction of the primary quinone converts the reaction centers into the closed state, and the fluorescence intensity increases. Simultaneously with this process, Q_A^- in the closed centers is reoxidized by oxidized molecules of the secondary quinone (Q_B) and, later, by electron carriers in the subsequent part of electron transport chain, which decelerates the fluorescence rise. After 2–5 ms of illumination, the rapid reduction of the acceptor is terminated because the rates of Q_A reduction and Q_A reoxidation become equal, and fluorescence attains the level J. Following the gradual reduction of consecutively positioned acceptors in the electron transport chain (the mobile plastoquinone PQ, the cytochrome b_6f complex and PSI acceptors), the intensity of chlorophyll FL increases from the point J to the point I; this process lasts from 3–5 to 30 ms. The reduction of the plastoquinone pool is accompanied by simultaneous oxidation of PQ due to the PSI operation. At the level I of the fluorescence induction curve, the rates of electron transport toward the PQ pool and from the PQ pool via PSI to PSI acceptors ($NADP^+$, etc.) become equal. After complete reduction of available PSI acceptors, fluorescence reaches its maximum (F_P) at the point P, which corresponds (at a given intensity of actinic light) to the maximum reduction of PSII acceptors and to the lowest yield of

photochemical reactions (Fig. 3). The area above the curve of the fast induction of chlorophyll FL (a gray shaded area A_M in Fig. 3) indicates the number of available electron acceptors in PSII [30]. The transition from the peak P to the steady state S includes an apparent decline in fluorescence, which is due to the oxidation of PSI acceptors, the enhanced transfer of excitation energy from PSII to PSI, the accumulation of protons in the thylakoid lumen, and the formation of the proton gradient (ΔpH) between the lumen and the chloroplast stroma. These changes promote photophosphorylation and the onset of the dark stage of photosynthesis. After 5 to 9 s of switching on the light, the fluorescence intensity increases again (transition from the point S to M), because the linear electron flow between the photosystems is decelerated as the proton gradient is built up and exerts its down-regulation effect via the negative feedback. Several seconds later (in 3–5 s), the Calvin–Benson cycle is mobilized because the ATP production increases and the flavin-containing ferredoxin–NADPH oxidoreductase (FNR) turns activated, thereby promoting the accumulation of reduced NADPH. Thus, the linear electron flow is accelerated and chlorophyll FL decreases for a few minutes toward its steady-state level T (terminal fluorescence). The decrease in fluorescence during this period is determined by photochemical and nonphotochemical quenching of excited chlorophyll *a* molecules in the antenna complexes of PSII. The time of establishing terminal fluorescence depends on the plant physiological condition and on the stage of plant development [5]. The steady-state level of chlorophyll fluorescence indicates that the equilibrium is achieved between the production of energy-rich compounds (NADPH and ATP) in photochemical reactions and the consumption of these products in dark biochemical reactions (Fig. 3).

Methods of Chlorophyll Fluorescence Measurements

Measurements of chlorophyll FL require separation of the measured signal from excitation light that drives photochemical reactions of photosynthesis and excites chlorophyll fluorescence in the photosynthetic apparatus; a part of unabsorbed light is reflected from the leaf surface or is scattered by the internal leaf structures. Fluorescence spectrometers used for the measurements of chlorophyll fluorescence operate on the principles of emission spectroscopy. To date, a number of methods for measuring FL and a wide range of fluorometers have been developed. The most practical and frequently used methods consist in direct acquisition of FL and the measurements of modulated fluorescence.

Direct Acquisition of Chlorophyll Fluorescence

Measurements are performed after preadaptation of a photosynthesizing sample to darkness for 20–30 min. The sample is illuminated by continuous light with the wavelengths shorter than 670 nm, which is achieved with the use of optical filters or special light-emitting diodes or diode lasers. When the excitation light is switched on, the photodetector monitors chlorophyll fluorescence in the wavelength range from approximately 680 to 760 nm. The shape of the fluorescence induction curve permits some conclusions to be made about the photosynthetic performance of the sample and the dynamics of photosynthetic reactions [5]. At the end of the induction phase of chlorophyll FL under continuous illumination (when fluorescence attains a constant level F_T), the fluorometer measures the stationary chlorophyll fluorescence (Fig. 3).

The above-described typical system for FL measurements is called continuous-excitation type of chlorophyll fluorescence system. It consists of an excitation light source providing the photon flux of approximately 3500 μmol/(m^2 s) with a wavelength ~625 nm and the detector with a long-pass filter transmitting only light with $\lambda > 700$ nm (Fig. 4). The detector of chlorophyll FL transmits the signal to the amplifier, and the output signal is digitized and fed into a microprocessor for calculating the required parameters of fluorescence.

This technique is used for determining the following parameters (they are described in detail in the next chapter):

F_O	initial (zero-time) fluorescence;
F_M	maximal fluorescence;
$F_V = F_M - F_0$	variable fluorescence;
F_V/F_M	maximal quantum efficiency of PSII;
t_{FM}	time to reach maximal fluorescence F_M ;
PI	performance index of photosynthetic apparatus (PSA);
A_M	area above the induction curve of chlorophyll fluorescence

Owing to rapid advances in technology, modern fluorometers can measure basic FL parameters from the shape of the initial part of the Kautsky curve. These fluorometers comprise a high-intensity light source (photon flux density above 2500 μmol quanta/(m^2 s)) and a photosensor with a microprocessor for fast and precise detection with the temporal resolution of 10 μs, which ensures fluorescence measurements at short times, including the initial induction phase (Fig. 5). Thus, by measuring fluorescence, one can trace the time course of primary reactions in photosynthesis. The detailed analysis of the measured signals (JIP-test) provides the means to assess the impact of various stress factors on plants [3, 29, 46].

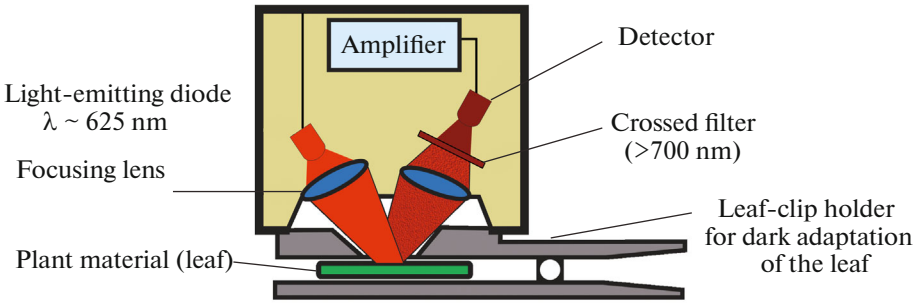

Fig. 4. Schematic view of the measuring unit in fluorometers designed for direct recording of chlorophyll *a* fluorescence, such as PEA, Handy PEA, and M-PEA (Hansatech Instruments Ltd.).

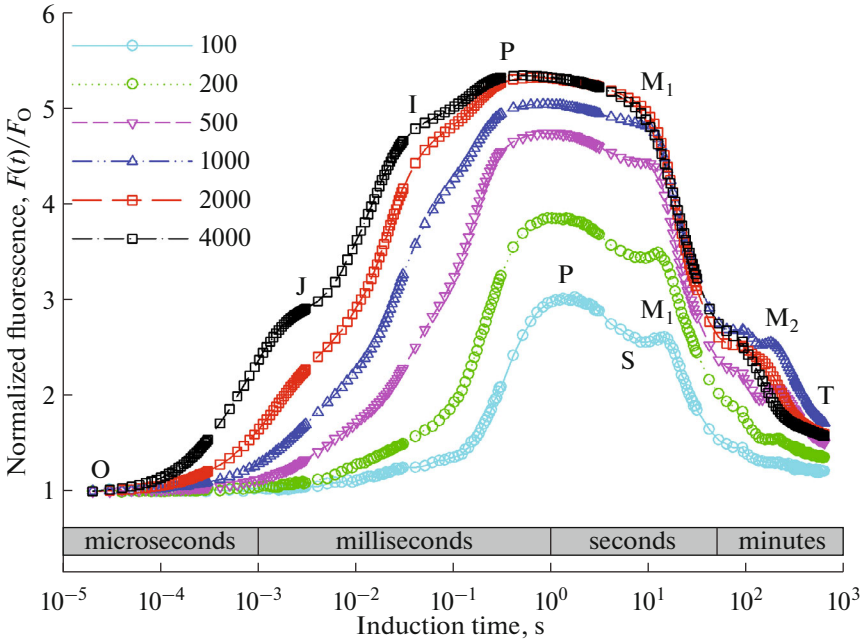

Fig. 5. Typical induction curves of variable fluorescence recorded with a Handy PEA fluorometer at different intensities of excitation light (in μmol photons/(m^2 s)).

Modulated Fluorescence of Chlorophyll a

Modulated chlorophyll fluorescence is measured on the basis of pulse amplitude modulation (PAM) system [47]. In this case, chlorophyll FL is excited with a light source that emits modulated light that is switched on and off at regular intervals. Accordingly, the detector monitors only the variable component of the fluorescence induced in the sample (Fig. 6). Thus, chlorophyll FL can be measured in the presence of an additional source of actinic light of any spectral composition, including sunlight.

