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Detailed Insight into the Behaviour of Chlorophyll *a* Fluorescence Transient Curves and Parameters during Different Times of Dark Adaptation in Sunflower Leaves

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Abstract: The reason for this examination is today's wide usage of chlorophyll *a* fluorescence (ChlF) among researchers worldwide to measure photosynthetic efficiency. Although the instructions of the ChlF measuring device clearly emphasize the need for methodology adjustments, depending on the specific plant species, many researchers use the usual 30 min of dark adaptation before measurement. Namely, before any ChlF measurement, it is necessary to determine the specific duration of the leaf adaptation to the conditions of darkness of each plant tissue. Because of the numerous uses of the ChlF measurements, we decided to conduct this research to determine whether the appearance of the curves and parameter values depend on the time of sunflower leaf tissue adaptation to dark conditions. Therefore, this research aimed to examine the optimal adaptation time of sunflower tissue to dark conditions to obtain timely precise measurements and credible appearance of ChlF transient curves as well as accurate parameter values. The research was carried out on the sunflower hybrid Luka with 0, 15, 30, 45, 60, 75 and 90 min of dark adaptation in the vegetative, budding and flowering stages in the field conditions. According to the analyzed transient curves and parameters, it was determined that sunflower leaves should be kept in dark conditions for at least 15 min before the measurement of ChlF, which leads to the complete oxidation of PSII and the electron transport chain prior to a saturating pulse of light.

Keywords: *Helianthus annuus* L.; photosynthetic efficiency; clips; dark conditions; minutes; parameters

Citation: Markulj Kulundžić, A.; Sudarić, A.; Matoša Kočar, M.; Duvnjak, T.; Liović, I.; Mijić, A.; Varga, I.; Viljevac Vuletić, M. Detailed Insight into the Behaviour of Chlorophyll *a* Fluorescence Transient Curves and Parameters during Different Times of Dark Adaptation in Sunflower Leaves. *Agronomy* **2024**, *14*, 954. https://doi.org/10.3390/agronomy14050954

Academic Editors: Xiaoying Liu, Tran Quoc Khanh and Junfei Gu

Received: 2 April 2024
Revised: 25 April 2024
Accepted: 30 April 2024
Published: 2 May 2024

1. Introduction

The presence of frequent weather changes, accompanied by challenges from year to year, forced plant breeders, agronomists and biologists to use faster, simpler, and sufficiently informative methods in agriculture. For this reason, the analysis of measuring chlorophyll *a* fluorescence (ChlF) parameters is widely used to detect plants' response to environmental change (detecting plant stress) and genetic variation in controlled environments or field conditions. Monitoring, identifying and quantifying crop response to changing environmental conditions from a technical and scientific perspective are extremely complex and difficult but highly desirable for screening tolerant and high-yielding genotypes in breeding programs [1]. Furthermore, the integrated use of the ChlF analysis has the potential to accelerate the progress in elucidating plant functions by linking gene functions and environmental responses to various biochemical pathways, metabolism and processes [2].

ChlF, more precisely OJIP fluorescence transient, has been widely used for more than twenty-five years to study photosynthesis in plants in the interactive approach of biologists

and physicists [3,4] in fundamental research as well as in applied sciences. Therefore, shortly after, ChlF was adopted as a useful tool in agronomy research [5,6]. The application of the ChlF method on sunflowers started immediately after its introduction [7,8]. Some of the resulting research on sunflower deal with the photosynthetic characteristics at different nitrogen inputs into the soil [9], differences under combined salt and drought stress [10], the impact of light and heat stress on sunflower genotypes [11,12], the effect of drought and heat stress on sunflower [13] and by examining the effects of other environmental stresses on sunflower plants.

