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METHODS

Effect of Light Intensity on Chlorophyll Fluorescence in Wheat Leaves: Application of PAM-Fluorometry

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Abstract—Application of pulse-amplitude-modulation (PAM) fluorometers for measuring slow stages of chlorophyll fluorescence induction (CFI) is considered. With an example of *Triticum aestuvum* L. plants grown under continuous illumination at a photon flux density of 600 μmol/(m^2 s) photosynthetically active radiation (PAR), the CFI curves were analyzed with leaves of various ages as a function of actinic light intensity. The fluorometer PAM-2100 was applied for measurements of CFI curves. The characteristic peaks of CFI curves in wheat leaves were most conspicuous and had the largest amplitudes at 600–800 μmol/(m^2 s) PAR, which corresponds to the middle range of actinic light intensities employed in PAM-2100 fluorometers. In plants exposed to favorable and stressful conditions, the developmental stages may proceed at different rates; thus, the comparison of fluorescence parameters for leaves of equal calendar age but having different physiological states may provide ambiguous data. Therefore, the feasibility of recording CFI curves of different types is quite important for rapid diagnostics of the age and state of plant leaves, as well as for adequate physiological conclusions.

Keywords: Triticum aestivum, induction of chlorophyll fluorescence, actinic light intensity, leaf ontogeny

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INTRODUCTION

The chlorophyll fluorescence induction (CFI) in plant leaves, also termed Kautsky effect, is a phenomenon that has been known for more than 80 years [1]. Over this period, the method employing CFI phenomenon proved to be a highly informative tool for assessing the state of plant photosynthetic apparatus (PSA) in applied and basic research [1, 2]. The method is based on measuring CFI curves that represent temporal changes of chlorophyll fluorescence intensity. The scientific interest resides in the shape of CFI curve [2–7], its amplitude, and temporal characteristics [8–10]. Characteristics of CFI slow phases (PSMT-transitions [1, 2]) are highly informative for understanding the mechanisms of PSA functions at the leaf level under various conditions, because these phases are related to biochemical steps of photosynthesis and to the rate of CO_2 exchange in leaves [10–14]. It is assumed that the slow phase of CFI curve results from oxidation of the photosystem II (PSII) primary acceptor Q_A due to the activation of dark reactions of photosynthesis (photochemical quenching of fluorescence) and from the increase in heat dissipation in the light-harvesting antenna of PSII (nonphotochemical quenching of fluorescence, NPQ) [2, 10]. The CFI kinetics is controlled by a series of interrelated factors considered in detail in several reviews [1, 2, 10, 11]. These factors include the redirection of electron flows between cyclic and noncyclic electron transport pathways in chloroplasts [13, 14], the redox-dependent activation of the Calvin cycle enzymes, and activation of the ATP synthase complex [2, 11, 12, 15].

In the last few years, pulse-amplitude-modulation (PAM) fluorometers have become a globally accepted tool for the research of plant organisms [1, 2, 10, 13, 14]. Owing to their advantages, these fluorometers have de facto provided the standard approach in plant physiology and ecology for studies of chlorophyll fluorescence. At the same time, some experimental results of earlier studies have been unwarrantably forgotten, despite retaining their scientific and practical significance [2, 11]. This remark refers to studies based on single-beam fluorometers, where required spectral regions were selected with a combination of filters. In these studies, the slow phases of CFI curves (staring from the peak P attained approximately at 1 s) were analyzed to assess the physiological state of leaves in normal and stressed higher plants [3, 5–7].

Modern pulse-modulated fluorometers have the facilities of recording and storing the Kautsky curve in the device memory (PAM-2100) [13, 14]. However,

Abbreviations: AL—actinic light; CFI—chlorophyll fluorescence induction; PAM—pulse-amplitude-modulation (fluorometry); PSII—photosystem II, PSA—photosynthetic apparatus; NPQ—nonphotochemical quenching of chlorophyll fluorescence.

technical features of fluorescence excitation and measurement in these new instruments differ significantly from those in classical single-beam fluorometers, where a single light source and a system of filters or a spectrometer were generally used [2, 11]. In PAM-fluorometers, the light emission from several sources is used simultaneously: the measuring pulse-modulated light, high-intensity saturating light pulses, and continuous actinic light (AL) from one of two sources. The AL intensity may vary according to the researcher's choice. This technical design not only increases the information capabilities of CFI method but also improves the sensitivity of PAM-fluorometers. Theoretically simulated data presented in [16] described the intensity of chlorophyll fluorescence as a function of excitation irradiance and duration of AL exposure. Theoretical analysis of the CFI kinetics revealed that "PAM-fluorometer data are related to PSA functional states."

