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RESEARCH PAPERS

Chlorophyll Fluorescence as an Indicator of Age-Dependent Changes in Photosynthetic Apparatus of Wheat Leaves

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Abstract—Wheat (*Triticum sativus* L.) seedlings of various ages (2- to 16-day-old plants) were used to study age-dependent changes in the chlorophyll fluorescence induction (CFI) at various light intensities during fluorescence measurements. Plants were raised in a growth chamber using hydroponics with expanded clay, controlled environmental conditions, and 690 μmol/(m^2 s) photon flux density (PFD) of photosynthetically active radiation (PAR). Parameters of CFI were determined under actinic PFD of 380, 580, 820, and 1340 μmol/(m^2 s) PAR. The fifth leaf from the stem base, exposed to uniform lighting, was sampled for measurements. This leaf emerged at the plant age of 16 days. Based on fluorescence data, we calculated the maximal photochemical quantum yield of photosystem II (F_v/F_m), the effective photochemical quantum yield of PSII (Yield), parameters of photochemical (qP) and non-photochemical (qN and NPQ) quenching of chlorophyll fluorescence, the F_p/F_t ratio, and the "vitality index" (fluorescence decrease ratio, R_{fd}). At moderate actinic PFD, applied commonly in PAM fluorometers (about 380 μmol/(m^2 s)), age-dependent changes in NPQ, F_p/F_t, and R_{fd} were observed. Analysis of CFI parameters in wheat leaves of different ages at PFD increasing from 380 to 820 μmol/(m^2 s) revealed that R_{fd}, NPQ, and qN are the most sensitive markers of the leaf age among all parameters tested. These suitable indicators can be used for rapid assessment of the leaf age.

Keywords: Triticum sativus, chlorophyll fluorescence parameters, actinic light intensity, leaf ontogeny

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INTRODUCTION

Analysis of chlorophyll fluorescence induction by means of pulse-amplitude modulated (PAM) fluorometers is widely applied nowadays for assessing the functional state of plant photosynthetic apparatus (PSA) and its resistance to stresses [1, 2]. Measurements of several fluorescence parameters (F_0, F_m, F_p, F'_0, F'_m, and F_t) enable determination of the effective photochemical quantum yield of photosystem II (PSII), parameters of photochemical (qP) and non-photochemical quenching of PSII fluorescence (NPQ, qN), the maximal photochemical quantum yield of PSII (F_v/F_m), the "vitality index" (fluorescence decrease ratio, R_{fd}), and other characteristics of PSA in plant leaves [1–3]. It is known that these parameters are affected by stress factors [3–6] and undergo changes during natural senescence of plant leaves [5, 7–12]. The age dependence of chlorophyll fluorescence parameters is a well-established fact. However, the extent of this dependence for individual parameters remains poorly examined; in many cases researchers were satisfied by simple comparison of fluorescence characteristics in mature and senescent leaves using rough estimates of leaf age [7–11, 13]. We showed earlier that the ratio F_v/F_m, characterizing the maximal quantum efficiency of PSII photochemistry, undergoes only slight (~8%) age-dependent changes under normal growth conditions [12, 14], whereas the ratio F_p/F_t (F_p and F_t are fluorescence intensities at the peak level P and under steady state, respectively [1]) exhibits its age-dependent changes with an amplitude of at least 40% [14]. Furthermore, the age dependence could be enhanced or weakened depending on the conditions of measurements [7, 12, 14].

Abbreviations: CFI—chlorophyll fluorescence induction; F_0 and F_m—minimal and maximal levels of chlorophyll fluorescence after dark adaptation; F'_0, F'_m, and F_t—minimal, maximal, and stationary levels of chlorophyll fluorescence in the light-adapted state; F_p—fluorescence intensity at the peak of CFI curve recorded under actinic illumination after preliminary dark adaptation; F_v/F_m—the ratio of variable ($F_v = F_m - F_0$) to maximal (F_m) fluorescence representing the maximal photochemical quantum yield of PSII; NPQ—non-photochemical quenching in PSII; PAM—pulse-amplitude modulated (fluorometry); PAR—photosynthetically active radiation; PFD—photon flux density; PSI and PSII—photosystem I and II; PSA—photosynthetic apparatus; qN—coefficient of non-photochemical quenching of chlorophyll fluorescence; qP—coefficient of photochemical quenching of PSII fluorescence; R_{fd}—vitality index; Yield = $(F'_m - F_t)/F'_m$—effective photochemical quantum yield, i.e., the fraction of light energy used by PSII complexes for electron transport.