In this measurement system, the light is switched on for a short time (1–3 μs); this time interval is sufficient to detect the pulse of chlorophyll FL. The sensor part of such apparatus is exposed to light signals of three types (Fig. 6):

(1) Actinic light scattered at the surface of the sample (continuous illumination);

(2) Fluorescence of the sample induced by the actinic light (continuous signal);

(3) Pulse-induced fluorescence signal that is excited by modulated light. It is this fluorescence signal that is electronically amplified, whereas other (unpulsed) signals are disregarded.

Measurements can be performed on whole leaves or leaf parts, as well as on suspensions of chloroplasts or algal cells adapted to darkness or light.

The technique with the use of PAM technology ensures reliable measurement of the initial fluorescence F_0, because the intensity of measuring light beam is so low that its does not initiate photochemical reactions. When the PAM system is applied, the measurement starts from switching on a very weak pulse-modulated measuring light (ML), which induces chlorophyll fluorescence (Fig. 6a). The source of ML is light-emitting diodes (LED) that emit radiation in the red spectral region (typically, $\lambda_{max} = 650$ nm). This

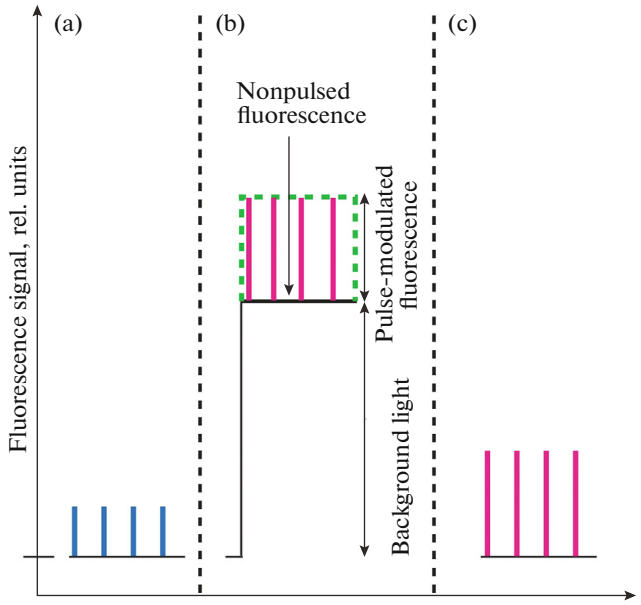

Fig. 6. Schematic illustration of light signals sensed by the detector at different operating regimes. (a) Fluorescence signal recorded when only modulated light is applied; (b) under combined application of actinic and modulated light; (c) fluorescence remained after filtering the constant actinic light component is indicative of chlorophyll photochemical reactions (Hansatech Instruments, Ltd.).

radiation is very weak and does not cause induction processes in leaves. Chlorophyll fluorescence in this case is mainly due to energy losses during migration of excitations over the antenna; this fluorescence value can be taken as F_0 (Fig. 7). Next, a saturating pulse (SP) is triggered. This is a comparatively short (e.g., 0.8 s) and very powerful pulse of light. Its intensity obtained, for example, from a halogen lamp can be as high as 20000 μmol/(m^2 s). The reduction of all PSII acceptors under the action of SP temporarily blocks the photochemical reactions, which results in a sharp increase in the fluorescence yield. The fluorescence intensity in dark-adapted samples attains its maximal value (F_M). After FL returns to the initial F_0 level, continuous actinic light (AL) is switched on. The photosynthetic photon flux density (PPFD) of actinic light provided by a halogen lamp ranges usually from 200 to 3000 μmol/(m^2 s). The actinic illumination elevates the fluorescence yield to the peak value F_P, whose value varies depending on the AL intensity. Over the next few minutes, the FL intensity decreases to the steady-state level F_T, which signifies the balancing of all reactions in the light and dark stages of photosynthesis [5].

In 240 s from the onset of actinic illumination, the second saturating pulse is applied, which reduces the PSII electron acceptors and temporarily blocks the photochemical reactions. During this pulse, the FL yield increases to the level of F_M' that is lower than F_M.

The difference between F_M and F_M' is determined by nonphotochemical quenching of fluorescence [48]. Measurements of chlorophyll *a* FL using PAM fluorometers allow researchers to rapidly evaluate the efficiency of conversion of photosynthetically active radiation (PAR) into chemical energy in photosynthesis and to determine several important parameters:

F_0'	initial (zero-time) fluorescence in light-adapted samples
F_M'	maximal fluorescence
$F_V'/F_M' = (F_M' - F_0')/F_M'$	maximal quantum efficiency of the primary photochemical reaction in a light-adapted sample;
$\Phi_{PSII} = (F_M' - F_T')/F_M'$	actual quantum yield of PSII photochemical reactions in the actinic light;
$q_P = (F_M' - F_T')/(F_M' - F_0')$	photochemical quenching;
$q_N = (F_M - F_M')/(F_M - F_0)$	nonphotochemical quenching;
ETR = $\Phi_{PSII} \times 0.50 \times$ PPFD$_a$ = $= 0.84 \times 0.50 \times$ PPFD$_a$	rate of linear electron flow (PPFD$_a$ designates the rate of light *absorption* by a sample expressed in μmol/(m^2 s))

Parameters of Chlorophyll a Fluorescence, Their Values, and Physiological Significance

Analysis of chlorophyll *a* fluorescence includes a series of characteristics. Despite the repeated efforts to introduce uniform terminology, the same parameters are often presented in different ways [49].

F_0, Initial Fluorescence of Dark-Adapted Samples

The parameter F_0 (**initial or zero-time fluorescence**) is an indicator of energy losses during excitation energy transfer in the antenna and from the antenna to the reaction center of PSII [5, 50]. Zero-time fluorescence of healthy leaves (F_{open}, F_{zero}, or F_{ground}) is the first point on the induction curve of chlorophyll *a* FL. It characterizes the fluorescence emission by excited chlorophyll molecules in the PSII antenna under conditions when Q_A acceptors (plastoquinone molecule) are fully oxidized, all PSII reaction centers are open (able to trap the excitation energy and irreversibly perform the primary photochemical reaction), and nonphotochemical quenching (qN) is absent. These conditions are fulfilled after preadaptation of photosynthesizing objects to darkness [51]. Radiative energy

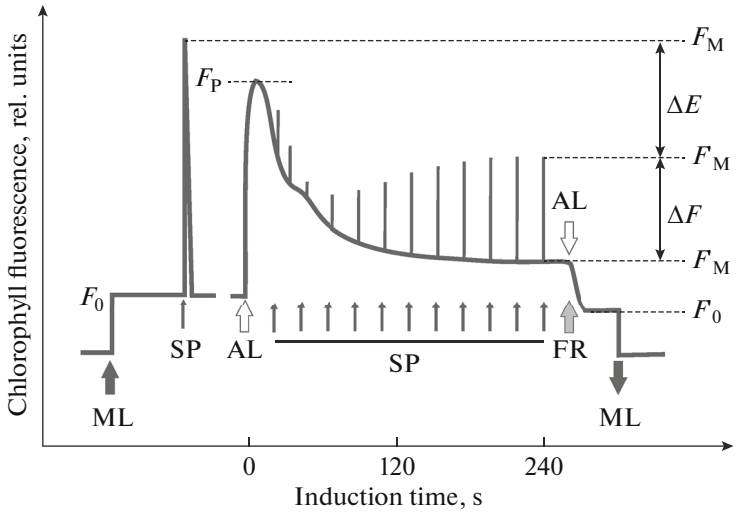

Fig. 7. Schematic representation of a typical experiment intended to determine the photosynthetic efficiency with the use of modulated fluorescence. See text for detailed description. ML—modulated measuring light; SP—saturating pulse(s); AL—actinic light; FR—near-infrared light (far-red light); F_P—maximum fluorescence of chlorophyll *a* under excitation with actinic light; F_0—minimum fluorescence of chlorophyll *a* in dark-adapted samples; F_0'—minimum fluorescence of chlorophyll *a* in light-adapted samples; F_M—maximum fluorescence of chlorophyll *a* in dark-adapted samples; F_M'—maximum fluorescence of chlorophyll *a* light-adapted samples; F_T—steady-state chlorophyll *a* fluorescence in light-adapted samples; $\Delta E = F_M - F_M'$; $\Delta F = F_M' - F_T$.

losses in the antenna depend not only on the intensity of the excitation light in the PAR region but also on the effectiveness of excitation energy transfer from the light-harvesting complex LHC II to the reaction center of PSII. Zero-time fluorescence is measured at low PAR intensities, for example, at 0.01 μmol/(m^2 s) [52].

High values of F_0 indicate a comparatively low efficiency of excitation energy transfer between pigment molecules in the light-harvesting antenna of PSII. Such a decrease in efficiency is observed, for example, under heat stress that damages thylakoids and inactivates the PSII [5]. The increase in F_0 can be also due to the decreased efficiency of energy transfer toward the reaction center of PSII after the dissociation of LHC II from the core of PSII [53]. A similar situation was also observed in salt-stressed plants [54].