Another important point to be considered in fluorescence measurements is age-related state of plant leaves. Previous studies [3, 5, 6] have shown that the shape of CFI curves, with certain peaks either lacking or evident in different proportions, depends critically on the leaf age. It is worth noting that researchers employing single beam fluorometers for CFI measurements do not always specify the leaf age for the compared plant groups [17], although the stage of leaf ontogeny is essential and should be taken into account for the comparison of fluorescence parameters [18]. When chlorophyll fluorescence is recorded with new techniques, e.g., video recording and analysis of kinetic parameters of spatially distributed fluorescence over the leaf surface [19], nonuniform illumination is thought to be the main reason for heterogeneity of the integral fluorescent parameter R_{fd} (vitality index [14]) over the leaf area. However, the age-related heterogeneity of chloroplasts within an individual leaf remains unnoted, and the age dependence of R_{fd} parameter is often overlooked [19]. At the same time, under certain measurement conditions (specifically, under proper choice of AL intensity range), the age dependence of CFI parameters and kinetics is manifested most clearly [20, 21]. The occurrence of individual peaks in the CFI kinetic curve is related to the activity and regulation of certain biochemical cycles in the plant leaf ontogeny. For example, the peak M was missing in CFI curves of young leaves where CO_2 fixation was almost absent [5, 8, 22]. With the advance in PSA development and activation of Calvin cycle enzymes, the M peak becomes evident and its amplitude increases [5–9, 22, 23].

Three types of CFI curves were classified [6]. These induction curves appeared in succession throughout the leaf development and aging: (1) single-peak curves with a rapid decline to a steady-state level without the PSM phase (young leaves); (2) two-peak curves with a distinct PSM transition; the peak P was recorded within 1–2 s and the peak M was attained 8–10 s after the onset of illumination (mature leaves); (3) single-humped curves with a slow decline to the steady-state T level (senescent leaves). Such changes of induction curves were observed for a wide range of plants with C_3 and C_4 types of carbon metabolism, like mono- and dicotyledonous plants [5–9, 22]. The ontogeny-related changes in CFI of plant leaves grown under controlled [5–8, 24] and natural [9] light conditions, together with experimental data on age-dependent changes in physiology of photosynthesis [24–26], suggest that the above CFI-based classification correlates with the leaf physiological age, because the distinguished groups of CFI curves corresponded to certain stages of leaf ontogeny. Based on the age-related changes in fluorescence characteristics, we proposed and tested the developmental approach to the assessment of plant leaf states under optimum and stressful growth conditions [7, 20, 27].

When conventional single-beam fluorometers were applied, the classical Kautsky curves with clearly distinct individual peaks were observed under actinic light intensity of 150–250 μmol quanta/(m^2 s) in the blue region of photosynthetically active radiation (PAR) (400–500 nm) [4, 5, 15, 18, 23]. The actinic light sources inducing CFI in PAM-fluorometers differ from those in single-beam fluorometers not only in technical but also in spectral characteristics. For example, Lichtenthaler et al. [14] used saturating "white light" (2000 μmol/(m^2 s) PAR) or a short-wavelength red irradiation with a He/Ne-laser (632.8 nm, 700 μmol/(m^2 s)). Therefore, in order to observe CFI curves with clearly resolved peaks using modern PAM fluorometers, it is important to determine the appropriate range of actinic light intensities.

Since different types of CFI curves correspond to separate stages of leaf ontogeny, the possibility of recording CFI curves of different types is essential for the rapid assessment of leaf age in plants under favorable and adverse growing conditions. Wheat leaves represent a convenient model for analysis of age-related changes in plants [25].

Because the major part of experimental data on age dependence of CFI curves in plant leaves was formerly obtained with single-beam fluorometers, the aim of this work was to study the influence of AL intensity on slow stages of CFI in leaves of different ages using a PAM fluorometer and to identify the AL optimal range for age-dependent distinctions in CFI curves.