Modern physiological and ecological studies employing the chlorophyll fluorescence induction (CFI) are mostly performed with mature leaves. As a rule, the leaf age is estimated by the calendar method, i.e., by counting the number of days since leaf emergence [7, 15]. Rough estimates of the leaf age can be made by taking into account the maximum leaf area [8, 11]. In a previous study [16] it was stated that accuracy and reliability of assessing the PSA state based on the ratio F_p/F_t, as an integral CFI parameter, depends on the stage of leaf ontogeny. Since leaf ontogeny is a complex process, while the general concept of leaf senescence is missing, reliable criteria for the transition of the mature leaf to the stage of senescence are currently absent [15, 17, 18]. Apparently, a set of physiological, biochemical, and biophysical traits is needed for the description of the leaf age condition [8, 15–18].

The F_p/F_t ratio is known to undergo consistent age-dependent changes toward the steady state; the duration of stationary state coincides with period of leaf active functioning [7, 19]. However, the F_p/F_t ratio does not provide precise assessment of the leaf age state at the stage of leaf maturity and beginning of senescence, because this ratio fluctuates in relation to metabolic and developmental changes of the whole plant [20]. Our studies with radish and wheat plants revealed a significant impact of leaf senescence on the light-response curves for F_p/F_t ratio. Leaf senescence was accompanied by changes in shape and quantitative parameters of the light-response curves for F_p/F_t [12, 14].

Age dependence of CFI parameters was mostly studied under constant intensity of actinic light [7–9] and only with young and old leaves [10, 11, 13]. Therefore, the goal of this work was to quantify the age dependence of CFI and to examine the impact of actinic irradiance on CFI parameters. Research in this area is essential for precise determination of the leaf age state and for assessment of leaf age-dependent plant resistance to various environmental factors.

MATERIALS AND METHODS

Wheat plants (*Triticum sativus* L., line 232 bred by Lisovskii [21]) were raised in a growth chamber using hydroponics with expanded clay, controlled environmental conditions, and 690 μmol/(m^2 s) photon flux density (PFD) of photosynthetically active radiation (PAR) [21]. Air temperature was automatically maintained at 24 ± 1°C over the growth period. The relative humidity was 65–75%. For CFI measurements we sampled uniformly illuminated fifth leaves from the upper leaf layer (the fifth leaf counting from the stem base; this numeration corresponds to the sequence of leaf appearance); the leaf ages ranged from 2 to 16 days. The plant age at the beginning of measurement was 16 days. We selected five reference points corresponding to different stages of leaf ontogeny [15]: the first three points (the second, fifth, and ninth days of leaf development) corresponded to the ontogeny stages at which the leaf area was 20, 50, and 80% of the maximal value. The fourth point coincided with the end of leaf growth (12th day), and the fifth point corresponded to 16-day-old leaves. Measurements were performed on the middle part of intact undetached leaf blades.

Parameters of CFI induced by actinic light were recorded by means of a PAM-2100 fluorometer (Walz, Germany) at photon flux densities of 380, 580, 820, and 1340 μmol photons/(m^2 s). Prior to fluorescence measurements leaves were adapted to darkness for 30 min. Four replicate measurements were performed for each light treatment. The measurement protocol employed in our study was similar to that described by others [2, 22]. The maximal and effective quantum yields of PSII photochemistry were determined using expressions $F_v/F_m = (F_m - F_0)/F_m$) and Yield = $\Delta F/F'_m = (F'_m - F_t)/F'_m$ [2]. The parameter Yield provides an approximate measure for the overall quantum yield of photosynthesis.