Determining the length of dark adaptation is necessary to accurately measure transient chlorophyll fluorescence, which can vary depending on the plant species [14]. Genty et al. [15] measured the initial minimum fluorescence (F_0) yield after at least 1 h of leaf adaptation to darkness in maize (*Zea mays* L.), red campion (*Silene dioica* L. *Clairv.*), barley (*Hordeum vulgare* L. *var. Clermont*) and chlorophyll-b-Iess barley (*Hordeum vulgare* L. *chlorina* F-2 mutant). In contrast, Mishra et al. [1] stated that ChlF in vivo should be measured after ~20–30 min of dark adaptation. Numerous researchers use that specified time of about 30 min in their research on maize, wheat, barley, soybean [16–19] and many other plants. Namely, the sufficient time for dark adaptation depends on the information it wants to receive. For *Pisum sativum* L., an adaptation time of 15 min was sufficient to study regulatory and photoinhibition-related processes that allowed ferredoxin-NADP+-reductase (FNR) [20], whereas, in a *Pinus brutia* Ten., 1 h is needed [21]. If you want to examine long-term adaptation responses of plant tissue to treatment, a much longer dark adaptation time that also allows recovery of regulatory processes and processes such as photoinhibition can be considered by Kalaji et al. [22]. From this, it can be concluded that the approach of measuring ChlF parameters without examining the necessary time of tissue adaptation to darkness is incorrect because the response of different plant species' tissue does not have to be in accordance. Dark adaptation is a complicated process that can be affected by several factors in the subsequent fluorescence measurement [22]. Namely, before implementation of any ChlF research, preliminary research should be carried out in order to correctly determine how much time the tested plant tissue needs to adapt to the conditions of darkness so that the obtained data are correct for comparison. According to Kalaji et al. [22], samples' dark adaptation for ChlF measurements is often associated with the re-oxidation of plastoquinone A (Q_A^-). By customized adaptation time to dark conditions of plant tissue, a high-quality application of the correct methodology is ensured, whereby the primary stable electron acceptor of the reaction center of the photosystem (PS) II, plastoquinone (Q_A), is completely oxidized, reaction centers (RCs) are open, which enables the measurement of minimum fluorescence (F_0). Conversely, maximum fluorescence (F_m) is considered when all Q_A and all electron carriers outside it are in a reduced state, and the RCs are closed. The steps between the two mentioned ChlF parameters (F_0 and F_m) describe the dynamics of the steps in PSII, PSI and further in photosynthesis [23].

Changes in the ChlF intensity in dark conditions, when the tissue is illuminated for one second, are called the Kautsky effect [24] and are shown on the OJIP logarithmic curve. Band O corresponds to the initial emitted fluorescence (F_0), while bands J and I correspond to the emitted fluorescence after 2 and 30 ms, respectively. Band P corresponds to F_m [25]. Under certain conditions (heat stress), the appearance of additional bands is possible when the K band appears at around 300 µs. Additionally, between 50 and 300 µs (usually 150 µs), also a shift of the induction curve can appear, called the L band [4]. H and G bands may also appear. H band between J and I at 2–40 ms and G band between I and P at 40–300 ms [26].

In leaves adapted to dark conditions, the inactivation of some enzymes (Rubisco, D-fructose-1,6-bisphosphatase, phosphoribulokinase and sedoheptulose-1,7-bisphosphatase, ATP synthase and FNR) occurs to prevent the wasting reaction. The mentioned enzymes were reactivated before steady-state photosynthesis was induced, affecting fluorescence induction's kinetics. Considering that the active FNR, which represents the activated acceptor side of the PSI, has an effect on the IP phase of the OJIP transients and on the F_m amplitude that can be reached by a strong light pulse [21], this method of adaptation time

to darkness is the simplest to use, where the tissue must be kept in the dark long enough for the FNR to lose its activity again [20–22].

Also, during the adaptation of samples to darkness for ChlF measurements, the length of the period without light should be considered. Namely, for the redox equilibration of the PQ pool and $CaMn_4O_xCl_y$ cluster, only a few minutes of darkness are needed. At the same time, a longer duration of the dark period can deplete the respiratory substrates through respiration in cyanobacteria and chlororespiration in higher plants and algae. In addition, it can deplete ATP pools and transmembrane ion concentration gradients to various extents [27].

Sunflower (*Helianthus annuus* L.) is a global oilseed that is adaptable to climate changes, whereby, according to some authors, it maintains stable yields in various environmental conditions, including drought [28], while others state certain stages of sunflower development as very sensitive to changes [29]. Instead of being a C_3 plant with net photosynthesis of 25–32 µmol CO_2 fixed m^{-2} s^{-1} of leaf, the domesticated sunflower has a high photosynthetic potential, which is similar to the C_4 plant maize. The sunflower plant's potential was manifested by higher tissue permeability for CO_2 diffusion and high RuBisCO activity, which resulted from stomata on both sides of the leaf [30]. Furthermore, studying the behavior of ChlF parameters by stages of sunflower development, it was observed that the values of photosynthetic parameters depend on the sunflower development stage [31].