MATERIALS AND METHODS

Wheat plants (*Triticum aestivum* L.) were grown in a growth chamber under controlled environmental conditions and continuous irradiance of 600 μmol/(m^2 s) PAR as previously described [28, 29]. The air temperature was maintained at 24 ± 1°C; relative humidity was 65–75%.

For CFI measurements, we used the fifth-story leaves at the age from 2 to 16 days, and performed experiments in four replicates. Five age points corresponded to different stages of leaf ontogeny [25, 29]. The CFI curves were recorded using a PAM 2100 fluorometer (Heinz Walz, Germany) at AL intensities of 380, 570, 810, and 1330 μmol/(m^2 s) PAR obtained from the installed halogen lamp. Prior to fluorescence measurements, the plant leaves were adapted to darkness for 30 min. In order to avoid distortion of slow changes in CFI curves, we did not apply saturating light pulses. A built-in halogen lamp was used as a source of actinic white light (wavelengths < 710 nm). Because growth characteristics of leaves differ depending on plant species and because some dicots are characterized by the intercalary type of leaf growth, there is no straightforward methods for selecting the leaves of identical physiological age [6]. Therefore, we have chosen wheat plants as representatives of monocotyledonous plants with a linear growth of leaf blades. This choice greatly simplifies the selection of leaf parts having identical age states [3, 5, 25]. We studied the slow stages of CFI, because there is a positive correlation between the photosynthetic rate (expressed per 1 mg of chlorophyll) and the ratio of chlorophyll fluorescence M peak to the steady-state fluorescence, as displayed on the classical CFI curves [23]. We assessed the relative decrease in chlorophyll fluorescence during the induction period from the peak P to the stationary level, a so-called "vitality index" R_{fd}, according to the formula [13]:

$$R_{fd} = (F_P - F_S)/F_S, \quad (1)$$

where F_P is the fluorescence intensity at the peak P and F_S is the steady-state fluorescence level.

RESULTS AND DISCUSSION

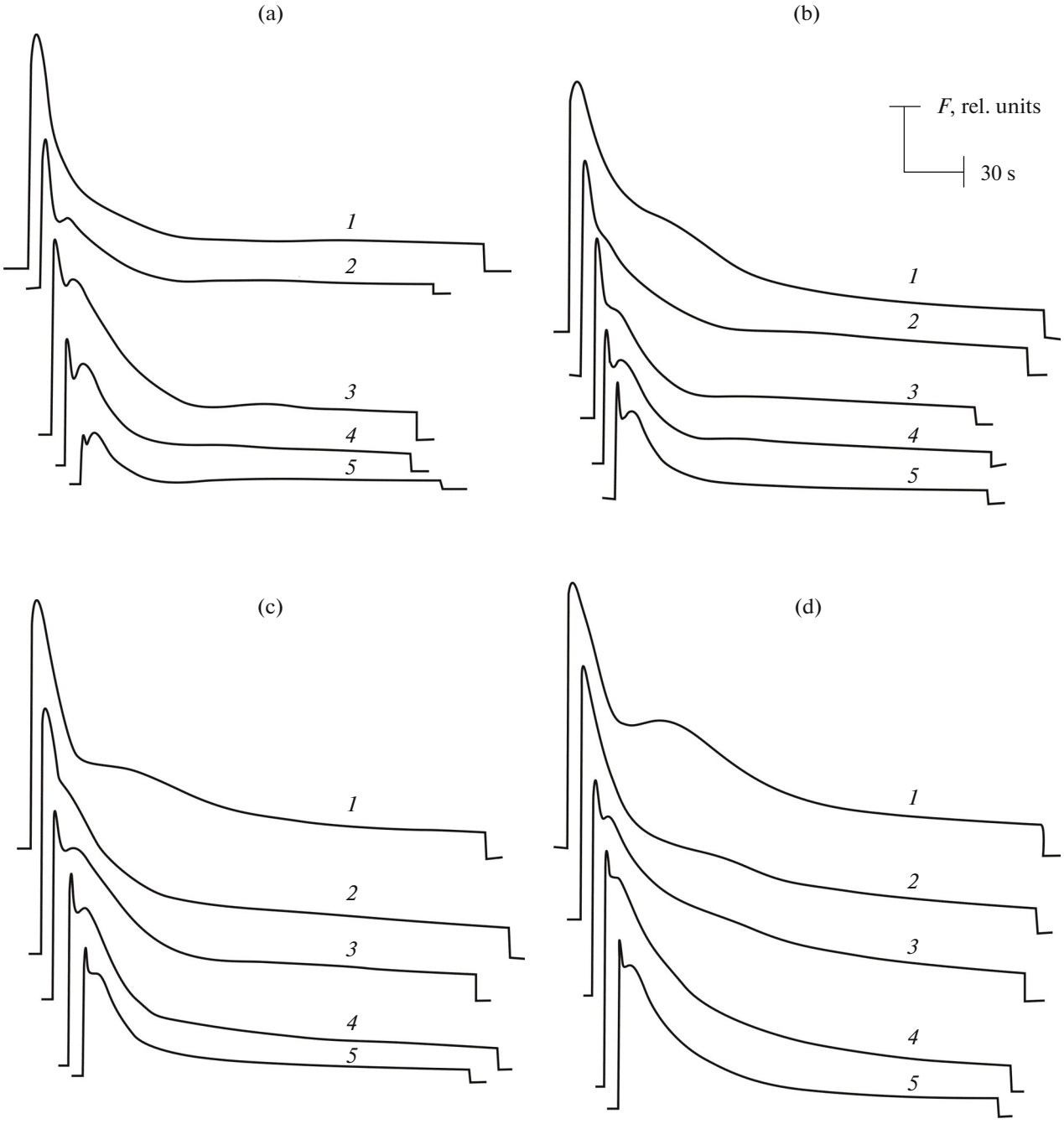
Our fluorescence measurements with a PAM-2100 fluorometer showed that, at AL intensity of 380 μmol/(m^2 s) PAR, which was almost twice as high as in single-beam fluorometers, the amplitude of the fluorescence P peak (F_P) on the CFI curve started to decrease when the wheat leaf achieved the stage of early senescence (Fig. 1a). When single-beam fluorometers were used, such CFI changes were only observed under insufficient duration of preliminary dark adaptation or under low AL intensities (<120 μmol/(m^2 s)) [3].