F_M, Maximal Fluorescence

F_M is determined after dark adaptation using a saturating light pulse that saturates photochemical reactions in PSII reaction centers within the period of pulse duration (0.8–2.0 s). The maximal intensity of fluorescence (F_M) can be obtained if PPFD of saturating pulse ranges from 3500 to 10000 μmol/(m^2 s). Under these conditions, all plastoquinone molecules in PSII become reduced, all PSII reaction centers are temporarily closed and cannot pass additional electrons. It should be noted that the parameter F_M is correctly determined only in the absence of nonphotochemical quenching (qN). Under application of a saturating pulse, the F_M level would depend, among other factors, on the chlorophyll content in the tissues examined. The decline in F_M indicates that the photosynthesizing object experiences stress, which means that the PSII electron acceptors cannot be fully reduced. After reaching the maximum fluorescence F_M, the efficiency of chlorophyll fluorescence decreases rapidly to the level F_T, which is close to F_0.

Sometimes, the ratio F_M/F_0 (the maximum fluorescence normalized to zero-time fluorescence) is useful. In healthy leaves, this ratio equals to 4–6; its values in plants depend on the action of certain stress factors. For example, the F_M/F_0 ratio during drought can diminish to 1.0 [55], which indicates the destruction of PSII.

t_{FM}, Time of Achieving the Maximal Chlorophyll Fluorescence F_M

The parameter t_{FM} designates the time from the start of measurement to the moment when chlorophyll FL reaches the maximum level F_M; it is usually equal to 500–800 ms [52]. The measurement of t_{FM} is an alternative method for determining the amount of pool of oxidized plastoquinones. Under the influence of stress factors hampering the transport of high-energy electrons from the reaction center to plastoquinones, this parameter increases substantially [56].

$F_V = F_M - F_0$, Variable Fluorescence

The parameter F_V (or $F_{variable}$) is the difference between chlorophyll FL values F_M and F_0 that are measured after dark adaptation. The extent of F_V is related to the maximum quantum yield of PSII. The

low value of this index indicates the decrease in PSII activity and dissipation of excitation energy as heat. The value of F_V decreases under the influence of environmental stress factors (low and high temperatures, freezing, etc.) that are damaging to the thylakoids [56].

$F_V/F_0 = k_p/k_n$, Ratio of Rate Constants for Photochemical Reaction and Nonphotochemical Deactivation of PSII Excitations

This parameter is also determined after dark adaptation. It reflects the efficiency of use of excitation energy in PSII and is equal to the ratio of rate constants for the primary photochemical reaction (k_p) and the total rate of nonphotochemical losses (k_n) [46]. Under the action of stress factors modifying the oxygen-evolving complex (OEC), the increase in nonphotochemical losses is usually associated with accumulation of oxidized pigment $P680^+$ that acts as a fluorescence quencher. In this case, the above parameter characterizes the changes in water-splitting efficiency (oxygen evolution) in PSII. The water-splitting complex is considered a very sensitive part of the photosynthetic electron transport chain [57].

F_V/F_M, Maximal Photochemical Efficiency of PSII

There is substantial evidence that the F_V/F_M parameter, i.e., the $(F_M - F_O)/F_M$ ratio measured in dark-adapted plants, reflects the potential quantum efficiency of PSII and can be used as a reliable indicator of the photochemical activity of photosynthetic apparatus. For the majority of fully developed plants under stress-free conditions, the maximum F_V/F_M value equals 0.83 [58]. The decrease in F_V/F_M means that the plant experienced stress before measurements that damaged the PSII functions and depressed the efficiency of electron transfer. This depression is often observed in plants exposed to various stressors, to bright light in particular. One example is the depression of F_V/F_M in potato plants grown under moderate drought at high light intensities. After watering these plants, the F_V/F_M parameter increased gradually to the level characteristic of control plants [59]. The change in F_V/F_M value is considered to be the most sensitive indicator of photoinhibition (suppression of photosynthesis and damage to the photosynthetic apparatus under high light intensity) [60].

The F_V/F_M ratio is not proportional to the photosynthetic rates, as measured from O_2 evolution or CO_2 assimilation, and it does not always correlate with the chlorophyll content in leaves and fruits [61]. Changes in F_V/F_M ratio can also be caused by nonphotochemical quenching of the excited states of chlorophyll molecules [48]. Studies have shown that the F_V/F_M ratio measured in thylakoid membranes decreases in the presence of inorganic anions, such as Cl^-, SO_4^{2-}, and PO_4^{3-}, which might be related to the increased fluidity (lowered viscosity) of thylakoid membranes. The presence of organic anions, such as acetate, succinate, and citrate, did not cause any changes in electron transport rates and the F_V/F_M ratio [62]. The F_V/F_M parameter can be also used as an indicator for degradation of D1-protein (an important PSII constituent) that is damaged by the excess light stress or modified by other impacts resulting in inactivation of photosynthetic reaction centers [63].

In some water-stressed plants, e.g., in subtropical plant species *Vigna unguiculata*, no significant changes in chlorophyll *a* fluorescence were observed [64], indicating that the photochemical activity of PSII remained unimpaired. Under prolonged exposure to stress, the parameter F_V/F_M was found to decrease, which resulted presumably from stress-induced nonradiative dissipation of excitation energy (i.e., the increased nonphotochemical quenching) and from the reduced efficiency of trapping the excitation energy by open reaction centers. However, during long-term stress, a significant decrease in F_V/F_M was observed, which was also accompanied by the decrease in the rate of photosynthesis. In 3 days after plant watering, the initial rate of photosynthesis was restored, and the fluorescence parameters become similar to those recorded before the stress treatment [64].

When potato plants were treated by freezing temperatures (−3°C) at high-intensity light, the F_V/F_M decreased substantially in all the genotypes examined. This decrease in F_V/F_M could be explained by inhibition of the dark enzyme reactions of photosynthesis on the background of comparatively efficient electron transport in the light reactions of photosynthesis. In this case, the high-energy electrons are transferred from PSI to oxygen, giving rise to the reactive oxygen species [65].

A_M, Area above the Induction Curve of Chlorophyll Fluorescence

The extent of A_M or S_M, the area above the FL induction curve, is proportional to the pool size of electron acceptors in PSII. Measurements of A_M are important for applied research, such as analysis of photosynthesis inhibition by herbicides, e.g., 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and the dynamics of herbicide delivery to leaves. The A_M can be measured in the conventional units of bms (bit–millisecond), which is the product of the fluorescence signal measured in bits and the length of time (in ms) elapsed from the point F_O to F_M. The faster F_M is attained (rapid reduction of the pool of PSII electron acceptors), the smaller is the area above the induction curve of chlorophyll FL. When electron transport from the reaction centers to the pool of plastoqui-

nones is inhibited under stress conditions, the A_M area decreases [51]. A parameter similar to A_M is its normalized presentation, S_M ($S_M = A_M/F_M$). This parameter also determines the amount of nonreduced electron acceptors in PSII, but it is linked to the total content of photochemically active chlorophyll *a* in the analyzed material [66].

F_T, Stationary Fluorescence

The parameter F_T (sometimes designated as F_S) denotes the intensity of chlorophyll fluorescence emitted by photosynthesizing objects under stationary lighting. After illumination of dark-adapted samples, the steady state on the induction curve of chlorophyll FL (F_T level) is established within 3–5 min. By this time, the equilibrium is achieved between the photochemical production of assimilative power (ATP and NADPH) and enzymatic reactions consuming these resources in the dark stage of photosynthesis. Any disturbance of photosynthetic reactions (e.g., by stress factors) delays the attainment of the steady state (F_T).

The F_T value depends on the photosynthetic efficiency and physiological condition of photosynthesizing objects, which are, in turn, determined by external environmental factors, such as actinic irradiance. After the treatment of leaves with a herbicide paraquat capable of capturing electrons from the reduced PSI acceptors with the resulting production of hydrogen peroxide, the F_T level is established immediately [67].

F_T/F_0, Ratio of Stationary and Zero-Time Fluorescence

This parameter is inversely correlated with nonphotochemical quenching. At high light intensities, it shows a negative correlation with the electron transport rate, assimilation of carbon dioxide, and the stomatal conductance. The parameter F_T/F_0 is a suitable indicator of the plant water status [6].

F_M', Maximal Chlorophyll a Fluorescence in Light-Adapted Leaves

This parameter is determined by applying saturating light pulses on the background of continuous photosynthetically active (actinic) illumination. The values of F_M' are lower than the F_M values determined in dark-adapted leaves, because the PSII acceptors are partly reduced under actinic illumination.

The difference $F_M' - F_T = \Delta F$ represents the portion of chlorophyll fluorescence that is quenched by the photochemical reactions.

The difference $F_M - F_M' = \Delta E$ corresponds to quenching of chlorophyll *a* fluorescence due to nonphotochemical events [48].

F_0', Minimal Fluorescence in Illuminated Samples

This parameter is usually measured when an additional pulse of far-red illumination with $\lambda \approx 735$ nm is applied on the background of photosynthetically active light, which rapidly oxidizes the PSII electron acceptors. The dominant excitation of PSI under these conditions removes electrons from PSII and opens the majority of PSII reaction centers. The fluorescence F_0' represents the minimal (zero) fluorescence in light-adapted samples; F_0' is a measure of fluorescence under complete oxidation of the acceptor Q_A in the presence of nonphotochemical quenching.

$F_V' = F_M' - F_0'$, Variable Fluorescence in Light-Adapted Leaves

The parameter F_V' is proportional to the actual quantum yield of PSII in the light (on the background of continuous actinic illumination). The magnitude of F_V' is affected by nonphotochemical quenching (qN).

