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REVIEW

Ontogenetic approach to the assessment of plant resistance to prolonged stress using chlorophyll fluorescence induction method

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Abstract

The review deals with objective reasons that limit the use of chlorophyll fluorescence induction kinetics (Chl FIK) method in plant ecology. Based on the ontogenetic approach (analysis and comparison of the dynamics of the Chl fluorescence ratio F_p/F_s and physiological characteristics of plant leaves in ontogeny) possible criteria for the estimation of general plant resistance of photosynthetic apparatus (PSA) to prolonged stress affects are proposed. One of these criteria is the presence or absence of a steady-state phase in the dynamics of Chl fluorescence ratio F_p/F_s (or R_{fd}) of plant leaves after they stop growing. We also propose to use the duration of the steady-state phase and variability of Chl fluorescence ratios R_{fd} and F_p/F_s in this period for quantitative assessment of plant PSA resistance to prolonged stress during plant leaf ontogeny.

Additional key words: leaf age; photosystems 1 and 2; radiation colour; specific leaf growth rate.

Introduction

The development of effective and precise methods for the estimation of plant resistance and transition from reversible to irreversible structural-functional changes resulting in the death of plants under effect of adverse factors is very important both for ecological monitoring and for basic research of plant stress. Besides, a viable solution of ecological monitoring of vegetative communities is only possible if we pass from the level of separate leaf or whole plant on to phytocenosis level. Thus, new methodical approaches are needed. The method of chlorophyll (Chl) fluorescence induction kinetics (Chl FIK) allows monitor one of the most important functional characteristics of plant state – activity of its photosynthetic apparatus (PSA) at different levels of organization (Lichtenthaler 1992, Lazár 1999). Chl FIK parameters can be used for getting necessary quantitative information about photosynthetic processes. These parameters were intensively elaborated and applied to photosynthetic research within the last three decades, *e.g.* F_v/F_m ratio (Kitajima and Butler 1975, Venediktov *et al.* 1999, Mohammed *et al.* 2003, Popovic *et al.* 2003, Sayed 2003), relations of Chl fluorescence decrease R_{fd} and F_p/F_s (Brown 1967, Nesterenko and Sid'ko 1980, 1993, Jenkins *et al.* 1981, Lichtenthaler and Buschmann 1984, Lichtenthaler *et al.* 1986, 2000, 2005b, Lichtenthaler and Rinderle 1988, Šiffel *et al.* 1988, Kharuk *et al.* 1994). These Chl FPs are most widely used for comparative studies of fully active and stressed plants (Lichtenthaler and Rinderle 1988, Kharuk *et al.* 1994, Lichtenthaler *et al.* 2000, Nesterenko *et al.* 2001, Sayed 2003,

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Abbreviations: A_p – stress adaptation index; AR – actinic radiation; ARI – adaptation irradiance of plants at which CO_2 exchange efficiency is maximal; Chl – chlorophyll; Chl F – Chl fluorescence; Chl FI – Chl fluorescence induction; Chl FIK – Chl fluorescence induction kinetics; Chl FP – Chl fluorescence parameter; ETC – electron transport chain; F_d – maximum variable fluorescence at the slow stage of Chl fluorescence; F_{m1}, F_{m2} – Chl fluorescence at peaks M1 and M2, respectively; F_0, F_m – minimal and maximal Chl fluorescence in darkness adaptation state; F_p – Chl fluorescence at maximum P; F_s – steady-state Chl fluorescence at stage T; F_v – maximum variable fluorescence at the fast stage of Chl fluorescence induction; MR – modulated radiation; O-I-D-P – the fast stage of Chl FI; PAM – pulse amplitude modulation; PAR – photosynthetically active radiation; P_N – net photosynthetic rate; PS – photosystem; PSA – photosynthetic apparatus; P-S-M-T – slow stage of Chl FI; RC – reactive centre; R_{fd} – vitality index; SP – saturating pulse; T_1, T_2, T_3 – critical leaf ages corresponding to leaf areas of 20, 50, and 80 % of the maximum value, respectively (Mokronosov 1981); T_4 – time of completion of leaf growth; T_5 – leaf age corresponding to the maximum rate of photosynthesis.

Mohammed *et al.* 2003, Nesterenko and Tikhomirov 2003, 2005). Numerous reviews describe application of Chl FIK method in plant stress physiology (Lichtenthaler and Rinderle 1988, Lichtenthaler 1990, Daley 1995, Roháček and Barták 1999, Schreiber *et al.* 2000, Roháček 2002, Korneev 2002, Fracheboud and Leipner 2003, Lichtenthaler *et al.* 2005b). Furthermore, the future of Chl fluorescence application in stress research lies in the new Chl fluorescence imaging technique (Babani and Lichtenthaler 1996, Lichtenthaler and Miehe 1997, Buschmann *et al.* 2000, Lichtenthaler and Babani 2000, Lichtenthaler *et al.* 2000, 2005b). However, despite the wide and successful application of Chl FIK method for the estimation of stress effects, application of the Chl FIK method in plant ecology is limited by several reasons:

(*1*) The state of RC of PS2 determines the level of fluorescence during the induction period (Govindjee 1995, Lazár 1999, Roháček 2002). Chl FPs based on F_0 can be affected in their absolute values due to the contribution of Chl F emission from photosystem 1 (PS1) pigment complex to this level. Although the Chl F emission from PS1 is insignificant at room temperature, its effect should be taken into account when analysing the data obtained with samples under strong stress or non-physiological conditions (low/high temperature, CO_2 starvation, excessive irradiance, *etc.*) (Roháček 2002).

(*2*) Non-specific character of Chl FIK changes caused by different stressors to plants. Chl F helps detect the presence of stress but it is not generally used to diagnose the type of stress, because many stresses influence photosystem 2 (PS2) function in similar way (Lichtenthaler and Rinderle 1988, Korneev 2002, Fracheboud and Leipner 2003, Popovic *et al.* 2003, Kolb *et al.* 2005). Some scientists believe that when applying Chl F to the detection of stress conditions in plants one should always measure several Chl FPs as a first fast screening test for stress and then combine it with other physiological measurements (*e.g.* photosynthetic gas exchange, leaf water potential, respiration activity) in order to determine the exact physiological state of plant and the degree of damage (Lichtenthaler and Rinderle 1988, Schindler and Lichtenthaler 1996).