In order to quantify thermal dissipation of excitation energy, we used the parameters qN and NPQ characterizing non-photochemical quenching of fluorescence. The coefficient of non-photochemical quenching qN and non-photochemical quenching NPQ were calculated from the equations: $qN = 1 - F_{v'}/F_v$ and $NPQ = F_m/F'_m - 1$. The NPQ value characterizes the rate constant of energy dissipation to heat in the antenna of PSII [1, 23]. In order to determine the proportion of PSII complexes in which Q_A was oxidized before the application of the saturating light pulse (4000 μmol/(m^2 s), we calculated the coefficient of photochemical quenching $qP = (F'_m - F_t)/(F'_m - F'_0)$ [1].

The "vitality" index, R_{fd} was calculated from the formula: $R_{fd} = F_p/F_t - 1$.

Presently, the above fluorescence parameters are commonly used for determining plant resistance to stress factors [4, 13, 24, 25] and for studying the leaf ontogeny [8–12, 14, 23].

Data in tables and figures are means values and their standard errors.

RESULTS

Analysis of the F_v/F_m ratio as an indicator of PSA condition in wheat leaves during their development revealed that this ratio remained quite high in leaves of various ages (Fig. 1), even at early stages of leaf senescence (12- and 16-day old leaves); the relative changes in F_v/F_m ratio were 2%. The highest F_v/F_m value (0.815) was detected on the 9th to 12th days of leaf development.

Fig. 2 shows that age dependences of the F_p/F_t ratio were similar at all intensities of actinic light (380–

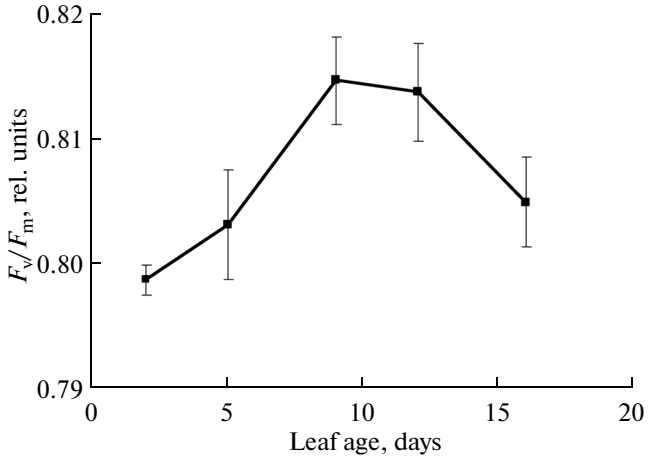

Fig. 1. Age dependence of the maximal photochemical quantum yield of PSII (F_v/F_m) in the fifth leaf of *Triticum sativus* (numeration from the stem base).
Bars in the graph designate mean values ± standard errors ($n = 16$).

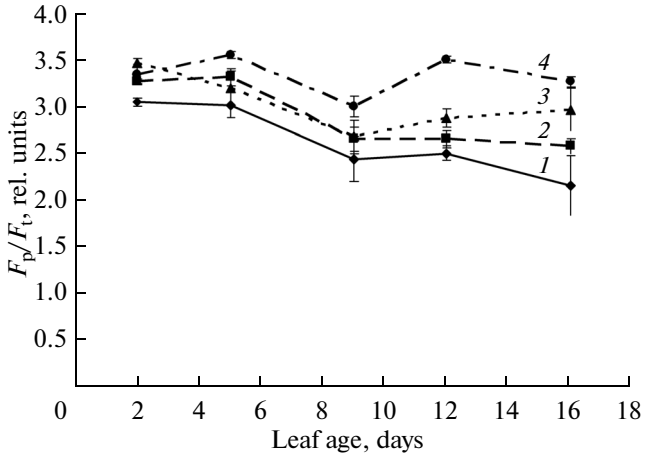

Fig. 2. Effect of actinic light intensity on the age dependence of F_p/F_t in the fifth leaf of *Triticum sativus*.
Numbers designate actinic light intensities: *1*—380, *2*—580, *3*—820, *4*—1340 μmol/(m^2 s). Bars in the graph represent mean values ± standard errors ($n = 4$).