So, in accordance with the text above, before using the ChlF measurement, every researcher must first get to know the material to determine the conditions that need respect to obtain valid and correct data. To determine the correct time for adapting a sunflower (*Helianthus annuus* L.) leaf to dark conditions, providing valid and comparable ChlF data, the sunflower hybrid Luka was tested in the vegetative, budding and flowering developmental stages. We hypothesized that the different adaptation times of sunflower leaves will affect the flow of electrons between the photosystems, which will be expressed differently according to the stages of sunflower development.

2. Materials and Methods

2.1. Cultivation of the Plants

The experiment was conducted on sunflower hybrid Luka at the Agricultural Institute of Osijek (45°32′ N, 18°44′ E; 94 m altitude). Characteristics of the hybrid Luka: two-line mid-early hybrid, vegetation length from 115 to 120 days, firm stems and high genetic potential for grain yield (6 t ha^{-1}) and oil content (51–53% DM). The hybrid is widely adaptable. It has a favorable position of the head, a high hectoliter mass of grain and extremely good fertilization of the central part of the head [32].

The experiment was conducted in the field. Sunflower seeds were sown by hand planters at 4 cm depth in five-meter long rows with 70 cm distance between rows and 23 cm distance within rows. The soil texture was sandy clay loam. The hybrid was part of the experiment sown in four rows in three repetitions.

2.2. Chlorophyll a Fluorescence (ChlF)

ChlF was measured on the fully developed upper leaf on 20 plants per row in the sunflower's three development stages using a plant efficiency analyzer (Handy PEA, Hansatech Instruments Ltd., Norfolk, UK). The first measurement was carried out in the vegetative stage of developed six pairs of leaves (V6), the second in budding (R3), and the third in the flowering (R5.7) stage of sunflowers [33]. The measurements were carried out during the period from 7:30–9:00 a.m. Before measurement, clips were placed for 0 (without dark adaptation), 15, 30, 45, 60, 75 and 90 min to achieve dark conditions. All clips were placed at the beginning of the experiment, after which we waited for a certain period. The leaves were exposed to a pulse of saturating red light at 3500 µmol m^{-2} s^{-1}.

Chlorophyll fluorescence transients were double normalized between O-P steps and presented as the relative variable fluorescence (V_t) on a logarithmic scale. The difference in the relative variable fluorescence was calculated and presented as the difference ΔV_{OP},

ΔV_{OK}, ΔV_{OJ}, $\Delta V_{JI,}$ and ΔV_{IP} normalized to the control (0 min or without dark adaptation) [34]. L, K, H and G Bands in the relative variable fluorescence curves appear at 0.15 ms, 0.3 ms, 20 ms and 100 ms, respectively [26].

2.3. Statistical Analysis

JIP-test parameters (Table 1) used for this research (F_0, F_m, φP_0, φD_0, φR_0, δR_0, ABS/RC, DI_0/RC, TR_0/RC, ET_0/RC, RE_0/RC, RC/CS_0, RC/ABS, TR_0/DI_0, $ET_0/(TR_0 - ET_0)$), PI_{ABS}, $\delta R_0/(1 - \delta R_0)$ and PI_{total}) were calculated in MS Excel from the recorded ChlF data according to Strasser et al. [23] and Yusuf et al. [34]. Mean values and standard deviations of ChlF parameters are shown in Table S1. The mentioned parameters were expressed as relative units. A one-way ANOVA was used to determine the statistical differences between time (minutes of dark adaptation) per developmental stage. Every developmental stage of the ChlF (n = 20) was processed separately, which was followed by the Fisher post hoc least significant difference (LSD) test at $p < 0.05$.

Table 1. Formulas and definitions of JIP-test parameters.