(*3*) One of the most important limiting reasons is the ambiguity in the interpretation of Chl F data, especially at transition from interpretation of results of Chl F measurement at a leaf level to the estimations of PSA status of the whole plant or phytocenosis. Solution of this problem is especially important for the estimation of plant general resistance to prolonged stress. Both the investigations in laboratory and field show that the time course of plant ontogeny is changed when cultivating plants under stress, while the ontogeny of plants growing in favourable conditions has a normal time course. As a rule, stress results in the acceleration of plant ontogeny (Udovenko 1979, Thomas and Stoddart 1980, Mikkelsen and Heide-Jørgensen 1996, Nesterenko and Tikhomirov 2003, 2005). On the other hand, Chl FIK parameters depend on leaf age (Kiknadze 1971, Nesterenko and Sid'ko 1980, 1985, 1986, 1993, Bradbury and Baker 1981, Jenkins *et al.* 1981, Šesták and Šiffel 1997, Santos *et al.* 1998, Šesták 1999, Nesterenko and Tikhomirov 2003, 2005). Moreover, senescence or reaction to stress is also characterized by a higher average statistical deviation of the measured Chl FIK parameters (Lazár and Nauš 1998). At present there are no methods allowing discern physiological changes during plant ontogeny from those associated with plant responses to changing climatic factors and various stressors. Chl fluorescence is most useful in detecting and indicating acute stress responses and much less useful for monitoring chronic or long-term changes, such as those that occur during senescence (Lichtenthaler and Rinderle 1988). Nevertheless, we believe that there is another approach, which we called ontogenetic, that implies the use of age dependence of FPs for the estimation of the general resistance of plants to long-term stresses (Nesterenko and Tikhomirov 2003, 2005).

There are several aspects of Chl FIK features that can be used for comparative studies of fully active and stressed plants:

(*a*) Changes of the shape of FIK during plant leaf ontogeny. This aspect was considered in reviews (Šesták and Šiffel 1997, Šesták 1999) and some articles (Nesterenko and Sid'ko 1980, 1985, 1986, 1993, Bradbury and Baker 1981, Jenkins *et al.* 1981, Nesterenko *et al.* 1985, Nesterenko and Tikhomirov 2003, 2005). Nesterenko and Sid'ko (1985) revealed three types of Chl FIKs (Fig. 1), appearing in turns during leaf development and ageing. Similar changes of the Chl FIKs were observed both for monocotyledonous and dicotyledonous plants with C_3 and C_4 carbon metabolism (Nesterenko and Sid'ko 1985, 1986, 1993).

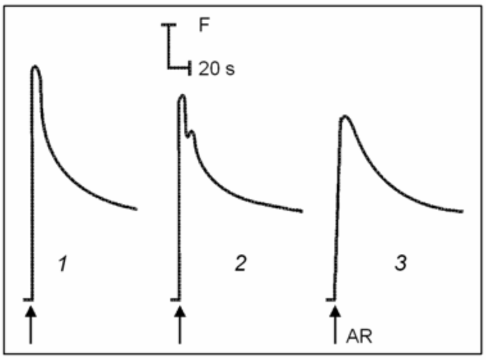

Fig. 1. Scheme of three types of the slow chlorophyll fluorescence induction patterns appearing during leaf ontogeny (Nesterenko and Sid'ko 1986): (*1*) one-peak curves with a rapid decline to stationary level without the SM phase (typical for young 2–4 d leaves); (*2*) double-peak curves with an expressed PSMT part (mature leaves); (*3*) one-peak curves with a slow decline to stationary level (senescing leaves).

(b) Age-induced changes in the shape of fluorescence emission spectra of plant leaf. This aspect was considered in the article of Šiffel *et al.* (1998) and reviews by Šesták and Šiffel (1997), Šesták (1999), and Buschmann *et al.* (2000).

(*c*) The dynamics of Chl FPs during the leaf ontogeny.

For successful solution of irreversible changes in structural-functional organization of plant PSA we propose to study the changes of Chl FP values and compare them with other physiological measurements during long-term plant development under stress.

This paper overviews Chl FPs applied for detection of the PSA status in plant leaves and checking general plant resistance to long-term stress factors, and the relevance of Chl FIK as a tool for determining transition from reversible to irreversible changes in the structural and functional parameters of plants induced by prolonged stress factors (possible criteria for the choice of integral Chl FIK Ps). On the basis of our experimental results and literature data we suggest the ontogenetic approach to the assessment of plant general resistance to prolonged stress factors. The experiment described here included the study of changes in Chl FIK parameter (F_p/F_s, for example) and physiological leaf characteristics (net photosynthetic rate – P_N, leaf growth characteristics) under controlled irradiation culture. Besides, we consider the effects of irradiation causing fluorescence in connection with the necessity of Chl FIK registration. The latter is necessary not only due to the possible activity of plant PSA, but also due to the optimal correlation of respiration and photosynthesis, which is important for the estimation of plant resistance to stressors.

Basic concepts

The wide use of Chl F in plant ecology on the phytocenosis level is connected with the determination of general plant resistance, which is the final result of the primary reactions of plants induced by stresses and secondary disorders developed during prolonged exposure to stress factors (Udovenko 1979). The most objective way to estimate the physiological state of the whole plant is through the ratio that reflects the interrelation of the basic physiological processes, such as relative growth rate of plant leaves, the share of respiratory expenses for adaptation, the share of total respiration in gross photosynthesis, *etc*. (Mokronosov 1980, Rakhmankulova *et al.* 2001). Cultivation of plants in optimal conditions is accompanied by the coordination of respiration and photosynthesis a day after the quantitative ratio of plants has started to approach minimal values (*ca*. 38–40 %). Different types of plants have similar values (Rakhmankulova *et al.* 2001). Under stress the ratio of total respiration and gross photosynthesis of plants, as a rule, increases, especially in plants that are unstable (Rakhmankulova 2002) and not adapted (Semikhatova *et al.* 1992) to this type of stress (about two times). Unstable species with more significant respiratory expenses at long adverse influence, as a rule, quickly finish their life cycle and pass to reproductive development. Steady species have a smaller respiratory "price" of adaptation, and these species are long vegetative (Lambers *et al.* 1981, Rakhmankulova *et al.* 2001, Rakhmankulova 2002). In this connection a question arises: is it possible, comparing ontogeny of plants (or their leaves) in favourable and stressful conditions, to define plant resistance to a certain kind of stress? If yes, what are the appropriate methods?