1340 μmol/(m^2 s) PAR) for leaves whose ages varied from 2 to 9 days. In older leaves the age dependences deviated appreciably at different intensities of actinic light. For example, at PFD > 580 μmol/(m^2 s), the F_p/F_t ratio increased with age in 12- and 16-day-old leaves (Fig. 2, curves *3*, *4*), whereas at PFD < 580 μmol/(m^2 s) this ratio decreased with age or remained unaltered with respect to the F_p/F_t values in 9-day-old leaves (Fig. 2, curves *1* and *2*, respectively). Age-dependent variations of F_p/F_t values at a given intensity of actinic light (380 μmol/(m^2 s) PAR) were about 30% (Fig. 2), whereas variations of R_{fd} amounted to 42% (Table 1). At actinic irradiance of 820 μmol/(m^2 s), the relative changes in F_p/F_t ratio were lowered to 23% and changes in R_{fd} reduced to 24% (Table 1).

The age-dependent plots of photochemical quenching qP were flattened at a wide range of actinic irradiance. For example, in the PFD range from 380 to 820 μmol/(m^2 s), age-dependent variations of qP did not exceed 16%; however, at PFD 1340 μmol/(m^2 s) these variations increased to 27% (Fig. 3). As for non-photochemical quenching of chlorophyll fluorescence (NPQ) at actinic irradiances from 380 to 1340 μmol/(m^2 s), the age-dependent variations of NPQ at any given PFD were 35–43% (Fig. 4). The increase in actinic irradiance modified the age dependences of NPQ.

The results presented in Figs. 2 and 4 indicate that the lowest PFD-dependent variations in F_p/F_t and NPQ were evident in the mature 9-day-old leaves. For

Table 1. Age dependence of the fluorescence characteristics Yield, R_{fd}, and qN for *Triticum sativus* leaves sampled from the top leaf layer

Treatments (various light intensities)	Leaf age, days			
	5	9	12	16
		Yield		
1	0.61 ± 0.01	0.58 ± 0.02	0.62 ± 0.02	0.62 ± 0.04
2	0.44 ± 0.04	0.50 ± 0.03	0.51 ± 0.01	0.51 ± 0.02
		qN		
1	0.44 ± 0.14	0.45 ± 0.01	0.42 ± 0.04	0.40 ± 0.05
2	0.61 ± 0.07	0.49 ± 0.04	0.56 ± 0.03	0.63 ± 0.02
		R_{fd}		
1	2.01 ± 0.13	1.44 ± 0.24	1.50 ± 0.08	1.16 ± 0.32
2	2.20 ± 0.18	1.68 ± 0.18	1.88 ± 0.10	1.96 ± 0.22

The fifth leaf counting from the stem base (the numeration corresponds to the succession of leaf emergence) was sampled for measurements. The table lists relative values obtained upon the increase in actinic irradiance from 380 (1) to 820 (2) μmol/(m^2 s).

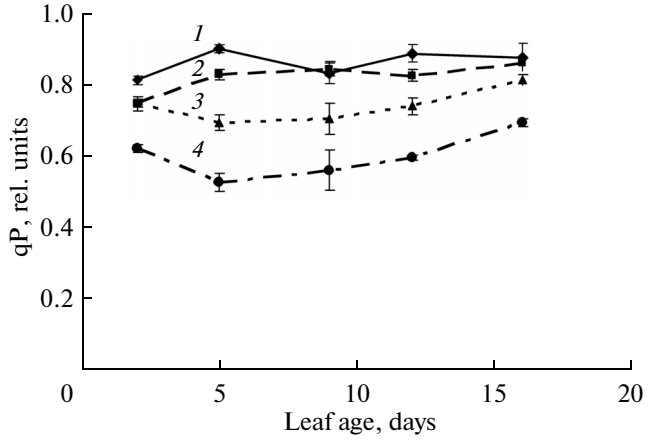

Fig. 3. Age dependence of the coefficient of photochemical PSII fluorescence quenching (qP) in the fifth leaf of *Triticum sativus* at various intensities of actinic light. Designations are the same as in Fig. 2. Bars in the graph are mean values ± standard errors ($n = 4$).