$Rfd = (F_M - F_T)/F_T$, Vitality Index of PSII

The parameter Rfd (relative fluorescence decrease) is indicative of interactions between the light-stage reactions activated by PAR absorption and the dark reactions of photosynthesis. This parameter is diminished when the balance between photochemical reactions in thylakoids and the rates of enzymatic reactions in the chloroplast stroma is disturbed [68]. Under variations of PAR level, the vitality index can be regarded as a measure of the potential photosynthetic activity. Lichtenthaler et al. [52] in experiments with tree leaves exposed to full light observed Rfd values above 2.7, while with shaded leaves this index varied from 1 to 2.7. A positive linear correlation between the Rfd values and the rate of photosynthetic CO_2 assimilation was found but only when the stomata were open.

The Rfd values of approximately 2.5 or higher indicate a high photosynthetic activity, whereas the values below 1.0 suggests strong suppression of CO_2 assimilation [69]. In plants exposed to various stresses, the fluorescence F_T is usually increased whereas the F_M remains comparatively constant; thus, the difference $F_M - F_T$ is lowered and, accordingly, the Rfd value decreases [52].

$Ap = 1 - (1 + Rfd_{730})/(1 + Rfd_{690})$, Stress Adaptation Index

The parameter Ap can be determined by simultaneous measurements of Rfd at two wavelengths, for example, 690 and 730 nm. This index is high in young healthy leaves exhibiting high photosynthetic activity. The Ap values ranging from 0.1 to 0.2 are indicative of stress experienced by plants [24, 70].

Parameters of Photochemical Fluorescence Quenching

The depression of chlorophyll *a* fluorescence signal is called quenching. The sharp decrease in the intensity of chlorophyll *a* FL associated with the increased use of excitation energy in photosynthetic reactions is called photochemical quenching. If the depression in fluorescence signal results from dissipation of excitation energy in the form of heat, it is called nonphotochemical quenching. Photochemical quenching parameters correspond to that part of the absorbed light energy that can be used in photochemical reactions of photosynthesis [48].

$$Quantum\ yield = (F_M' - F_T)/F_M' = \Delta F/F_M'$$

The quantum yield parameter (Genty parameter, Φ_{PSII}, or Yield) delineates the quantum yield of the photochemical reaction in PSII. This is the most popular and important parameter; it represents the number of photons used in photochemical transformations normalized to the total amount of the absorbed PAR quanta [71]. Under laboratory conditions, the values of Genty parameter are linearly related to the assimilation rate of carbon dioxide in photosynthesis. These two characteristics may diverge under stress conditions when the efficiency of carboxylation is altered due to activation of photorespiration or pseudocyclic electron transport [72]. In plants experiencing drought stress, the effectiveness of CO_2 assimilation reactions usually decreases because of stomata closing, which, in turn, reduces the consumption of ATP and NADPH. This retardation of electron transport in photosystems I and II should result in the decrease of Genty parameter.

However, activation of photorespiration and acceleration of the Mehler reaction can also normalize the electron transport in plants grown under stress-free conditions. For example, Flexase et al. [73] found in experiments with grape leaves that closing of the stomatal aperture by 75% depressed photosynthetic rate by 54%, whereas the PSII quantum yield was diminished by only 19%.

The PSII yield decreases in plants stressed by low temperature, because cooling suppresses the activity of the Calvin–Benson cycle reactions known to consume ATP and NADPH; the retarded consumption of these products slows down electron transport in the light stage of photosynthesis [74]. Interruption of the cooling period and the increase in temperature led to the slight decrease in photochemical efficiency of PSII in maize leaves, which indicates that other factors, apart from photochemical efficiency of PSII, could be responsible for the overall suppression of photosynthesis upon cooling [75]. According to Fracheboud [67], the cooling-induced decline in the PSII yield might also be due to the decreased fluidity of thylakoid membranes.

In some cases, the photochemical efficiency (yield, Φ_{PSII}) and photochemical quenching (qP) do not show positive correlation with the amount of assimilated carbon dioxide, CO_2. Schindler and Lichtenthaler [76] observed that Φ_{PSII} and qP in maple leaves exposed to direct sunlight decreased to nearly zero, while photosynthesis was inhibited by only 30%. The authors explained this discrepancy as being due to the fact that fluorescence measurements were limited to the chloroplasts located on the upper leaf side.

Another complication is that the photosynthetic rates are low at dim irradiance at which the efficiency of light conversion into the energy of chemical bonds in photosynthetic products is very high, while the converse relations hold true at high-intensity light.

On the other hand, some studies revealed strong positive correlation between the photochemical efficiency of PSII and the yield of CO_2 fixation (Φ_{CO_2}) [77].

ETR = Yield × 0.84 × 0.50 × PPFD, Electron Transport Rate through Photosystems

The parameter ETR is the product of the quantum yield (Φ_{PSII}) and the photon flux density (PPFD) expressed in μmol photons/(m^2 s) multiplied by a factor of 0.50.

Thus, it can be calculated that the electron flux through the photosystems constitutes one half of the photon flux trapped by the reaction centers (the coefficient of 0.84 stems from the assumption that 84% of the photons absorbed by a standard leaf are delivered to the reaction centers). The factor 0.5 takes into account that the transfer of one electron through electron transport chain requires the energy of two quanta of the absorbed PAR (one quantum for PSII and the second for PSI).

Stressful factors reduce the value of this parameter. For example, the electron transport rate in potato under drought conditions was significantly lower than in the control plant group [59]. The parameter ETR decreased in barley plants exposed to nitrogen deficiency [78] and salinity treatment [54].

$$qP = (F_M' - F_T')/(F_M' - F_0),\ Photochemical\ Quenching$$

The parameter qP is the fraction of light energy consumed by the open centers for photosynthetic reactions with respect to the total amount of energy absorbed by PSII (table). Changes in qP are caused by the closure of the reaction centers due to the saturation of photosynthesis with actinic light. The parameter qP quantifies the fraction of PSII open reaction centers, and the expression (1 – qP) corresponds to the fraction of closed centers [48].

Relationship between the measured chlorophyll *a* fluorescence parameters qP and qN (photochemical and nonphotochemical quenching), the functional state of PSII, and the proton concentration gradient across the thylakoid membrane [80]

Parameter	Reaction center of PSII	ΔpH	qP	qN
F_O	All RCs are open	Absent	1	0
F_O'	Open	Low	1	$0 < qN \leq 1$
F_M	All RCs are closed	Absent	0	0
F_M'	All closed	Low	0	$0 < qN \leq 1$
F_V	Closing of RCs during the saturating pulse after dark adaptation	–	$1 \rightarrow 0$	0
F_V'	Closing of RCs during the saturating pulse under actinic light	–	$X \rightarrow 0$	$0 < qN \leq 1$
F_T	Some RCs are closed	Low	$0 \leq qP \leq 1$	$0 < qN \leq 1$

$F_V'/F_M' = (F_M' - F_0)/F_M'$, *Efficiency of Open PSII Reaction Centers in the Light*

The decrease in F_V'/F_M' ratio might be caused by deepoxidation of xanthophyll pigments in the xanthophyll cycle, which elevates the pigment ratio (A + Z) / (V + A + Z). The observed relations indicate the role of this cycle in regulation of PSII activity in natural conditions. In bright light (e.g., at noon), zeaxanthin (Z) and antheroxanthin (A) are the most abundant pigments, whereas violaxanthin (V) is predominant at nights [67].

Parameters of Nonphotochemical Fluorescence Quenching

Nonphotochemical quenching (qN, NPQ) results from the dissipation as heat of the part of the energy absorbed during the light stage of photosynthesis [48]. Such processes are activated to prevent the excess light absorption and photoinhibition in normal plants or in plants damaged by other stress factors. In these cases, the rate of PSII damage exceeds the rate of PSII repair.

$qN = (F_M - F_M')/(F_M - F_0) = (F_M - F_M')/F_V$, *Nonphotochemical Quenching*

The values of qN vary from 0 to 1 [77]. Hartel et al. [79] supposed that nonphotochemical quenching originates mainly from structural changes caused by the operation of the xanthophyll cycle. This ratio is regulated by small pH changes on both sides of the thylakoid membrane [67].

The pretreatment of leaf samples with the protonophore nigericin capable of eliminating the proton gradient across thylakoid membranes inhibited nonphotochemical quenching of chlorophyll *a* fluorescence under exposure to saturating light. In the leaves untreated with nigericin, the major part of fluorescence was quenched due to the dissipation of excitation energy as heat, which was evident from the large difference between F_M and F_M' and, accordingly, from the increase in qN [67]. Rosenqvist and Kooten [80] found that values of photochemical (qP) and nonphotochemical (qN) quenching depend on the functional condition of PSII and on the difference of proton concentrations on the opposite sides of the thylakoid membrane (Table).

Illumination of leaves with the high intensity light is accompanied by the increase in the content of zeaxanthin (Z) and antheraxanthin (A) as a result of violaxanthin (V) deepoxidation to A and Z in the xanthophyll cycle. These conversions promote the dissipation of absorbed energy as heat and, accordingly, diminish the excitation energy transferred to PSII reaction centers, thus protecting the plants from photoinhibition [81]. Conversely, under low light conditions, A and Z are epoxidized to form V, which reduces the heat losses of energy and increases the efficiency of PAR utilization [82].