Physiological characteristics reflecting the ratios of basic physiological processes and the analysis of their changes during the ontogeny in favourable and stressful conditions can be used for estimation of the physiological state of plants and prediction of their resistance to the changing environment (Rakhmankulova 2002). But the methods of registration are not efficient, which makes difficult ecological monitoring analysis, and forecast of the plant state. Another side of the plant resistance topic is the registration of transition from reversible changes to irreversible damages in the structure and function of PSA which also can not be solved by classical physiological methods (Bulgakov 2002).

All stress factors affecting a certain part of the plant eventually cause direct or indirect effect on photosynthesis. Since functional disorders are usually ahead of structural disorders, the dynamic stability of PSA is of highest interest for the early diagnosis of general plant resistance (Veselovsky *et al.* 1993). Apart from the initial and final plant PSA states, the duration and pattern of the transition from one state to another also determine general resistance of plants (Udovenko 1979, Veselovsky *et al.* 1993). Conventional gasometric measurements of photosynthesis are often destructive to the plant. Chl F is a valuable tool to detect the induction of stress and damage as well as the regeneration of plant PSA when the stress factor is removed. In contrast to other eco-physiological methods, *e.g.* gasometric methods, the advantage of Chl FIK is that it is very fast, highly sensitive, and nondestructive. The interrelation between Chl F and photosynthetic activity, first observed and described by Kautsky and Hirsch (1931), is successfully applied to studies of potential photosynthetic activity of plant leaves (for theoretical reviews and photosynthetic application see Kraus and Weis 1991, Govindjee 1995, Lazár 1999, Schreiber *et al.* 2000, Roháček 2002). The fluorescence ratios F_v/F_m, R_{fd}, and F_p/F_s, widely used for comparative studies of fully active and stressed plants, change with leaf age (Šesták and Šiffel 1997, Šesták 1999, Santos *et al.* 1998, Yang *et al.* 1999, Nesterenko and Tikhomirov 2003, 2005). Age dependence of Chl FPs is an important reason of ambiguity in the interpretation of the results of Chl F measurements. This peculiarity limits further application of the Chl FIK method to plant stress research. Chl F methods, as a rule, determine the age of leaves not by their physiological state, but by the number of days from their appearance (Hong *et al.* 1999), *i.e.* ontogenetic approach that considers certain developmental stages is not used. In this connection there are several restrictions of the use of Chl FPs for diagnostics of plant responses to different stresses. Certainly, there is a correlation between FPs and the photosynthetic activity of plant leaves (Ireland *et al.* 1984, Nesterenko and Sid'ko 1993, Šesták and Šiffel 1997, Karavaev and Polyakova 1998). It is believed that their values and P_N decrease if the stress factor increases (Lichtenthaler *et al.* 1986, Lichtenthaler and Rinderle 1988, Roháček 2002, Sayed 2003). However, these parameters also decline during plant ontogeny (Nesterenko and Sid'ko 1980, 1985, 1986, 1993, Šesták and Šiffel 1997, Santos *et al.* 1998, Šesták 1999).

There is a problem of distinguishing between the changes in these parameters during normal plant ontogeny (*i.e.* under favourable conditions of growth and development) and the changes of these parameters induced by the long exposure of plant PSA to stress factors. On the other hand, the formation of plant leaves under extreme environmental conditions (drought, virus infection, unfavourable irradiance, temperature, *etc.*) is accompanied by a modification of the temporal characteristics of their ontogeny (Udovenko 1979, Thomas and Stoddart 1980, Nesterenko *et al.* 1985, Mikkelsen and Heide-Jørgensen 1996, Nesterenko and Tikhomirov 2003, 2005). Therefore, this process is accompanied by modification of the dynamic resistance of plant (Udovenko 1979, Veselovsky *et al.* 1993, Shabala and Voinov 1994). Analyzing the experimental facts mentioned above and taking into account the fact that stable species have a longer life cycle than the unstable ones (Lambers *et al.* 1981, Rakhmankulova *et al.* 2001, Rakhmankulova 2002), we suggest ontogenetic approach consisting in the use of age dependence of FPs for the estimation of the general plant resistance to long-term stresses. Another aspect of this problem is the necessity to define conditions optimal for Chl FIK registration at which the resistance of plant PSA reaches its maximum. We are interested not in the potentially possible photosynthetic activity of plant PSA, but in the most optimal correlation of photosynthesis and dark respiration of plants. One of the important conditions for Chl FIK registration of plant leaves is the irradiance exciting Chl F (AR intensity).

Chl FIK parameters used for estimation of PSA resistance of plant leaves to stresses

F_v/F_m: Chl FIK method is based on the processes that affect PS2 activity, as Chl, a part of this photosystem, is the major contributor to Chl F at room temperature (Govindjee 1995, Lazár 1999, Roháček 2002).