Formula	Description
F_0	Minimum fluorescence
F_m	Maximum fluorescence
$\varphi P_0 = 1 - (F_0/F_m)$	Maximal photochemical quantum yield
$\varphi D_0 = F_0/F_m$	Quantum yield of energy dissipation
$\varphi R_0 = \varphi P_0 \times \delta R_0$	Quantum yield for the reduction of terminal electron acceptors at the photosystem I acceptor side
$\delta R_0 = (F_m - F_I)/(F_m - F_J)$	Probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the photosystem I acceptor side
$ABS/RC = M_0 \times (1/V_J) \times (1/\varphi P_0)$	Absorption flux per active reaction center (RC)
$DI_0/RC = ABS/RC - TR_0/RC$	Dissipation flux per active RC
$TR_0/RC = M_0 \times (1/V_J)$	Trapping flux per active RC
$ET_0/RC = M_0 \times (1/V_J) \times (1 - V_J)$	Electron transport flux per active RC
$RE_0/RC = M_0 \times (1/V_J) \times \psi E_0 \times \delta R_0$	Electron flux reducing terminal electron acceptors at the PSI acceptor side per RC
$RC/CS_0 = \varphi P_0 \times (V_J/M_0) \times (ABS/CS_0)$	Density of active photosystem II reaction centers (RCs) per cross-section
$RC/ABS = 1 - ABS/RC$	Density of RC on chlorophyll *a* basis
$TR_0/DI_0 = F_V/F_0$	Flux ratio trapping per dissipation
$ET_0/(TR_0 - ET_0) = (F_m - F_J)/(F_J - F_0)$	Electron transport from Q_A^- to intersystem electron acceptors
$PI_{ABS} = RC/ABS \times TR_0/DI_0 \times ET_0/(TR_0 - ET_0)$	Performance index on absorption basis
$\delta R_0/(1 - \delta R_0) = (F_m - F_I)/(F_m - F_J)$	Electron transport from PQH_2 to final photosystem I (PSI) acceptors
$PI_{total} = PI_{ABS} \times (RE_0/ET_0/(\delta R_0/(1 - \delta R_0))$	Performance index for energy conservation from exciton to the reduction of PSI terminal acceptors

3. Results and Discussion

3.1. OJIP Transients

The measurements in this study were conducted on the youngest developed leaf at the top of the stem. In the first two stages of development, V6 vegetation stage and budding, the plant still develops its leaf mass, in contrast to flowering, when the sunflower reaches its maximum leaf mass development [35]. For this reason, we chose the upper leaf developed towards the light source in all three development stages, which was equally developed per stage for ChlF measurements. The difference in the speed of photosynthesis is created with regard to the angle of the positioning of the leaf on the stem in relation to the horizontal plane [36].

Results in Figure 1a–c clearly show the separation of the control curve in the P band (0 min of dark adaptation), which was determined under light conditions from curves obtained from measurements at different dark adaptation times. The resulting separation from other measurements that were carried out under dark adaptation conditions was most likely caused by the retention of the activity of ferredoxin-NADP+ oxidoreductase. From

this, it can be concluded that the time of adaptation to darkness lasting 15 min and more in sunflower plants was sufficient to achieve a decrease in ferredoxin-NADP+-reductase, which agrees with the estimate of Schansker et al. [20,21] on peas and pine. Observing the curves with normalized data (Figure 1d–f) it is noticeable a different pattern of behavior of the curves per the development of sunflowers, i.e., the aging of the plants, which Viljevac Vuletić et al. [37] and Sitko et al. [38] also determined on wheat and grapevine, respectively. Aging caused changes in the direction of some bands' amplitude per the sunflower development stages. The most pronounced visible changes were observed on band J.