$NPQ = (F_M - F_M')/F_M'$, *Nonphotochemical Quenching*

NPQ (SV_N, Stern–Volmer type of quenching) is a parameter alternative to qN that quantifies nonphotochemical quenching [83]. This parameter is associated with heat losses; it can range from zero to infinity. For the majority of healthy plants, NPQ values are 0.5–3.5, although they differ in various species or plants cultivated at different growth conditions [48, 52].

The plants grown under high light intensities can lose 50–70% of the absorbed photon energy as heat, with the involvement of the xanthophyll cycle [84]. Niyogi et al. [85] found that the xanthophyll cycle accounts for 80% of NPQ in *Arabidopsis thaliana*. Using barley plants adapted to high and low photon flux densities (1500 and 375 μmol/(m^2 s)), Kovar et al. [86] observed the enhancement of NPQ at increasing PAR levels, with NPQ being stronger in plants adapted to low light and weaker in plants adapted to high-intensity light. These changes were paralleled by

the decrease in qP, which was larger in plants grown at high irradiance and smaller in plants grown at low irradiance.

JIP (OJIP) TEST

General Description of the Test

This chapter describes relationships between photosynthetic reactions and quantitative characteristics of the chlorophyll fluorescence signal (JIP-test parameters), as well as the practical application of these parameters. The JIP-test parameters are based on the theory of energy fluxes in thylakoid membranes [87, 88]. This theory offers the basis to derive simple algebraic equations expressing the balance between energy influx and efflux for any system of photosynthetic pigments; it provides information about the probable fate of the absorbed energy. These equations characterize the energy-based interaction between individual components of PSII for various types (models) of photosystem organization, known as "grouping" or "connectivity" or "overall grouping probability," and describe all the ways underlying these interactions [11].

The JIP-test is based on measurements of chlorophyll *a* FL and on analysis of signals providing detailed information on the structure and function of PSA (primarily PSII). The models underlying the JIP-test describe the primary photosynthetic reactions by taking into account the structure of PSA in full consistency with the theory of energy fluxes occurring in the thylakoid membrane between the complexes of photosynthetic pigments in PSII. In the past few years, determinations of JIP-test parameters have been increasingly used in various fields of plant biology as a means to assess physiological conditions of PSII [46, 89].

The physiological condition of PSA is evaluated by analyzing several groups of biophysical parameters that are measured and calculated in various ways [22, 90]:

(1) ***Phenomenological energy fluxes*** are calculated per unit area of the photosynthetic object (e.g., per unit area of illuminated leaf surface).

(2) ***Specific energy fluxes*** are calculated per one operating reaction center of PSII.

(3) ***Quantum yields*** represent the number of electrons transferred at a certain step of the light stage of photosynthesis in proportion to the number of photons absorbed by PSII.

(4) ***Efficiencies*** reflect the probability of electron transfer via the given site of electron transport chain.

(5) ***PSA performance*** indexes represent the products of specific potentials at the successive stages of energy transduction.

(6) **The fraction of reaction centers** in the total chlorophyll pool is used to distinguish the relative number of reaction centers that are *able* to reduce the PSII primary acceptor Q_A and those that are *unable* to reduce Q_A. The latter RCs are termed heat sinks or silent reaction centers (RCsi); they do not reduce Q_A and, at the same time, do not return the excitation energy to the light-harvesting antenna. The respective PSII structures do not contribute to the variable fluorescence and have an FL yield as low as in open PSII reaction centers. These centers can be activated after the recovery of a plant from stress that caused their inactivation. This test allows researchers to analyze the function of that part of the reaction centers (reopened centers) that cannot reduce the secondary plastoquinone acceptor Q_B (the so-called slow reopening Q_B-nonreducing RCs) and to evaluate the probability of energy fluxes between various PSII components.

The name OJIP is derived from the adopted names for characteristic points on the curve of the fluorescence induction, reflecting the change in FL signal after illumination of the photosynthesizing object (Figs. 8, 9). During the O–J phase, the primary electron acceptor Q_A in PSII is gradually reduced, and the dynamic equilibrium (quasi-steady state) at the point J is achieved between the Q_A reduction and its oxidation. The increase in chlorophyll *a* FL during the J–I phase reflects the shift of the quasi-stationary state at the Q_A level toward further reduction of Q_A, which is caused by the light-induced reduction of the plastoquinone pool. The further increase in the quantum yield of chlorophyll *a* FL in the I–P phase reflects the gradual reduction of PSI acceptors and the full reduction of the PQ-pool.

Analysis of OJIP transients allows researchers to better understand relationships between the structure and function of the photosynthetic apparatus and quickly assess the plant viability [22, 29, 46].

The test is accomplished by measuring chlorophyll *a* fluorescence signal digitized at short separation intervals, starting from 40–50 μs up to 1 s. During measurements, the most important FL values are recorded at specified times of 50 μs, 100 μs, 300 μs, 2 ms, 30 ms, and 1 s. These values can be used to calculate several JIP-test indices, such as ABS/CS, TR/CS, ET/CS, RC/CS, and DI/CS, which are used to assess the functioning of PSII. The electron transport rates in PSII depend on the rate of NADPH consumption in dark reactions of photosynthesis, as well as on the rate of assimilate utilization by the whole plant. This, in turn, depends on the plant physiological condition and on the influence of various external factors, including stress factors.

The JIP-test extends possible applications of fluorescence studies and ensures rapid assessment of photosynthesis under impact of various stress factors [2, 29, 46, 91].

Under certain stress conditions (high temperature, high intensity irradiation, or extreme nitrogen defi-

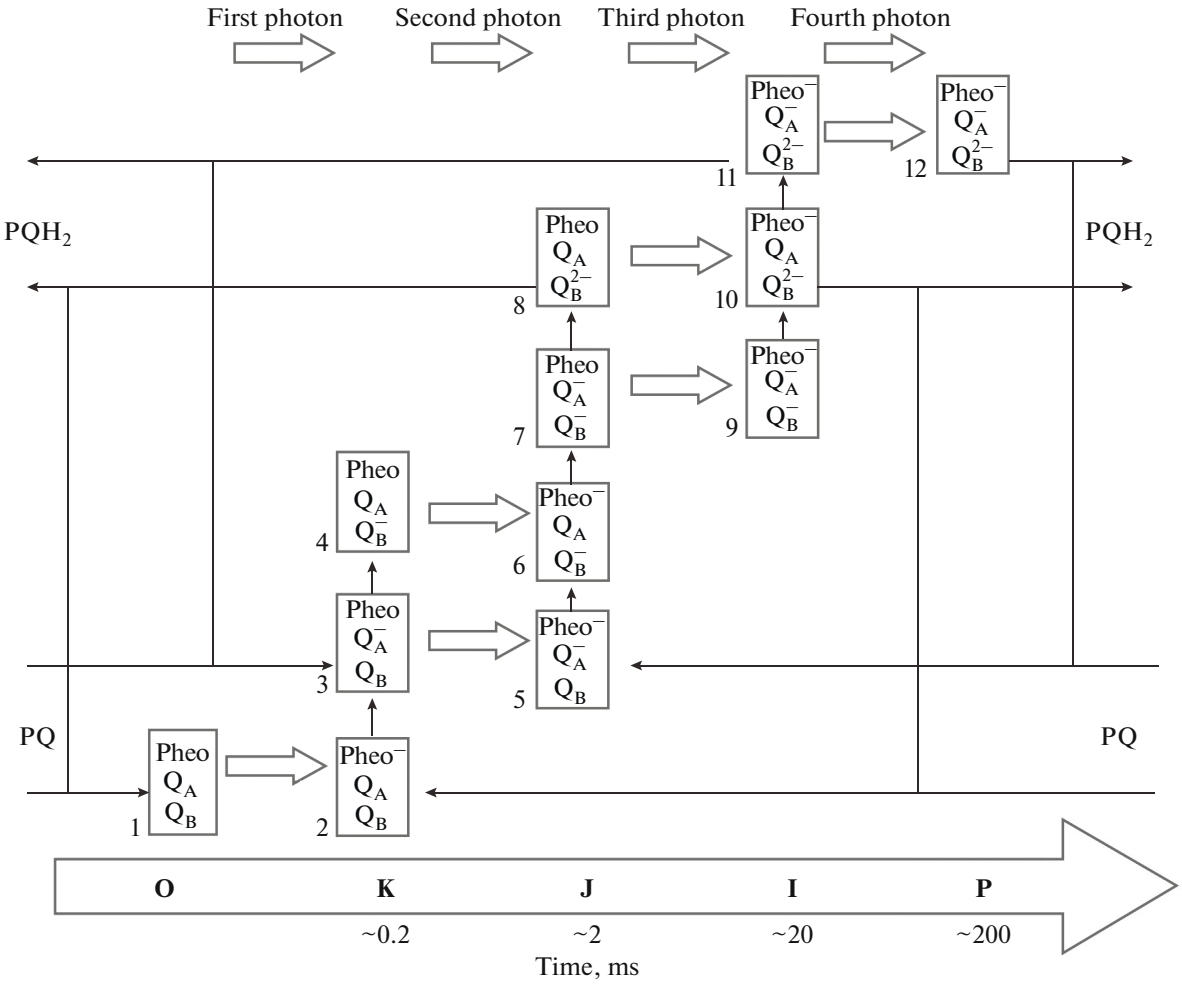

Fig. 8. Redox state of the electron carriers, pheophytin (Pheo) and quinones (Q_A and Q_B) on the acceptor side of PSII at various moments of OJIP induction curve (modified from [91]).