During the registration of Chl FIK fast phase, the relation of variable fluorescence $F_v = F_m - F_0$ to the maximum level F_m (F_v/F_m), which indicates potential photosynthetic activity of a leaf, is often used for the description of PSA at the leaf level under stress (Lichtenthaler and Rinderle 1988, Roháček 2002, Sayed 2003, Mohammed *et al.* 2003). The higher are F_v values with the corresponding F_0 and F_m, the better are the conditions for PSA functioning (Roháček 2002). However, the possibilities of F_v/F_m parameter are limited (Lichtenthaler and Rinderle 1988), as it is only a primary photochemical process of PS2 during the first 100–500 ms and can not give information about the whole process of photosynthesis. Another disadvantage of the F_v/F_m parameter is the dependence of its values on leaf age (Šesták and Šiffel 1997, Šesták 1999). The stabilization stage of F_v/F_m ratio was discovered after the initial decrease and further increase (Mészáros *et al.* 1995). Nevertheless, today the F_v/F_m parameter is actively applied to the estimation of plant resistance to different adverse influences, because during strengthening of the effect and after-effect of the extreme factor, F_v/F_m decreases, which may be the criterion of certain disorders in the electron transport chain (ETC) of PS2 (Venediktov *et al.* 1999, Roháček 2002). For examples of application of this ratio to the solution of some ecological and physiological questions connected with plant resistance to stresses see Mohammed *et al.* (2003), Popovic *et al.* (2003), or Sayed (2003). In particular, this parameter was applied to reveal the mechanism of photosynthesis limitation of wheat caused by water stress and to distinguish the water stress effect from photoinhibition (Sayed 2003), as well as to study the effect of water stress on PS2 and xanthophyll cycle components. Another application of this parameter is the studying of thermal stress in different wheat cultures, cold stress in oats, ageing of rice leaves induced by salt stress, *etc.* (Sayed 2003). As a rule, this parameter is successfully applied together with other Chl FIK parameters (components of suppression of Chl FIK slow phase) for the revelation of stress influence mechanisms and monitoring plant PSA status (Lichtenthaler and Rinderle 1988). However, it is difficult to unequivocally forecast long-term stresses based on the values of this parameter. For example, using this parameter for woods in city ecosystems based on Chl FIK measurements in the bark of annual shoots showed that the average value of F_v/F_m became larger and the statistical dispersion was less significant, if the plant was in favourable conditions (Venediktov *et al.* 1999). It is difficult to estimate unequivocally the degree of tree damage with intermediate values (0.60–0.74) of the measured F_v/F_m parameter (Venediktov *et al.* 1999).

R_{fd} and F_p/F_s: The parameters of Chl FIK slow stage are of major interest to studying of stress physiology and plant ecology, because they are closely connected with the biochemical processes of the leaf (Lichtenthaler and Rinderle 1988, Nesterenko and Sid'ko 1993, Schindler and Lichtenthaler 1996, Karavaev and Polyakova 1998). For description of plant leaf PSA, it is reasonable to use the relation of fluorescence decrease ($F_d = F_p - F_s$) to the standard fluorescence level (F_s) (Brown 1967), which is called vitality index:

$$R_{fd} = F_d/F_s = (F_p - F_s)/F_s = F_p/F_s - 1 \quad (1)$$

(Lichtenthaler *et al.* 1986, Lichtenthaler and Rinderle 1988), or the relation of maximal fluorescence F_p to the steady-state level of fluorescence F_s (F_p/F_s) (Nesterenko and Sid'ko 1980, 1993, Šiffel *et al.* 1988, Veselovsky and Veselova 1990, Lichtenhaler *et al.* 2000, Nesterenko *et al.* 2001a, Korneev 2002) (all nomenclature according to van Kooten and Snel 1990). These relations indicate the potential photosynthetic activity of leaves and are widely applied in physiological and ecological studies for estimating plant resistance (Lichtenthaler and Rinderle 1988, Veselovsky and Veselova 1990, Kharuk *et al.* 1994, Babani and Lichtenthaler 1996, Lichtenthaler and Miehe 1997, Nesterenko *et al.* 2001, Sayed 2003). For example, the Chl modulated fluorescence method was used for screening wheat cultures for water stress, using the easily measured R_{fd} parameter (Havaux *et al.* 1988, Flagella *et al.* 1996, Tambussi *et al.* 2002). The review of Lichtenthaler and Rinderle (1988) illustrates both its advantages (easy to measure, integrity of characteristics) and its limited nature, caused by the dependence of its values on the leaf age. The value of R_{fd} decreased with leaf age of needles (Lichtenthaler and Buschmann 1984, Lichtenthaler and Rinderle 1988, Lichtenthaler *et al.* 1989, Kharuk *et al.* 1994) and leaves (Bach and Lichtenthaler 1983, D'Ambrosio *et al.* 1992, Buschmann *et al.* 2000). As a rule, F_p/F_s, close to R_{fd}, increases and decreases simultaneously with the development of the leaf (Nesterenko and Sid'ko 1980, 1985, 1986, Šiffel *et al.* 1988, Šesták and Šiffel 1997, Šesták 1999, Nesterenko and Tikhomirov 2003, 2005).

Stress-Adaptation Index A_p: R_{fd} parameter can be measured at two wavelengths (690 and 730 nm). Combination of R_{fd}' (at 690 nm) and R_{fd}'' (at 730 nm) values leads to the establishment of an additional fluorescence parameter, that was called stress adaptation index (Strasser *et al.* 1987, Lichtenthaler and Rinderle 1988) and is determined by the following equation:

$$A_p = 1 - (1 + R_{fd}')/(1 + R_{fd}''). \quad (2)$$

This index measures the possibilities of leaves to reorganize PSA structure for the better adaptation in case the irradiance will change. Young photosynthetically active leaves, having high R_{fd} (for example, at 690 and 730 nm 3.10 and 3.61, respectively), as well as high A_p (0.25 and 0.26, correspondingly), have the highest index values (Lichtenthaler and Rinderle 1988). A_p decreases with leaf ageing and senescence. A_p value of 0.1 indicates that the leaf system has a week, if any, ability to react to the stressful changes of the environment. A series of examples of using the adaptation index A_p is given in the review of Lichtenthaler and Rinderle (1988).

Besides, it is a common practice to use parameters based on calculation of the area above the curve of fluorescence induction slow phase (Nesterenko and Sid'ko 1993) and Chl F halftime from peak P to the steady-state level T ($T_{0.5}$) (Kharuk *et al.* 1994) for description of Chl fluorescence induction slow phase in plant leaf ontogeny. These parameters also depend on leaf age (Nesterenko and Sid'ko 1993, Kharuk *et al.* 1994).