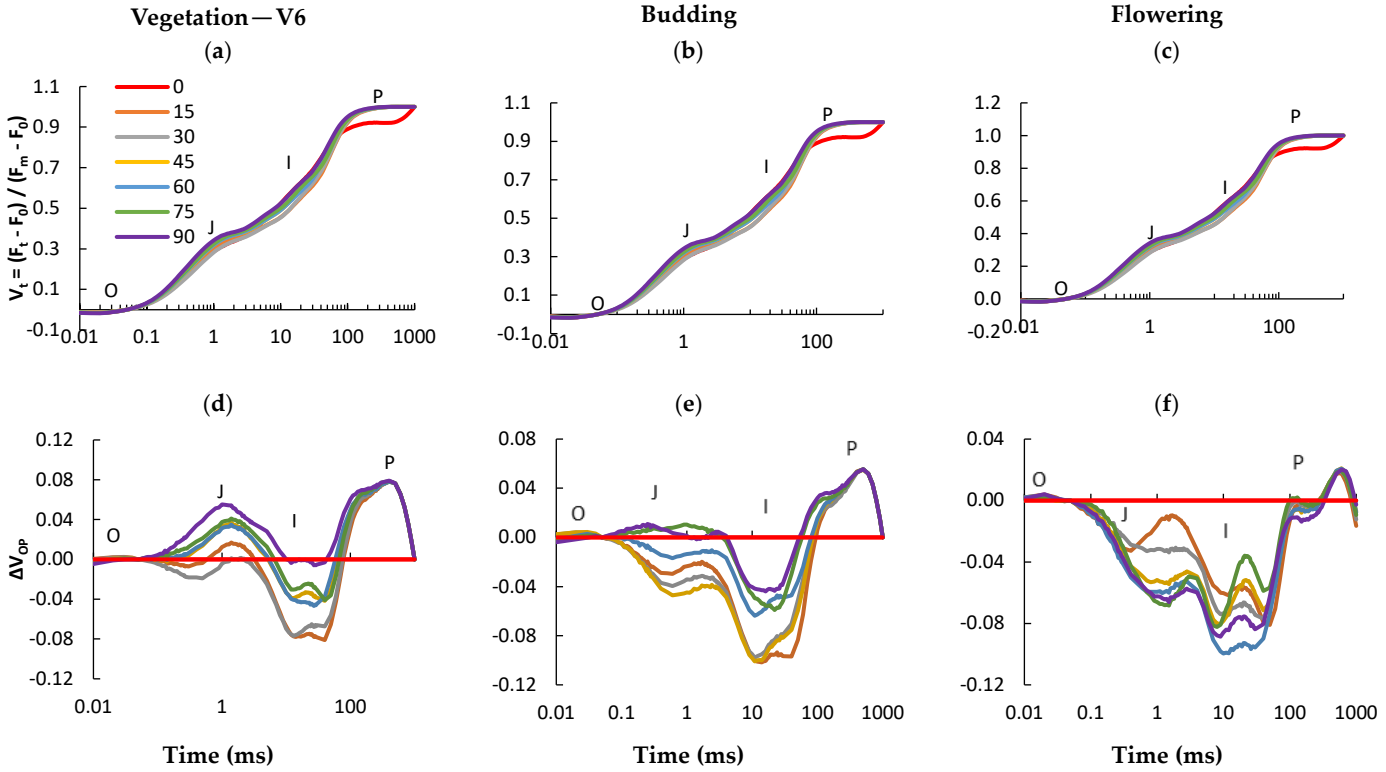

Figure 1. Shapes of the chlorophyll *a* fluorescence transient curves determined in sunflower vegetation—V6, budding and flowering stage after exposure to 0, 15, 30, 45, 60, 75 and 90 min of dark adaptation are shown as relative variable fluorescence V_t (**a**–**c**) and as difference kinetics ΔV_{OP} (**d**–**f**). The difference kinetics in the relative variable fluorescence was calculated as $\Delta V_t = V_t$ (0) − V_t (adaptation time) for each adaptation time and developmental stage.

The decreased level of fluorescence at 0.15 ms indicates a negative L band (Figure 2a–c) in vegetative development at all dark adaptation times. The dark adaptation time of 15 and 30 min in budding and the dark adaptation time of 15 min in the flowering stage retained a negative L band in contrast to the other dark adaptation times in those two measurements, which revealed the appearance of a positive L band. The K band (Figure 2d–f) is also negative in all three stages except in the vegetative developmental stage at 90 min of dark adaptation and in the budding stage at 75 and 90 min of adaptation. According to Yusuf et al. [34], the negative values of bands L and K are indicators of the high connection of PSII parts and increased stability of the system. H bands (Figure 2g–i) in all developmental stages, regardless of the duration of dark adaptation, were negative. That indicates the sustainability of the PQ capacity, which maintains the normal transfer of electrons between the photosystems. Therefore, the relative volume of the set of PSI acceptors was increased [26]. The shape of the G band depends on the efficiency of the electron flow and the rate of PSI reduction of the final electron acceptors. G bends (Figure 2j–l) were positive during

adaptation time in all developmental stages. When the pool of PSI electron end acceptors decreases, the electron transition is faster, and the positive peak of the G band appears [26].