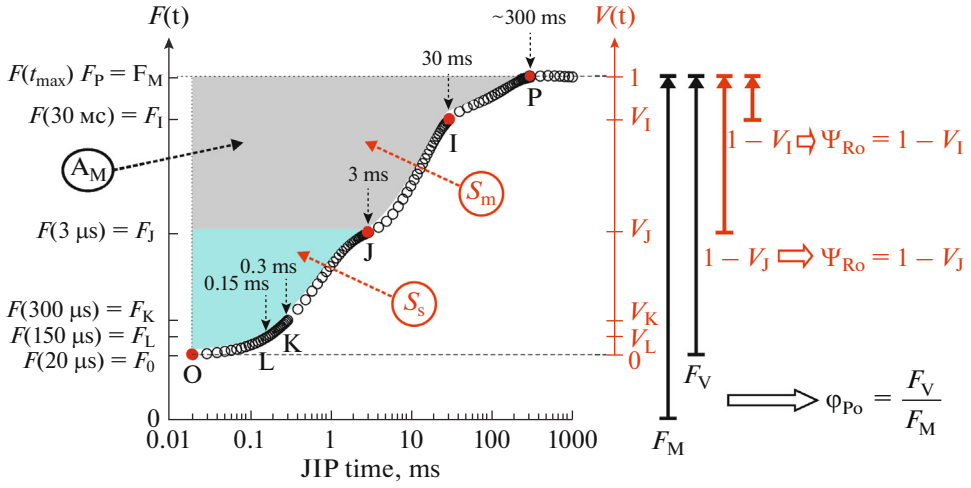

Fig. 9. Induction curve of variable chlorophyll *a* fluorescence recorded over 1-s illumination with red light at PFD of 4000 μmol/(m^2 s) (modified from [2, 91]). Arrows indicate the time of achieving the characteristic points during the OJIP transition. The left scale shows the measured fluorescence values; the right scale shows relative values of variable fluorescence.

ciency) the water-splitting complex is inhibited, which blocks the electron transfer from OEC active center to tyrosine [92, 93]. In this case, the induction curve of chlorophyll *a* FL acquires a specific phase at 200–300 μs, i.e., a local maximum called the K-peak (point K in Fig. 9). The relative increase in fluorescence at this point indicates some disorders in redox reactions associated with the decomposition of water.

Parameters Used in the JIP (OJIP) Test

Parameters characterizing the absorption of PAR energy and electron transport are called *specific* when they are calculated on a per RC basis, and they are termed *phenomenological* if calculated per unit area (cross section, CS) of the photosynthesizing object. In order to comprehend the key relationships used in the test, the following abbreviations are introduced:

RC—the photochemically active reaction center of PSII capable of reducing Q_A;

CS—unit cross section area (CS) of photosynthetic objects exposed to incident excitation light;

O and M (P)—indices referring to extreme values of chlorophyll *a* fluorescence (F_0 and F_M);

ABS—photon flux absorbed by the pigment molecules in the antenna.

Data Obtained Directly from Fluorescence Measurements [29]

F_t—fluorescence emitted at time t counted from the onset of actinic illumination (AL);

$F_{50\,\mu s}$ or $F_{20\,\mu s}$—minimum fluorescence signal (corresponding to F_O) recorded at 50 μs or 20 μs;

$F_{100\,\mu s}$—fluorescence at $t = 100$ μs;

$F_{300\,\mu s}$—fluorescence at $t = 300$ μs;

$F_J \equiv F_{2\,ms}$—fluorescence at $t = 2$ ms, during the J phase;

$F_I \equiv F_{30\,ms}$—fluorescence measured 30 ms after the onset of illumination (at the I phase);

$F_P = (F_P \cong F_M)$—maximum fluorescence during the P phase;

T_{Fm}—time (in ms) required to reach the maximum fluorescence F_M;

A_M—the area above the fluorescence induction curve.

Fluorescence Parameters Derived from the Measured Data

F_O—minimum fluorescence recorded when all PSII reaction centers are open;

F_M (F_P)—maximum fluorescence emitted when all PSII reaction centers are closed;

$F_V = F_t - F_O$—variable fluorescence at any time instant t;

F_V/F_O—ratio of rate constants for photochemical and nonphotochemical use of RC excitation energy;

$V_t = (F_t - F_O)/(F_M - F_O)$—normalized variable fluorescence at any time instant t;

$V_J = (F_J - F_O)/(F_M - F_O)$—normalized variable fluorescence at the stage J (2 ms after switching on the light); it represents the number of closed RCs relative to the total number of RCs that can be closed;

$V_I = (F_I - F_O)/(F_M - F_O)$—normalized variable fluorescence at the stage I (30 ms) that is related to the intermediate steady-state reduction of the plastoquinone pool. It reflects the ability of PSI and its acceptors to oxidize reduced plastoquinone;

$W_{OJ} = (F_t - F_O)/(F_J - F_O)$—relative variable fluorescence normalized to the amplitude of the J phase $(F_J - F_O)$;

$W_{OK} = (F_t - F_O)/(F_K - F_O)$—relative variable fluorescence normalized to the amplitude of K phase $(F_K - F_O)$;

$W_{E,100\,\mu s} = 1 - (1 - W_{300\,\mu s}) \times 1/5$—the W value at $t = 100$ μs, which simulates the exponential growth of fluorescence in the sample in the absence of connectivity between individual photosynthetic units of PSII;

$M_0 = (\Delta V/\Delta t) = 4(F_{300\,\mu s} - F_{50\,\mu s})/(F_M - F_O) =$ TRo/RC – ETo/RC—averaged initial slope (expressed in ms^{-1}) of relative variable fluorescence of chlorophyll *a*, $V = f(t)$. This parameter reflects the rate of closing the PSII reaction centers. It represents the maximum rate of Q_A reduction on the initial stage, whereas the reduced Q_A molecules can be reoxidized at later stages by electron carriers located beyond Q_A in the electron transport chain.

$S_M = (A_M)/(F_M - F_0)$—normalized total area above the OJIP curve; it reflects the capacity of the intersystem pool to accept electrons until full reduction of Q_A;

$S_S = V_J/M_0$—normalized total area above the OJ curve, which reflects a single act of Q_A reduction; the minimum S_S values are observed when each Q_A is reduced by only one electron (e.g., in the presence of DCMU);

$N = (S_M/S_S) = S_M M_0\,(1/V_J)$—the number of turnovers, i.e., the sum of Q_A reduction acts taking place during the FL induction from $t = 0$ to t_{FM}; N is number of electrons needed for complete reduction of all acceptors located beyond Q_A;

$V_{av} = 1 - (S_M/t_{Fm})$—variable fluorescence averaged over the time frame from $t = 0$ to t_{Fm};

S_M/t_{Fm}—index quantifying the average excitation energy of open reaction centers in the period from $t = 0$ to t_{Fm}, i.e., over the period required for complete closing of RCs [29].

Specific Energy Fluxes per Q_A-Reducing PSII Reaction Center

ABS/RC = M_0 $(1/V_J)$ $(1/\varphi_{Po})$—absorption energy flux per active reaction center (RC); it reflects the proportion between the amounts of chlorophyll *a* molecules in fluorescence-emitting antenna complexes and in the active reaction centers [22, 29];

RC/ABS = $Chl_{RC}/(1 - Chl_{RC})$—indicator of efficiency expressed as the concentration of reaction centers (RC) in the total pool of chlorophylls (Chl);

$TR_0/RC = M_0$ $(1/V_J)$—flux of excitation energy trapped per active reaction center (RC) at the start of illumination of a dark-adapted sample, i.e., at $t = 0$;

$ET_0/RC = M_0$ $(1/V_J)$ ψ_0—the electron flux transferred per active reaction center (RC) at $t = 0$;

$RE_0/RC = M_0$ $(1/V_J)(1 - V_I)$—the electron flux transferred per active reaction center (RC) and reducing terminal acceptors on the acceptor side of PSI (at $t = 0$);

DI_0/RC = (ABS/RC) − (TR_0/RC)—total energy dissipated per reaction center (RC) as heat, fluorescence, and energy transfer to PSI (at $t = 0$).

Quantum Yields or Flux Ratios

$\varphi_{Po} \equiv TR_0/ABS = [1 - (F_O/F_M)] = F_V/F_M$—maximum quantum yield of primary photochemical reactions (at $t = 0$) that indicates the probability of trapping the energy of absorbed photons (or excitons migrating over the antenna) by PSII reaction centers. In plants stressed by heat or high intensity light, the φ_{Po} values are usually lowered.