Necessary experimental conditions for Chl FIK registration aimed at the estimation of general plant PSA resistance

Chl FIK method possesses the following advantages: high information density, expressivity, high sensitivity, possibility to make experiments in the open environment, and intactness. As a result of intensive development of pulse-modulated technique with the help of the installations developed recently it has become possible to register Chl FIK in field conditions (Pukacki and Modrzynski 1998), to carry out remote Chl FIK measurements (Ounis *et al.* 2001), to obtain leaf fluorescent images (Lang *et al.* 1994, Kramer and Crofts 1996, Buschmann *et al.* 2000, Roháček 2002), *etc*. However, along with incontestable advantages, the Chl FIK method has a number of essential imperfections connected with difficulties of both theoretical and methodical character (Schindler and Lichtenthaler 1996, Šesták and Šiffel 1997, Roháček and Barták 1999, Roháček 2002). Today, the methods of interpretation of Chl FIK parameters do not allow to take into account the effect of PS1 and the related processes on Chl FIK (Pfündel 1998), especially in stress conditions, when the role of PS1 in cell metabolism increases (Bukhov and Egorova 2005). Interpretation of data is accompanied by application of the conditions connected with the PSA of a mature leaf (Roháček and Barták 1999, Roháček 2002). However, passing onto the level of the leaf and the whole plant, we deal with the heterogeneous age population of chloroplasts with a large variety of properties (Mokronosov 1981, Nesterenko and Sid'ko 1980, 1985, 1986, 1993, Šesták 1985, Babani and Lichtenthaler 1996, Šesták and Šiffel 1997, Polyakova *et al.* 2003). Distinctions of fluorescent characteristics were observed also for the top and bottom parts of the leaf (Šesták and Šiffel 1997, Šesták 1999, Lichtenthaler *et al.* 2000). Besides, the following peculiarities of Chl FIK can also be named among the methodical difficulties:

(*1*) The use of intensive excitation of Chl F radiation leading to photoinhibition, or the influence of other stressors can change Chl FIK parameters (for example, F_m and F_0) and ratios based on these parameters, including the parameter F_v/F_m widely used in ecological investigations (Demmig-Adams *et al.* 1996). In a similar way, both experimental conditions and measuring installations for Chl FIK (*i.e.* irradiance during plant cultivation, environment temperature, CO_2 concentration, dark-adaptation period and irradiance inducing fluorescence, *etc.*) should always be defined precisely, as variations of physical parameters at the time of measurement result in significant variations of results (Schindler and Lichtenthaler 1996, Roháček and Barták 1999, Schreiber *et al.* 2000, Roháček 2002).

(*2*) Application of Chl FIK parameters has certain limits, and perfunctory attitude towards their application can lead to wrong interpretation. As a rule, to receive trustworthy information about plant PSA state of plants it is necessary to use not one, but some independent fluorescence parameters (Lichtenthaler and Rinderle 1988, Roháček 2002).

(*3*) Using Chl FIK method in the field requires take into account the circadian cycle of changes of PSA activity (Roháček and Barták 1999, Roháček 2002).

(*4*) The situation becomes even more complicated when Chl FIK method is used for solution of such ecological problems as the estimation of plant resistance to long stressful influences and determination of the beginning of irreversible changes in PSA state. In this case, apart from the other difficulties, we face the problem connected with the age dependence of Chl FIK parameters and, as a result, the high degree of variability. However, a certain approach can help overcome this imperfection (high sensitivity of Chl FIK parameters to the leaf age) to become one of the advantages of Chl FIK method.

Let us consider the methodical peculiarities allowing determine the age state of plant leaf PSA more precisely. The most important factor is the irradiance inducing fluorescence. Up to the 1980-s, the majority of researchers used single-beam fluorimeters for Chl FIK registration, in which one source of radiation was used both for fluorescence stimulation and for photosynthesis maintenance. This method of continuous registration of fluorescence allows to obtain Chl FIK curve, which is conventionally divided into a fast phase, the so-called O-I-D-P transition (includes the rise from fluorescence level F_0, when all PS2 reaction centres are "open", to the maximum P) and a slow one, P-S-M-T transition (decrease of fluorescence after the peak P to the quasi-steady level T; F_t or F_s according to the generally accepted nomenclature by van Kooten and Snel 1990). Sometimes in the process of fluorescence slow decrease of additional maximum M1 is registered as connected with Calvin cycle activation delay (Krause and Weis 1991), as well as M2 supposedly connected with nitrogen metabolism (Moskvin *et al.* 1998). In chloroplast suspension no P-S-M-T decrease is observed and the peak P coincides with the fluorescence maximum level F_m, when all PS2 RC are "closed", *i.e.* $F_p = F_m$ (Korneev 2002, Roháček 2002). In native plant tissues this registration method reveales F_m values higher than F_p due to the possibility of activation of biochemical reactions, in which reduced ferredoxin is used (Korneev 2002). These values coincide when the irradiation by "active light" is high or when the processes responsible for Chl fluorescence decrease are inhibited (Korneev 2002). The detailed interpretation of physico-chemical mechanisms, forming the basis of Chl FIK phenomenon and its separate stages, is given in reviews (Kraus and Weis 1984, Lichtenthaler 1992, Lazár 1999, Korneev 2002, Roháček 2002, *etc.*). As a rule, for fluorescence excitation single-beam devices use radiation within the spectrum range of 400–500 nm and the dozens of W m^{-2} (Lichtenthaler and Rinderle 1988), F_m being higher than F_p (Roháček 2002). However, application of average irradiance (30–50 W m^{-2}) allows obtain induction curves with well-defined maxima M1, M2, *etc.* (Nesterenko and Sid'ko 1980, 1985, 1986, 1993, Kharuk *et al.* 1994, Karavaev and Polyakova 1998, Nesterenko and Tikhomirov 2003, 2005, Polyakova *et al.* 2003), which is important for qualitative estimation of Chl FIK. In stress situations, for the estimation of plant leaf PSA resistance it is reasonable to use not saturating AL in the order of 100 W m^{-2} and more which is described in some papers where PAM and other equipment is used (Roháček and Barták 1999, Roháček 2002), but irradiance close to that of adaptation radiation (ARI) of plants, *i.e.* to such PAR value, at which the efficiency coefficient of leaf CO_2 gaseous exchange is maximal, as it makes the correlation of photosynthesis processes and leaf dark respiration optimal (Tooming 1967). We recommend to use irradiance of about 30–50 W m^{-2} (the majority of agricultural plants have ARI within the range of 28-84 W m^{-2} – Tooming 1967, 1970) in case of diagnostics of leaf PSA state in stress situations due to the following reasons: (*1*) Such irradiances causing Chl F do not lead to photoinhibition of leaf PSA, and thus it is possible to estimate the current PSA state more appropriately; (*2*) Chl FIK is registered in the optimal combination of photosynthesis and dark respiration – the necessary condition for high productivity of plants. Tooming (1967, 1970) showed that intensive photosynthesis of separate leaves is not enough to provide for a high-yielding crop. It is necessary to ensure intensive photosynthesis and gaseous exchange of the crop as a whole. This goal is possible to achieve by creation of a valid crop, in which the leaves work not with the maximal photosynthesis intensity, but with the optimal one, corresponding to the ARI zone and maximal efficiency coefficient. High energy generating capacity of plants is very important in terms of biotic potential, so it is such structures and functions of plants that, other factors being equal, provide a higher productivity or, possibly, a higher efficiency coefficient of PAR usage, that are considered optimal. (*3*) Irradiance within the range of 30–50 W m^{-2} allows Chl FIK curves to give the fullest qualitative and quantitative information about the age state of leaf PSA (Karavaev and Polyakova 1998, Karavaev *et al.* 2001, Nesterenko and Tikhomirov 2003, 2005, Polyakova *et al.* 2003). (*4*) The form of fluorescence spectrum and the ratio F_{690}/F_{730} do not change at irradiances approaching 40 W m^{-2} (in spectral range of 400–500 nm). The form of fluorescence spectrum changes substantially when fluorescence is induced within the range of 525–600 nm (Lichtenthaler and Rinderle 1988).