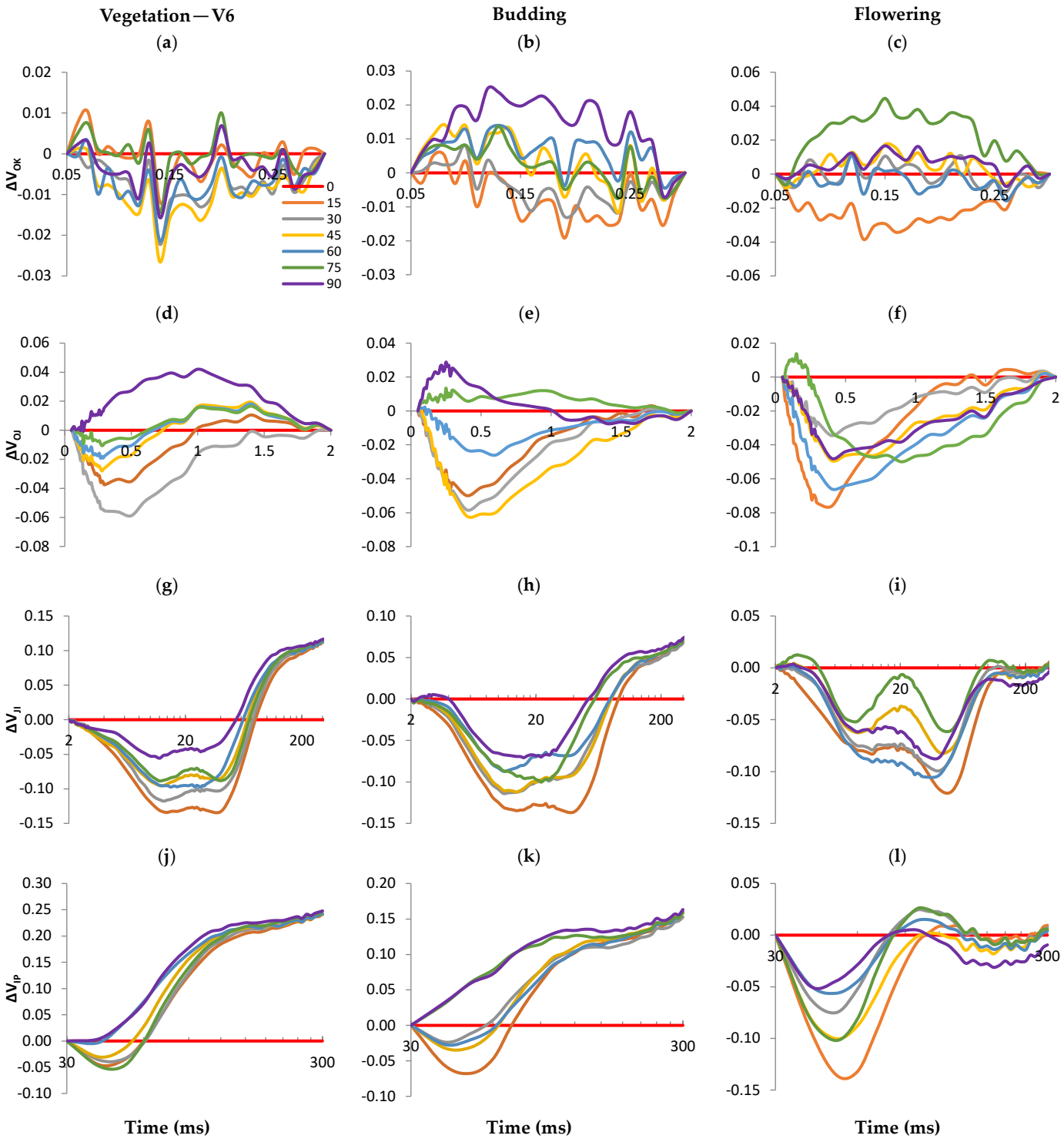

Figure 2. Shapes of the chlorophyll *a* fluorescence transient curves determined in sunflower vegetation—V6, budding and flowering stageafter exposure to 0, 15, 30, 45, 60, 75 and 90 min of dark adaptation are shown as difference kinetics ΔV_{OK}—band L (**a**–**c**), ΔV_{OJ}—band K (**d**–**f**), ΔV_{JI}—band H (**g**–**i**), ΔV_{IP}—band G (**j**–**l**). The difference kinetics in the relative variable fluorescence was calculated as $\Delta V_t = V_t(0) - V_t$(adaptation time) for each adaptation time and developmental stage.

From the curves shown, we can conclude that the L band was the most sensitive to dark adaptation duration. The K band follows it. According to bands L and K, adaptation to darkness longer than 30 min for the L band and up to 75 min for the K band is appropriate for all developmental stages. According to H and G beds, all adaptation times (15–90 min) are acceptable in all developmental stages.