$\varphi_{Po}/(1 - \varphi_{Po}) = F_V/F_O$—indicator of the effectiveness of primary photochemical reaction (electron transfer to Q_A^-);

$\varphi_{Eo} = ET_0/ABS = [1 - (F_O/F_M)]$ $\psi_0 = \varphi_{Po}$ ψ_0—quantum efficiency of electron transfer from Q_A^- to electron transport chain beyond Q_A^- (at $t = 0$);

$\psi_0 \equiv ET_0/TR_0 = (1 - V_J)$—probability of electron transport beyond Q_A^- (at $t = 0$); i.e., the efficiency with which the exciton trapped by RC drives the electron along ETC beyond Q_A;

$\psi_0/(1 - \psi_0) = ET_0/(TR_0 - ET_0)$—by definition ψ is expressed as ET/TR; hence, $\psi_0/(1 - \psi_0) = ET/(TR - ET)$, where (TR − ET) is the number of electrons delivered and accumulated on Q_A^-. Thus, the expression $\psi_0/(1 - \psi_0)$ is the ratio of electrons removed from the system and electrons accumulated in the system. The net accumulation of Q_A^- (dQ_A^-/dt) is the factor responsible for the increase in fluorescence signal. This parameter describes the capacity of electron transport to proceed beyond Q_A^-.

$\delta_{Ro} = RE_0/ET_0 = (1 - V_I)/(1 - V_J)$—probability with which the electron residing on intersystem carriers is able to reduce the terminal electron acceptors on the acceptor side of PSI (RE);

$\varphi_{Ro} = \varphi_{Po}$ φ_{Eo} $\delta_{Ro} = RE_0/ABS = \varphi_{Po}(1 - V_I)$—quantum yield for the reduction of terminal electron acceptors on the acceptor side of PSI (RE);

$\gamma_{RC} = Chl_{RC}/Chl_{total} = RC/(ABS + RC)$—probability that a given chlorophyll molecule functions as the reaction center of PSII;

$\varphi_{Do} \equiv 1 - \varphi_{Po} = (F_0/F_M)$—quantum efficiency of energy dissipation (at $t = 0$);

$\varphi_{Pav} = \varphi P_o = (1 - V_{av}) = \varphi_{Po}$ (Sm/t_{Fm})—average quantum yield of primary photochemical reactions (in the time frame from 0 to t_{Fm}).

Phenomenological Energy Fluxes per Excited Cross Section of a Photosynthesizing Object

ABS/CS_x—energy absorption per unit cross section (CS) of a photosynthesizing object and chlorophyll (CHL) at time zero ($t = 0$) or at the time of achieving the maximum fluorescence F_M (M). This quantity represents the amount of photon energy absorbed by the antenna associated with active and inactive reaction centers of PSII. This amount depends on the chlorophyll concentration in the tested sample, because the rate of energy absorption (ABS) by antenna molecules becomes equal at equilibrium to the sum of rate constants for disposal of excitation energy through all possible quenching pathways, multiplied by chlorophyll concentration, i.e.:

$$ABS = (k_n + k_p) \times (CHL), \quad (6)$$

where k_p is the rate constant of photochemical reactions and k_n is the rate constant for nonphotochemical dissipation of excitation energy [29, 94].

ABS/CS_{Chl}—absorbed excitation energy per unit cross section (CS) of a photosynthesizing object and the amount of chlorophyll *a* determined by reflectance measurements (parameter Chl/CS);

$ABS/CS_0 \approx F_0$—energy absorbed per unit cross section (CS) of a photosynthesizing object at the onset of measurement (at $t = 0$), equal approximately to F_0;

$ABS/CS_M \approx F_M$—energy absorbed per unit cross section (CS) of a photosynthesizing object at the moment of achieving the fluorescence maximum ($t = t_{Fm}$), equal approximately to F_M;

$TR_0/CS_x = \varphi_{Po} \cdot (ABS/CS_x)$—energy flux trapped by PSII reaction centers per unit cross section (CS) of a photosynthesizing object at $t = 0$;

$ET_0/CS_x = \varphi_{Eo} \cdot (ABS/CS_x)$—electron flux through PSII per unit cross section (CS) of a photosynthesizing object at $t = 0$;

$DI_0/CS_x = (ABS/CS_x) - (TR_0/CS_x)$—thermal dissipation of energy in PSII per unit cross section (CS) of a photosynthesizing object at $t = 0$.

Performance Indexes at t = 0 and Density of Active PSII Reaction Centers

$$PI_{ABS} = \frac{RC}{ABS}\frac{\varphi_{Po}}{1-\varphi_{Po}}\frac{\psi_0}{1-\psi_0} = \frac{\gamma}{1-\gamma}\frac{\varphi_{Po}}{1-\varphi_{Po}}\frac{\psi_0}{1-\psi_0}$$

—performance index, an indicator of PSII functional activity normalized to the absorbed energy;

$$PI_{CS} = PI_{ABS}\frac{ABS}{CS_x} = \frac{RC}{CS_x}\frac{\varphi_{Po}}{1-\varphi_{Po}}\frac{\psi_0}{1-\psi_0}$$

—performance index, an indicator of PSII functional activity expressed per unit cross section of illuminated area;

$$PI_{total} = PI_{ABS}\frac{\delta_{Ro}}{1-\delta_{Ro}}$$

—total performance index, indicating the integral functional activity of PSII, PSI, and intersystem electron transport chain. All parameters used for calculating the three performance indexes were described above.

$RC/CS_x = \varphi_{Po}(V_J/M_0)(ABS/CS_x)$—density of reaction centers capable of Q_A reduction.

Driving Forces—Logarithms of Performance Indexes at t = 0

$$DF_{ABS} = \log(PI_{ABS}) = \log\left(\frac{RC}{ABS}\right) + \log\left(\frac{\varphi_{Po}}{1-\varphi_{Po}}\right) + \log\left(\frac{\psi_0}{1-\psi_0}\right)$$

—indicator of driving forces in PSII with respect to absorption. This index can be used to estimate the sum of individual components (indexes) involved in the PSII-driven processes. The terms in the equation represent the contributions of driving forces (DF) with regard to the concentration of PSII active reaction centers, the primary photochemical reaction, and reoxidation of reduced quinone Q_A^-, respectively:

$$DF_{ABS} = DF_{RC} + DF_{light\ reactions} + DF_{dark\ reactions}, \quad (7)$$

where $DF_{RC} = \log(RC/ABS)$,

$DF_{light\ reactions} = \log(\varphi_{P_0})/(1-\varphi_{P_0})) = \log F_V/F_O$, and

$DF_{dark\ reactions} = \log(\psi_0/(1-\psi_0)) = \log((1-V_J)/V_J)$.

$$DF_{CS} = \log(PI_{CS}) = \log\left(\frac{ABS}{CS_x}\right) + \log\left(\frac{RC}{ABS}\right) + \log\left(\frac{\varphi_{Po}}{1-\varphi_{Po}}\right) + \log\left(\frac{\psi_0}{1-\psi_0}\right)$$

—indicator characterizing the driving forces in PSII per unit cross section (CS) of the photosynthesizing object.

Overall Grouping Probability of the Reaction Centers

As mentioned above, the energy of photons absorbed by chlorophyll molecules in PSII antenna complexes is transferred with a high probability to the reaction centers. The energy trapped by open RCs is used for photochemical reactions, while closed RCs radiate it as heat or fluorescence. When the object under study contains a mixture of open and closed RCs, the efficiency of photosynthetic reactions (and variable fluorescence intensity) will depend on the possibility of excitation energy transfer from the antenna complexes of closed RCs to open centers. The interaction between photosynthetic units is reflected in the shape of the FL induction curve. The interaction of PSII centers at the level of antenna complexes can be quantified by the p_{2G} coefficient, called the grouping probability. Its value can be calculated from the parameters of FL induction curve in its initial segments:

$$p_{2G} = \frac{(W_{E,100\ \mu s} - W_{100\ \mu s})F_O}{W_{100\ \mu s}(1 - W_{E,100\ \mu s}V_J)V_J F_V}, \quad (8)$$

where:

$$W_{E,100\ \mu s} = 1 - (1 - W_{100\ \mu s}) \times 1/5,$$

$$W_{100\ \mu s} = \frac{F_{100\ \mu s} - F_{50\ \mu s}}{F_{2\ ms} - F_{50\ \mu s}}, \text{ and}$$

$$W_{300\ \mu s} = \frac{F_{300\ \mu s} - F_{50\ \mu s}}{F_{2\ ms} - F_{50\ \mu s}}.$$

The overall grouping probability takes into account all possible ways of energy transfer between neighboring antenna complexes of PSII and indicates the probability for the use of the absorbed energy in photochemical reactions.

Structure–Function Indexes

$$SFI_{P_0(ABS)} = (Chl_{RC}/Chl_{tot})\varphi_{P_0}\psi_0.$$

This index provides structural and functional information about the strength of the influence of internal factors promoting the reactions in PSII.

The inverse parameter, $SFI_{No\ (ABS)}$ reflects the processes related to inhibition of reactions in PSII:

$$SFI_{N_0(ABS)} = (1 - Chl_{RC}/Chl_{tot})(1 - \varphi_{P_0})(1 - \psi_0), \quad (9)$$

where Chl_{tot} is the total content of chlorophyll *a*: $Chl_{tot} = Chl_{antenna} + Chl_{RC}$.