In the case of single-beam fluorometers [9, 30, 31], the increase in AL intensity to 600–800 μmol/(m^2 s) simplified the shape of CFI curve; i.e., the peaks M and M1 disappeared, while the peak P became sharpened. Alekseev et al. [16] reported that an increase in AL intensity enhanced fluorescence and that "the ratio of the first to the second peak increased nonmonotonically." Using a PAM 2100 fluorometer, we observed the classic Kautsky curve with a well-resolved M peak in mature wheat leaves at AL intensities of 570 and 810 μmol/(m^2 s) PAR (Figs. 1b and 1c). The amplitude of fluorescence changes was rather high for leaves of all ages tested.

Only at AL intensities of 1330 μmol/(m^2 s) PAR, discernible simplification of CFI curves was noticed (Fig. 1d). But even in this case, the young wheat leaves displayed an additional peak M1. According to the position of M1 along the time axis, this peak could be rather interpreted as a third peak becoming evident due to the smoothed second peak. It is supposed that the origin of this peak involves nitrogen metabolism, whose reactions represent a powerful sink for ATP and NADPH [2, 15].

While analyzing the results, we should first note that the range of AL intensities at which CFI curves were best resolved was almost equal to PAR level used for plant growing. This notion is consistent with the methodological approach described in [19], although the authors used single-beam fluorescence detection rather than PAM-fluorometry. Zavoruev et al. measured fluorescence with an originally designed fluorometer using AL intensity of 840 μmol/(m^2 s) [31], which is very close to light intensity employed in the present study. However, the CFI curves obtained in [31] had a simplified shape consisting of only fluorescence peaks P and M. Therefore, we believe that, in addition to light conditions during plant growing, the fluorometer type used for measurements should be definitely taken into account while choosing the AL intensity for CFI recordings.