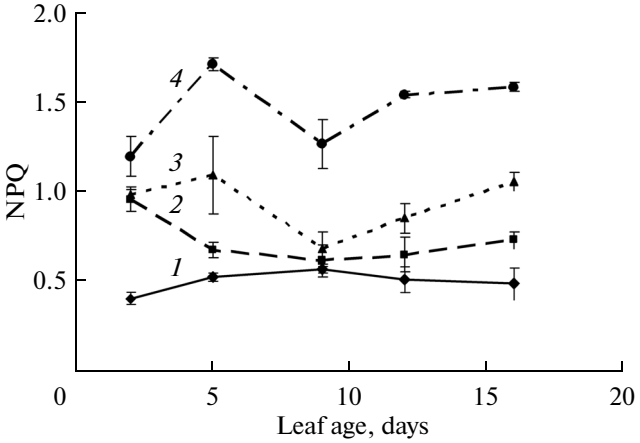

Fig. 4. Influence of actinic light intensity on the age dependence of non-photochemical quenching (NPQ) in the fifth leaf of *Triticum sativus*.
Designations are the same as in Fig. 2. Bars in the graph represent mean values ± standard errors ($n = 4$).

older samples (12- and 16-day-old leaves), the variations of fluorescence characteristics increased with PFD in the range from 380 to 820 μmol/(m^2 s). This fact is also substantiated by data in Table 1 for the parameters R_{fd} and qN. Comparative analysis of CFI characteristics in wheat leaves at actinic irradiance increasing from 380 to 820 μmol/(m^2 s) PAR identified the vitality index R_{fd} and non-photochemical quenching (NPQ, qN) as the parameters that are most sensitive to leaf age (Table 2).

DISCUSSION

In experiments with PAM fluorometers, the actinic light intensities vary in a wide range, from 140 to 1500 μmol/(m^2 s) PAR [1–4, 23]. Since PSA characteristics are evaluated with individual leaves, the question arises as to whether it is possible to reduce age-dependent variations of fluorescence parameters by adjusting experimental conditions during measurements. In this connection, two approaches are feasible. The first one is to conduct measurements with mature leaves or with samples at a definite stage of leaf ontogeny. These requirements are often difficult to accomplish because of the lack of precise criteria for leaf age and due to approximate ways of leaf age determination (the leaf age is usually counted by days from the moment of leaf emergence [5, 15, 16]). The second approach consists in careful adjustment of experimental conditions, especially actinic PFD, in order to minimize the influence of leaf age factor. For example, Nesterenko et al. [26] found that age-dependent variability of CFI amplitude and temporal parameters was largely reduced at actinic irradiances above 200 μmol/(m^2 s) PAR.

Data presented in Figs. 2–4 and Table 1 suggest that, at actinic PFD of 380 μmol/(m^2 s), the age-dependent condition of the leaf PSA cannot be determined with

Table 2. Changes in characteristics of CFI (Δ_{rel}, %) in *Triticum sativus* leaves sampled from the top leaf layer

Fluorescence parameters	Leaf age, days			
	5	9	12	16
R_{fd}	9.5	16.7	25.3	69.0
NPQ	47.7	11.1	36.6	61.4
qN	39.1	8.4	33.4	56.7
qP	−22.2	−16.6	−16.9	−8.0
Yield	−27.1	−14.6	−19.2	−18.0

The fifth leaf counting from the stem base (the numeration corresponds to the succession of leaf emergence) was sampled for measurements. The table lists relative values obtained upon the increase in actinic irradiance from 380 to 820 μmol/(m^2 s).