A modern and widely-spread Chl FIK registration method, using pulse amplitude modulation (Schreiber 1983, Roháček and Barták 1999, Korneev 2002) involves four sources of radiation for the four types of object lighting. Faint pulsed radiation is used as measuring light (ML), as it does not cause photochemical reaction (photon flux density 0.2–1.0 μmol m^{-2} s^{-1}). Chl FIK is registered with the help of active or actinic radiation (AR), the intensity of which is usually several hundreds of μmol m^{-2} s^{-1} (more than 200), which is, as a rule, higher that the irradiance used by single-beam registration of Chl FIK.

Application of ontogenetic approach to estimation of general plant resistance using Chl FIK method

In the process of strengthening of the effect and after-effect of the stressful factor the values of F_v/F_m, F_p/F_s, and P_N decrease. Such changes of Chl FPs are the criterion of damages in ETC of PS2 (Lichtenthaler and Rinderle 1988, Veselovsky and Veselova 1990, Roháček 2002). However, the decrease in values of these parameters is also observed during the natural course of leaf ontogeny of plants at different stages (Jenkins *et al.* 1981, Nesterenko and Sid'ko 1993, Šesták and Šiffel 1997). In this connection there is a problem of differentiation of changes in the examined parameters during normal ontogeny (*i.e.* in favourable conditions of growth and development) and changes caused by stressful influences on plant PSA. As functional changes occur before the structural ones, the greatest interest for early diagnostics of the general stability of plants is represented by the dynamic stability of systems at different levels of PSA organization. Such approach emphasizes, apart from the importance of registration of initial and final PSA state, the duration and character of system transition from one state to another (Udovenko 1979, Veselovsky *et al.* 1993, Shabala and Voinov 1994). The development of plant leaves in adverse environmental conditions (drought, virus infection, radiation mode of a certain spectral structure and intensity, *etc.*) leads to certain changes in time characteristics of ontogeny. For example, patho-physiological changes caused by a virus infection of potato declared themselves by a longer vegetation period (about 70–75 d), while the vegetation period of healthy plants was 62 d, and by a later occurrence of "complex" kinetics of slowed fluorescence in virus plants, which is the evidence of their slow development (Tsoglin *et al.* 1987). The ontogenetic approach also becomes necessary when a degree of irreversibility of extreme influences on plant leaves should be solved. According to Thomas and Stoddart (1980), ageing is influenced by the following environmental factors: irradiance, temperature, water system, mineral nutrition, and pathogen invasion. In particular, low concentrations of tropospheric ozone accelerate leaf ageing (Mikkelsen and Heide-Jørgensen 1996). Therefore, the estimation of a degree of irreversibility of plant damage can be made by registrating the starting moment of leaf senescence, because, basically, senescence deteriorates the leaf state and leads to its death (Thomas and Stoddart 1980, Mokronosov 1981). In this connection, one of the urgent problems of plant ecology is studying the ontogenetic features of PSA activity of plant leaves under long stressful influences with the help of fluorescence methods, in particular the Chl FIK method. For the solution of these problems experimental modelling in controllable environmental conditions has been successfully applied (Nesterenko and Sid'ko 1980, Tikhomirov and Sid'ko 1982, Nesterenko *et al.* 2001, Nesterenko and Tikhomirov 2003, 2005). For example, we made experiments on cucumber plant leaves, studying the age dynamics of Chl FIK ratio F_p/F_s and physiological parameters (P_N and growth characteristics of leaves) under conditions unfavourable for this plant – "red" radiation (spectral range 600–700 nm, test) and "white light" (spectral range 400–700 nm, control) (Nesterenko and Tikhomirov 2003, 2005). The excitation irradiance during Chl FIK registration on leaf surface level was 30 W m^{-2}. As a result, several amplitude-time differences were revealed in the dynamics of Chl fluorescence and physiological, *i.e.* leaf surface and P_N, parameters (Fig. 2*A,B*) for cucumber leaves cultivated in control and test irradiation modes: (*a*) differences were observed in the values of characteristic times of leaf ontogeny for time points T_1, T_4, and T_5 and coincidences for time points T_2 and T_3 (Table 1); (*b*) after the termination of leaf growth the control variant showed a steady-state phase in F_p/F_s dynamics, while the steady-state period was absent in the test variant (Fig. 2*B*). At the initial and final stages of leaf ontogeny, points T_1 and T_4, as well as at point T_5 that corresponds to the maximal leaf photosynthesis, F_p/F_s for both irradiation variants practically coincided. Substantial differences in the values of the discussed Chl FIK parameter (25–30 %) were observed during the transition period for leaf PSA in points of maximal growth rate (T_2) and leaf growth deceleration (T_3). The specific leaf growth rate for test variant was 22 % less than for the control one (Table 1). Another