There is also a noticeable difference in the size and behavior of the amplitude of the bands according to the stages of sunflower development. The most pronounced changes are visible in the ΔV_{OP} curves, the K and the G bands.

3.2. Chlorophyll a Fluorescence Parameters

Studying the individual parameters of chlorophyll *a* fluorescence in the vegetative and budding stages, from the initial low minimum fluorescence (F_0) values, F_0 increased during the dark adaptation time (Figure 3a). The opposite was true for F_0 values in the flowering stage, where F_0 decreased with the longer dark adaptation after the initial high values and raised only after 60 min of dark adaptation. Similar results to those in the flowering stage were confirmed by Cahyo et al. [39], examining the assessment of factual measurement times for chlorophyll *a* fluorescence in rubber (*Hevea brasiliensis* Muell. Arg.) clones. The F_0 value in this research shows that the lowest required dark saturation of sunflower leaf is for about 15–30 min.

Furthermore, the lowest maximum fluorescence (F_m) was obtained by measurement of ChlF conducted without dark adaptation (Figure 3b), most likely because the correct value of F_0 and F_m cannot be detected without adjusting for darkness [22]. After the initial low F_m values, the F_m increased with the dark adaptation duration in all developmental stages. During the first 45 min of dark adaptation, statistical change was estimated in the vegetative stage compared to the other two stages (budding and flowering stages). In the budding and flowering stages, no significant differences between dark adaptation from 15 to 90 min were determined. To determine the correct F_m values, keeping the clips on the sunflower leaf for a minimum of 15 min is necessary.

In all three developmental stages, maximal photochemical quantum yield (φP_0) values increased from the initial low values and differed by dark adaptation time points. Values 0.802, 0.797 and 0.796 of φP_0 for the vegetation, budding and flowering stages without dark adaptation, respectively (Figure 3c), confirm that the sunflower tissue needs to be adapted to dark conditions before measuring the ChlF. The same was reported by Cahyo et al. [39] in rubber. φP_0 values differed within dark adaptation time points, but in all three stages, they increased by elongation of the dark adaptation time. It is generally known that φP_0 values in healthy tissue, without stress, should be above 0.75 [40]; of course, the values depend on the tested tissue. The φP_0 values were the highest in the sunflower flowering stage when the entire leaf apparatus of the sunflower plants had fully developed and reached its maximum [35]. According to the φP_0 values, it is noticeable that the measurements should be carried out after 15 min to above, which was also confirmed by Cahyo et al. [39].

The quantum yield of energy dissipation (φD_0), represented by the ratio of F_0 and F_m, according to the results, also confirmed the need for dark adaptation of sunflower tissue prior to the ChlF measurement (Figure 3d). If the leaves were not adapted to the darkness, the φD_0 values were high. Values of φD_0 decreased as the leaves adapted to dark conditions. Although the φD_0 is directly related to the F_0 and F_m values, their results for determining sunflower leaves' adaptation to darkness were inconsistent (Figure 3a,b). The adaptation time did not affect the φD_0 result.

The quantum yield for the reduction of terminal electron acceptors at the PS I acceptor side (φR_0) and the probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the photosystem I acceptor side (δR_0) showed a decrease in values with the elongation of dark adaptation beginning with the 15 min dark adaptation point (Figure 3e,f). According to statistical analysis, valid data on φR_0 and δR_0 have been obtained after about 15 min of dark leaf adaptation.