sufficient accuracy based on individual fluorescence parameters or their combination. However, at actinic PFD > 580 μmol/(m^2 s) the parameters of non-photochemical quenching, NPQ and qN (Table 1) were clearly age dependent, making it possible to distinguish the stages of leaf maturity from the beginning of leaf senescence. For example, mature sugar beet leaves exposed to actinic PFD > 380 μmol/(m^2 s) were characterized by high effective quantum yields of PSII photochemistry and by intermediate NPQ values [8]. The old leaves exhibited high NPQ values. It was found that variations in photochemical quenching qP in young and old leaves become higher with the increase in PFD and that non-photochemical quenching (qN) is saturated at markedly lower light intensities in old leaves [27]. The latter observation suggests that the loss of excitations to heat is higher in old leaves than in young ones.

The accuracy of leaf age determination can be increased by comparing NPQ, qN, and R_{fd} (vitality index) at two actinic irradiances, e.g., at 380 and 820 μmol/(m^2 s) (Table 2). In mature 9-day-old leaves, this shift in PFD led to the lowest changes in parameter values, whereas it elevated the R_{fd} values by a factor of 1.5 and 4.0 in 12- and 16-day-old leaves, respectively; the NPQ values increased 3 and 6 times, respectively; and the values of qN increased nearly 4- and 7-fold, respectively (Table 2).

One physiological method for assessing the leaf age condition prior to leaf maturation is to measure the growth plot for leaf area [15]. The growth curve of leaf area reveals the period at which the leaf becomes fully expanded or achieves a certain level of expansion. The transition to maturity is evident from the inflection points on age dependences of all structural and functional indices [9, 15, 18]. The highest functional activity of the leaf, as assessed from biochemical and photosynthetic parameters, is usually attained in advance to full leaf expansion, when the leaf area is about 80% of the maximal value [14, 15, 19]. The normalized curves of leaf senescence represent the right-side halves of bell-shaped curves of leaf ontogeny plotted for many physiological traits. For example, such temporal changes were observed for chlorophyll *a* content, photosynthesis, the maximal fluorescence F_m, and the F_v/F_m ratio in *Arabidopsis* plants [9]. In studies of physiological properties and fluorescence in sugar beet leaves of various ages [8], a remarkably higher stability of fluorescence data, compared to biochemical parameters, was noticed. The dynamics of chlorophyll content and the sum of soluble carbohydrates was paralleled by age-dependent changes in the efficiency of photosynthetic energy utilization (parameter Yield) [8].

The light response curves for F_p/F_t ratio and variations of this parameter at different actinic irradiances are used as a biophysical criterion for the transition of higher plant leaves from maturity to senescence. In our previous work [14] we showed the advantage of this approach compared to the classical assessment of leaf PSA condition from F_v/F_m and F_p/F_t ratios determined at fixed actinic irradiance. Comparison of age-dependent changes in F_p/F_t, R_{fd}, NPQ, and qN at a series of actinic PFD revealed that parameters NPQ and qN are advantageous compared to the former ones. Although qN is more sensitive than NPQ to leaf age (leaf senescence, Table 2), the parameter NPQ is preferable for application in plant physiology for several reasons. In experiments with mono- and dicotyledonous plants, the parameter NPQ was not species-specific in the response to actinic light intensity [27]. On a comparative basis, qN characterizes the extent of non-photochemical quenching from F_v to F_v', whereas NPQ quantifies the decrease of F_m to F_m' [1, 23]; both parameters are attributed to PSII. It should be noted that NPQ can be calculated without determination of F_0', the minimal fluorescence in light-adapted leaves [28]. According to some reports [28, 29] the contribution of PSI into F_0 fluorescence can be as high as 30%, whereas the PSI contribution to F_m, according to simulation models, may constitute 13–18% [30].

It is known that non-photochemical quenching of chlorophyll fluorescence depends on a variety of processes having at least three molecular mechanisms: (1) ΔpH-dependent quenching, (2) transition of the pigment system from state 1 to state 2, and (3) photoinactivation of PSII [1, 13, 23]. The contribution of each mechanism to the total extent of non-photochemical quenching depends on light intensity [27] and the leaf age [13, 14, 23].