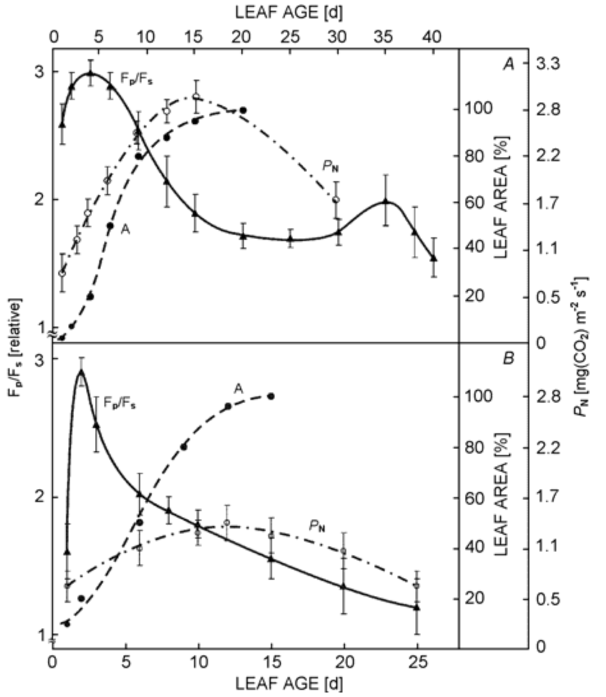

Fig. 2. Age-related changes in Chl fluorescence ratio (F_p/F_s), leaf area (A), and net photosynthetic rate (P_N) of cucumber leaves of the third stratum grown in two irradiation variants: (*A*) control ("white light", 400–700 nm) and (*B*) experiment (red radiation, 600–700 nm) (Nesterenko and Tikhomirov 2003). The irradiance in the two variants was 100 W m^{-2} PAR. T_1, T_2, and T_3 are the critical leaf ages corresponding to the leaf area of 20, 50, and 80 % of the maximum value, respectively (Mokronosov 1981); T_4 is the time of completion of leaf growth; T_5 is the leaf age corresponding to the maximum P_N.

effect in cucumber leaf ontogeny influenced by red radiation was the shortening of the final leaf growth stage, as compared to the control (T_3–T_4; Fig. 2*A,B*, Table 1). The maximum P_N (Fig. 1*A,B*) and its decrease also occurred earlier for the test variant; P_N at the maximal point was almost two times lower than for the control variant. All these facts point at the acceleration of leaf ontogeny at the last stage of its development in the test variant. Since the general mechanism of age changes in the character of Chl FIK curves of higher plant leaves consists in correspondence of separate curve types to certain ontogeny stages (Fig. 1), we compared Chl FIK curves for both irradiance variants at different leaf ontogeny stages. The analysis of the character of the curves (Fig. 3) showed qualitative uniformity of induction curves for the leaves of both variants at the first two stages of leaf development (0–T_3, Fig. 2, 3 and 7 d) and different curve types at stages T_3–T_4 (Fig. 3, 14 d), which was consistent with the data from Table 1 and proved the fast ageing of leaves cultivated in the test variant. According to the literature (Jenkins *et al.* 1980, Nesterenko and Sid'ko 1986, 1993, Tsoglin *et al.* 1987, Karavaev and Polyakova 1998, Nesterenko and Tikhomirov 2003, 2005), there was a qualitative correlation of the basic types of Chl FIK curves with the physiological age of the leaf that could be used for a quick comparative evaluation of the changes in leaf ontogeny and for the selection of plant material of the same physiological age.

Fig. 2 shows that the result of the comparative estimation of leaf PSA resistance to the extreme factors depends on the choice of time points of CFI registration, *i.e.* on the physiological age of the leaf. Based on the analysis of F_p/F_s dynamics and its comparison with the dynamics of the integral physiological characteristics (Fig. 2), we presume that the estimation of leaf PSA resistance to stress influences can develop in the following main directions:

Table 1. Dynamic characteristics of the growth curve of cucumber leaves for two variants of the irradiation regime of plant growth: "white light" (400–700 nm) and red radiation (600–700 nm) (Nesterenko and Tikhomirov 2003). The irradiance was 100 W m^{-2} PAR. For comparison see values of T_1, T_2, T_3, T_4, and T_5 in Fig. 2.

Irradiation	Characteristic time [d] T_1	T_2	T_3	T_4	T_5	Specific leaf growth rate [%]
White	4.0±0.5	6.0±1.0	9.0±1.0	20.0±1.5	15.0±1.5	0.23±0.02
Red	2.0±0.5	6.0±1.0	8.5±1.0	15.0±1.5	12.0±1.5	0.18±0.02

(*1*) The use of the presence or absence of a steady-state of the changes in FPs after the termination of the leaf growth as a criterion of leaf PSA resistance. If the steady-state stage is absent, the changes in the PSA state may be irreversible and even cause plant death.

(*2*) Evaluation of the degree of PSA resistance to a prolonged exposure of cultivated plants to a stress factor by the duration of the steady-state stage and variability of the fluorescence parameter during this period. We suggest that the duration of the steady-state stage and changes in the fluorescence parameter should be measured from the moment of attaining a certain level to the onset of a continuous decline of F_p/F_s parameter. For example, in the case shown in Fig. 2*A*, this period continued for about 15 d starting from the age of 20 d.