Analysis of CFI curves presented in the figure reveals another important point, namely, the possibility of errors in calculating the vitality index R_{fd}, whose value increases linearly with the height of peak P (F_P) in the CFI curve (see equation 1), where F_S is the steady-state fluorescence [13]. It is important to note that Lichtenthaler et al. [14] used AL intensity of 2000 μmol/(m^2 s) to measure R_{fd}, whereas AL intensities of 1000–1200 μmol/(m^2 s) are recommended to prevent the photoinhibition of PSA. While inspecting the influence of AL intensity on the parameter R_{fd} in wheat leaves of different ages, one can see (table) that R_{fd} values in mature (9-day-old) leaves remain at approximately constant level at irradiances up to 810 μmol/(m^2 s). Higher values of R_{fd} were characteristic of young (2- and 5-day-old) leaves. At 1330 μmol/(m^2 s) the difference in R_{fd} values for leaves of different ages was found to decrease. At first glance, it seems convenient to use high-intensity AL to reduce the contribution of age-related leaf heterogeneity into uneven distribution of R_{fd} values over the leaf area. However, when AL intensity increases to very high levels that saturate photosynthesis; i.e., when F_P approaches F_{max} (maximum fluorescence of dark-adapted leaves), the parameter R_{fd} becomes equal or close to the parameter NPQ, as documented by the analysis of relations between various parameters



Slow stages in chlorophyll fluorescence induction curves in wheat leaves of different ages. Leaves were exposed to actinic light of various intensities: (a) 380, (b) 575, (c) 810, and (d) 1330 μmol/(m^2 s). (*1*) 2 days, (*2*) 5 days, (*3*) 9 days, (*4*) 12 days, and (*5*) 16 days.

of fluorescence [13]. At intermediate AL intensities, the parameter R_{fd} is expressed by a simple formula containing two fluorescence parameters (equation 1).

At high intensity of AL applied for a period of approximately 5 min, the F_S fluorescence does not reach its steady-state level. In this case, it is preferable to calculate R_{fd} from a somewhat more sophisticated formula containing six fluorescence parameters. In total, R_{fd} is represented as a complex function of the parameters that reflect both photochemical and nonphotochemical processes causing quenching of chlorophyll fluorescence [13].

Thus, the shapes of CFI curves turn simplified at a high-intensity AL. Apparently, intermediate AL intensities ranging from 600 to 800 μmol/(m^2 s) PAR not only produce the classic curve of CFI slow stages with a distinct peak M in mature wheat leaves but also provide the means to differentiate the leaves of differ-

Parameter R_{fd} in *T. aestivum* leaves of various ages as a function of actinic light (AL) intensity

AL, μmol/(m^2 s) PAR	Leaf age, days				
	2	5	9	12	16
380	2.09 ± 0.01	1.97 ± 0.13	1.48 ± 0.24	1.38 ± 0.12	1.28 ± 0.23
570	2.43 ± 0.03	2.34 ± 0.06	1.77 ± 0.13	1.64 ± 0.12	1.66 ± 0.12
810	2.49 ± 0.04	2.40 ± 0.20	1.72 ± 0.33	2.00 ± 0.10	2.11 ± 0.14
1330	2.40 ± 0.10	2.56 ± 0.03	2.10 ± 0.10	2.49 ± 0.06	2.42 ± 0.05

Data are mean values ± standard errors, $n = 4$.

ent ages according to the shape of CFI curves. This diagnostic facility is important for rapid assessment of the leaf age state in plants grown under favorable and adverse conditions [7]. Unfortunately, the plants exposed to normal and stressful environmental conditions are often compared without attention to age-related distinctions of their leaves. In some cases, data from different publications can hardly be reconciled in the absence of full information on the age states of the materials used. Our paper does not aim at criticizing such publications but rather emphasizes that plant physiologists studying chlorophyll fluorescence should pay close attention to the stage of leaf ontogeny. For example, the leaf ontogeny may proceed at different rates under normal and stressful conditions. Consequently, comparison of fluorescence parameters for leaves having identical nominal age but different age states can yield ambiguous results. In our chlorophyll fluorescence studies [18], we pointed out the benefits of the ontogenetic approach to the comparative analysis of physiology of photosynthesis in various environmental conditions.

The optimal choice of AL intensity may depend on the irradiance used for plant growth [14] and on the conditions of mineral nutrition [15]. The light intensity during plant growth for cereal crops is usually higher than that for vegetable crops [28]. Further studies are warranted to clarify the influence of light conditions during plant growth on the choice of optimal AL intensity during CFI measurements in plant leaves.

We conclude that obtaining CFI curves of distinct types using PAM-fluorometers depend on the proper choice of AL intensities. The actinic irradiance should fall into the intermediate range of PAR values, e.g., 600–800 μmol/(m^2 s) in the case of wheat plants.

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