The application of high-intensity light leading to photoinhibition may disturb the amplitude parameters of CFI (e.g., F_m and F_0) and derivative ratios, including the F_v/F_m ratio used widely in environmental studies [4, 22, 28]. For this reason, the plant growth conditions, as well as conditions of CFI measurements should be strictly defined (actinic PFD, etc.). Variations of physical parameters during measurements may give rise to considerable variability of results [4, 22, 28]. Notwithstanding the recommended regimes of PAM fluorometry application [2, 22, 28], actinic PFD employed in various studies ranged from 140 to 1500 μmol/(m^2 s) PAR. For example Wang and Chen [10] determined F_m' and F_t at PFD equal to 200 and 1400 μmol/(m^2 s). The vitality index, R_{fd} is usually determined under saturating white light at PFD of 2000 μmol/(m^2 s) or under irradiation with a short-wavelength red light of He/Ne laser (632.8 nm) at PFD of 700 μmol/(m^2 s) PAR [2].

The present results show that, under comparatively low actinic irradiance, applied in experiments with PAM fluorometers (380 μmol/(m^2 s) and lower), the parameters F_p/F_t, R_{fd}, and NPQ are age dependent. Other fluorescence parameters, assessed at sufficiently low actinic irradiance, were almost independent of the leaf age. However, the increase in PFD of actinic light could result in appreciable influence of leaf age on the

fluorescence characteristics (e.g., qP), especially in leaves at early stages of senescence (Fig. 3, Table 1).

The choice of optimal actinic irradiance may depend on the light intensity used during plant growth. Nath et al. [9] cultivated *Arabidopsis thaliana* plants at PFD of 100 μmol/(m^2 s) and applied the actinic light with PFD of 1500 μmol/(m^2 s). In our work we employed PFD of 690 μmol/(m^2 s) for growing wheat plants. Light intensities used for growing cereal crops are usually higher than for growing vegetable crops [21].

Analysis of the literature data and our own results indicate that intensities of actinic light used for PAM fluorometry should be selected by taking into account growth irradiance and the purposes of the experimental study [4, 5, 9]. For example, in an earlier work [5] we proposed that actinic light of near-saturating intensity (about 1500 μmol/(m^2 s)) is not the optimal choice for assessment of PSA resistance under stress conditions. The light intensity applied for this purpose should be close to the so-called irradiance of plant adaptation; i.e., the PAR value corresponding to the maximal efficiency of CO_2 exchange in leaves. The reason beyond this proposal was that photosynthesis and leaf respiration are optimally balanced at such irradiance. The question of how light conditions during plant growth affect the optimal actinic irradiance during CFI measurements warrants further investigation. It should be noted that analysis of data from various publications should take into account not only the leaf age but also the stage of plant development. This point is important because fluorescence parameters may undergo temporal synchronous changes at some stages of plant development, as it was shown with an example of flag leaf of wheat [20].

Under prolonged impact of moderate stresses, the response of PSA in plant leaves is often nonspecific and consists in accelerated leaf aging [16, 19]. In this context, the assessment of age-dependent leaf condition and determination of the beginning of leaf senescence under the action of stress factors is actually equivalent to the assay of PSA resistance in plant leaves to these factors [5]. The problem of clear-cut differentiation of the leaf age conditions is particularly challenging for studying plant responses to long-term action of stress conditions. For example, Ghanem et al. [13] showed differential alterations of CFI parameters (F_v/F_m and NPQ) in young and old tomato leaves in response to prolonged (over several days) salt stress.

The development of new, undamaging methods for determining the leaf age is important for further advance in the theory of aging. Changes in chlorophyll fluorescence characteristics that are highly sensitive to the leaf age, e.g., the vitality index (R_{fd}) and parameters of non-photochemical quenching of PSII chlorophyll fluorescence of (NPQ, qN), can be used as suitable tests for rapid determination of the leaf age.

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