Apparently, for the estimation of plant PSA resistance it is necessary to determine the normal duration of the steady-state period in the dynamics of Chl FIK parameter F_p/F_s (or R_{fd}) under favourable growth conditions. Any deviation from the normal duration of the steady-state period for the given plant shows patho-physiological changes in plant PSA (acceleration of ontogeny when exposed to "red" radiation of 100 W m^{-2} PAR and high

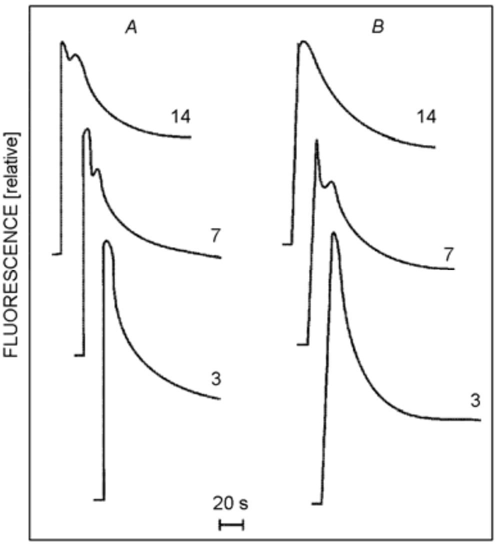

Fig. 3. The curves of chlorophyll fluorescence induction at different stages of ontogeny of cucumber leaves of the third stratum grown under two variants of the irradiation regimes of 100 W m^{-2} PAR: (*A*) control ("white light", 400–700 nm) and (*B*) experiment (red radiation, 600–700 nm). 3, 7, and 14 are the leaf ages [d] (Nesterenko and Tikhomirov 2003).

temperature – Nesterenko *et al.* 2001b, Nesterenko and Tikhomirov 2003, 2005), as well as deceleration of ontogeny in case of a virus infection (Tsoglin *et al.* 1987). We assume that the criteria for the estimation of general plant resistance worked out for the unfavourable irradiation influence (spectral range 600–700 nm, 100 W m^{-2}), based on the ontogenetic approach (Nesterenko and Tikhomirov 2003, 2005), can be used for the estimation of plant resistance to other stress effects.

The analysis of obtained results shows that with the help of registration of CFI slow phase it is possible qualitatively estimate leaf ontogeny, which allows to make a qualitative and quantitative estimation of general plant PSA resistance to stress factors or to determine if the growing conditions are favourable for plants or not. However, it is difficult to estimate the degree of irreversible changes of PSA caused by ageing, based on the nature of changes of F_p/F_s, as well as on the values of R_{fd} and F_v/F_m (Lichtenthaler and Rinderle 1988, Venediktov *et al.* 1999). It is necessary to search for more integral parameters of Chl FI, which could be used for the estimation of the degree of the irreversibility of leaf PSA changes caused by prolonged stress influences.

Fluorescence parameters for estimation of plant PSA damage degree (reversible and irreversible changes) caused by prolonged stress

Choosing the parameters to control the plant PSA state, it is necessary to accurately establish the basic and correlative ones. Basic parameters meet the criteria of biosystem normality, for example, the steadiness and stability of development (Bulgakov 2002). Correlative parameters are represented by the leading indicators of changes in basic parameters (Lichtenthaler and Rinderle 1988), which reflect different plant PSA organization. Among many variables that are used simultaneously and compete to become the most adequate, one should prefer those that meet the following requirements: integrity, non-specificity of response to influence, measurement cost minimization, accuracy of results, and the possibility of unambiguous physiological interpretation on the basis of this parameter with the aim of understanding, if we deal with deep irreversible changes or register some plant adaptation reaction of a reversible nature (Bulgakov 2002).

On the basis of obtained results and literature data, we can formulate the criteria which should be met by Chl FI integral parameters to solve the given problem: (*1*) correlation with the main energetic functions of plants, photosynthesis and respiration or their ratio; (*2*) correlations with the structural changes of PSA resulting in destruction of vegetative cells; (*3*) small variability of parameter values on a leaf plate of the top and bottom parts of a leaf; (*4*) relative stability of values of the integral parameter during the period of leaf maturity; (*5*) relative stability of the range of its age changes irrespective of the structure of cenosis; (*6*) opportunity of unequivocal physiological interpretation and the forecast of PSA state of plants.

Among the used FPs there are some candidates for integrated basic parameters which could be applied to solve the considered ecological problem. One of them is stress adaptation index A_p (Strasser *et al.* 1987, Lichtenthaler and Rinderle 1988) and the parameters introduced on the basis of the area above the curve of the slow phase (Nesterenko and Sid'ko 1993).

Conclusions

Based on the literature and our model experiments, we conclude that the ontogenetic approach combined with the application of Chl FIK method in plant ecology allows to estimate the PSA of leaves and the whole plant. This is important for investigation of various prolonged adverse effects on phytocenoses. Besides, the ontogenetic approach is necessary for expansion of fluorescence methods not only for monitoring of phytocenosis PSA state, but also for different stages of the analysis of ecological monitoring data, in particular for diagnostics of plant PSA using the scale "norm-pathology" during the detection and ranging of adverse factors of anthropogenic nature, able to cause damages in phytocenoses and other ecosystems, for ecological standardization of stress factors, and determination of plant tolerance borders. With regard for age-related changes of fluorescence parameters of plant leaves provided for in the ontogenetic approach, we recommend to eliminate the main problem limiting the application of Chl FIK method in plant ecology – the ambiguity of interpretation of the physiological state of plant PSA based on fluorescence parameters. Besides, the changing of these parameters during plant leaf ontogeny is an additional integral characteristic for the estimation of general plant resistance, which is important for plant ecology and physiology. Thus, the ontogenetic approach in fluorescence research is a necessary and promising method both for definition of plant resistance to prolonged stress effects of various nature and their combinations and for estimation of primary reactions of plant leaf PSA because of the necessity to determine the physiological age of leaves and conifer needles more precisely and to obtain dynamic characteristics of PSA stability at different levels of its organization. We believe that the qualitative and quantitative estimates of general plant PSA resistance we propose can be used for the estimation of general plant resistance in natural environment. However, despite encouraging results, our work is only preliminary.

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