The parameters of JIP-test can be divided into two groups. The parameters F_O/F_M, $V_{100\ \mu s}$, M_0, and V_J provide information about the elementary act of Q_A reduction, whereas the parameters V_I, S_m, and t_{Fm}

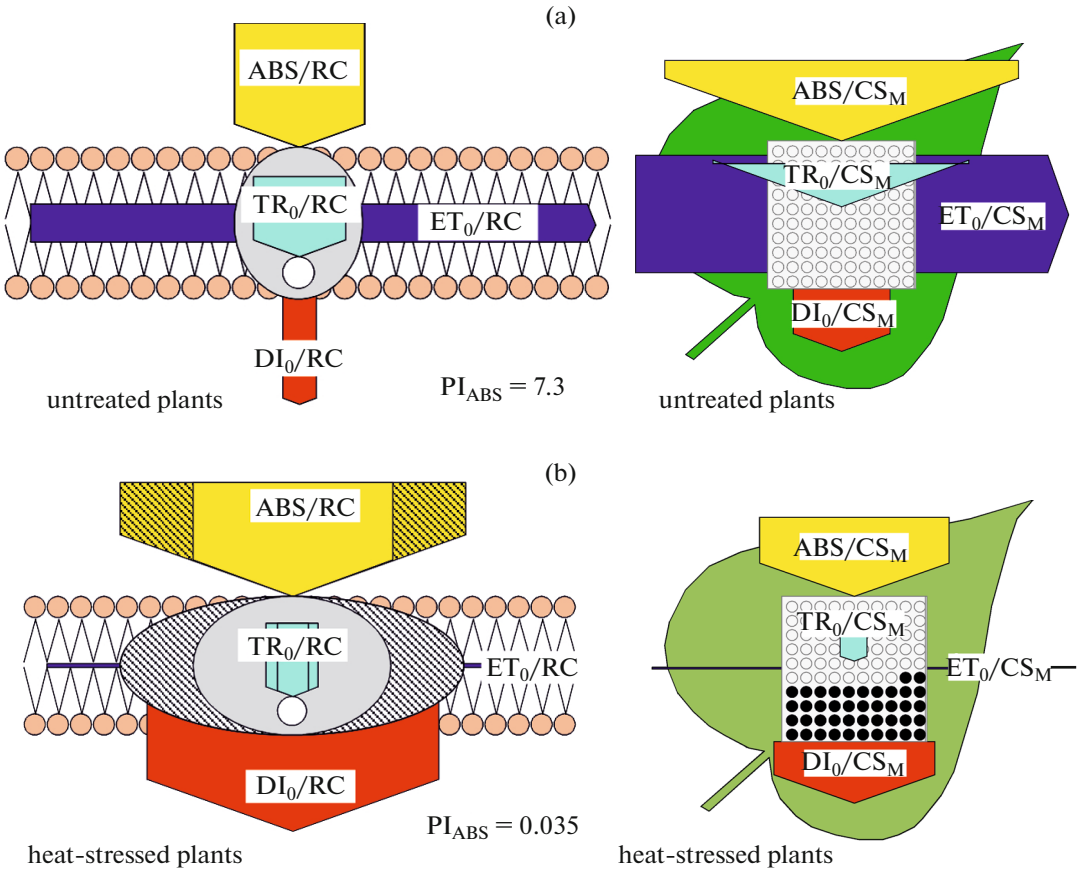

Fig. 10. Energy flux models for the leaf sample under (a) optimal (control) physiological conditions and (b) in the stressed leaf. The relative value of each parameter is proportional to the width of the arrows. The membrane model (on the left side) represents the specific activity on a per single RC basis. The average size of the antenna is represented by the ABS/RC parameter. The fluxes of absorbed energy that were trapped by nonreducing PSII centers are shown as diagonally hatched areas in ABS/RC and TR/RC contours. In the leaf model (on the right side), the phenomenological parameters are normalized per unit cross section (CS) of a photosynthesizing object. The Q_A-nonreducing reaction centers are shown with black circles; the Q_A-reducing active centers are depicted with white circles. The deepness of green color of leaves in the "leaf model" indicates the concentration of chlorophyll per unit area of illuminated photosynthesizing objects. All these values can be determined at minimum (index 0) or maximum (index m) levels of chlorophyll fluorescence; for example, ET_0 or ET_m. The fluxes presented in the scheme are normalized to the maximal fluorescence level (m).

reflect the processes involving multiple events of Q_A reduction.

The main chlorophyll fluorescence parameters (JIP test parameters), as measured with a Hansatech Instruments fluorometer, can be calculated and presented in a graphical form using Biolyzer software (copyrights belong to the Bioenergetics Laboratory, University of Geneva, Switzerland). Examples of graphical representation can be the "membrane model" of energy fluxes in a single RC of PSII and the "leaf model" formulated on a per unit cross section of the analyzed material (Fig. 10). Other ways of data presentation for simultaneous demonstration of a number of important parameters are *the spider plot* (Fig. 11) (see also [1]) and *the carpet plot* (see, e.g., [1, 95]).

CONCLUSIONS

Plant stress responses are always accompanied by changes in structural and functional characteristics of the photosynthetic apparatus. Measurements and analysis of fluorescence emitted by molecules of the antenna chlorophyll *a* become a powerful source of information on actual physiological condition of plants [96]. On the one hand, analysis of JIP-test parameters helps to reveal specific changes in different parts of photosynthetic apparatus and clarify the mechanism of stress impact on the plant material [46, 97, 98]. Presently, a large diversity of fluorometric equipment is offered by different manufacturers. These instruments range from portable devices capable of conducting automated screening of plant materials in the field to highly sensitive analytical systems where fluorescence signals are combined with the

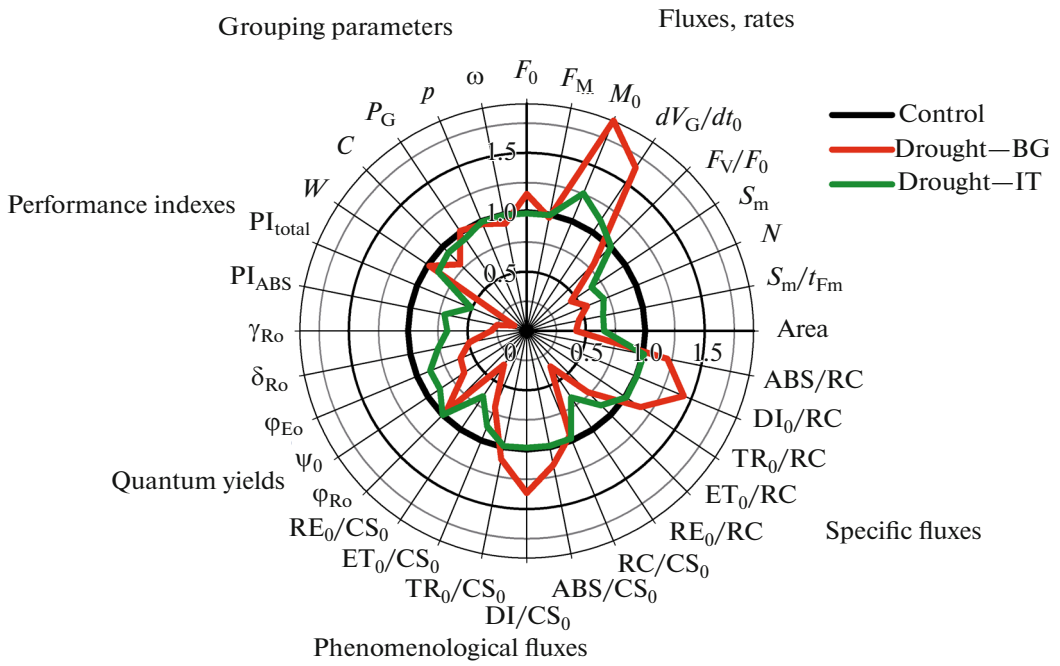

Fig. 11. Spider plot showing relative deviation of JIP parameters in leaves of water-stressed plants from the control values in untreated plants (black circle). The leaves were sampled from seedlings of two ecotypes of *Platanus orientalis:* Bulgarian ecotype (BG) and the Italian ecotype (IT). Prior to measurements, watering of seedlings was limited for 12 days. All parameters were normalized to the control values. The data were normalized to the control values determined separately for each ecotype. The parameters are divided into the following groups: energy fluxes, quantum yields and efficiencies, specific energy fluxes (calculated per single RC), relative phenomenological fluxes (calculated per unit of the illuminated leaf cross section), the grouping parameters, and generalized parameters of PSII functional activity, i.e., performance indexes. Authors' unpublished data.

detection of other important characteristics of the photosynthetic apparatus [98]. On the other hand, new approaches employing the methods of artificial neural networks are being developed for the secondary processing of fluorescence data [99]. These approaches provide general information on plant characteristics that, at first glance, have no direct relation to fluorescence, such as water content in plant tissues [97] or deficiency of certain minerals in a culture medium [100].

The present review summarizes information on the modern biophysical method for studying physiology of plants in vivo, based on the measurement and analysis of chlorophyll fluorescence. The authors hope that the article will help interested readers to apply this method as a simple, routine, and daily used technique for continuous monitoring of physiological condition of the plants used in their studies.

ACKNOWLEDGMENTS

V. Goltsev and M. Paunov are grateful to the Bulgarian National Scientific Fund (project no. DFNI B02/8) and the company Astea Solution for financial and material support. This work was also supported by the Russian Foundation of Basic Research and by the "Molecular and Cell Biology" Research Program of the Russian Academy of Sciences.

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Translated by A. Bulychev