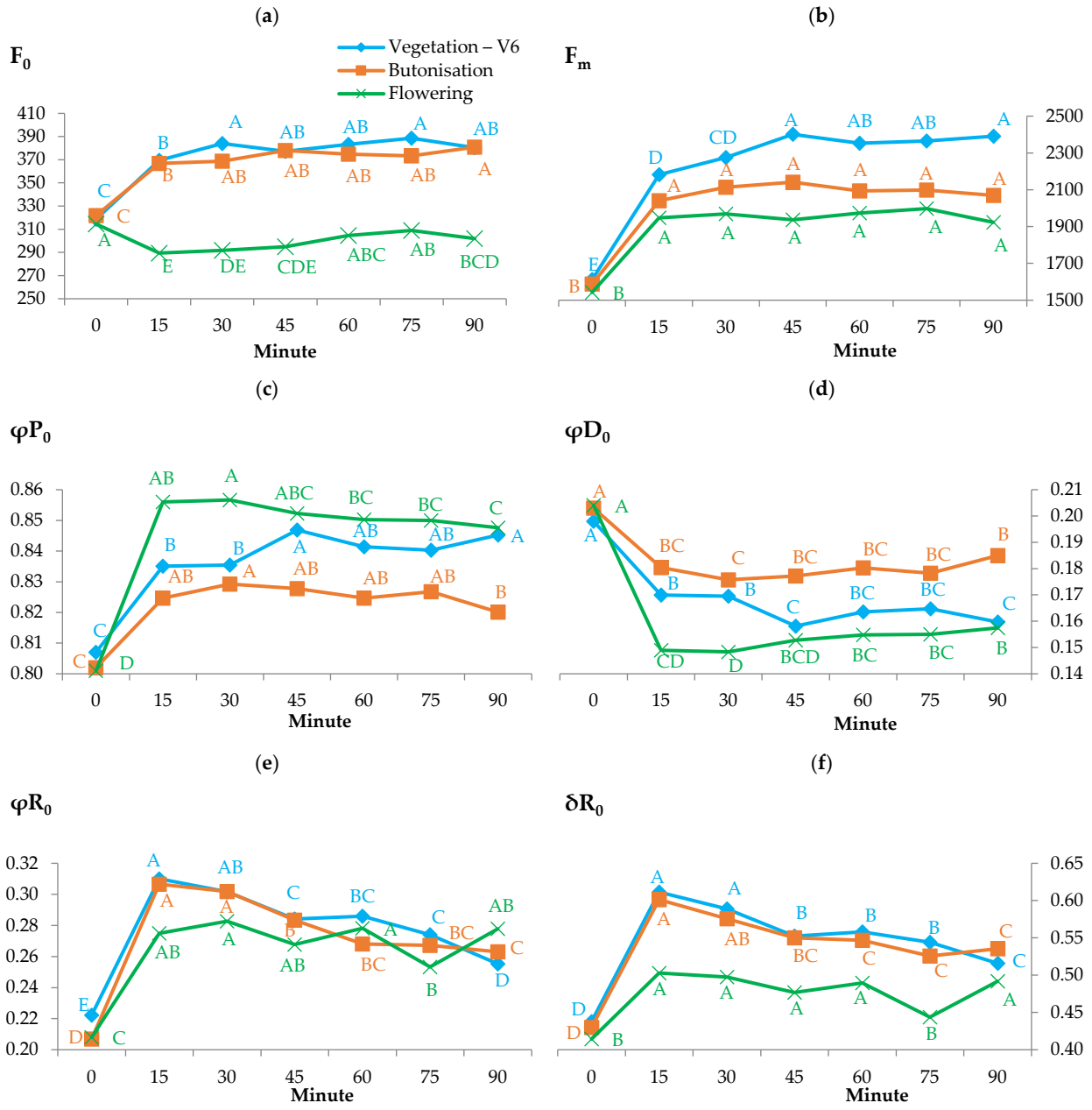

Figure 3. Chlorophyll *a* fluorescence parameters: (**a**) minimum fluorescence (F_0); (**b**) maximum fluorescence (F_m); (**c**) maximal photochemical quantum yield (φP_0); (**d**) quantum yield of energy dissipation (φD_0); (**e**) quantum yield for the reduction of terminal electron acceptors at the photosystem I acceptor side (φR_0); (**f**) probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the photosystem I acceptor side (δR_0) in sunflower vegetation—V6, budding and flowering stages after exposure to 0, 15, 30, 45, 60, 75 and 90 min of dark adaptation. The same letters do not show statistical differences within the developmental stage.

The high values of absorption flux per active reaction center (RC) (ABS/RC), dissipation flux per active RC (DI_0/RC), trapping flux per active RC (TR_0/RC), electron transport flux per active RC (ET_0/RC) without dark adaptation dropped sharply after 15 min of adaptation to dark conditions, after which the values raised with the elongation of dark adaptation time (Figure 4a–d). In contrast, electron flux reducing terminal electron acceptors at the photosystem I acceptor side per RC (RE_0/RC, Figure 4e) and decrease of density of active photosystem II reaction centers (RCs) per cross-section (RC/CS_0, Figure 4f), the values without dark adaptation were lower than values in dark-adapted sunflower leaves

in all three developmental stages. At 15 min of dark adaptation, the values increased compared to no adaptation, and afterward, they slightly decreased with the progression of the dark adaptation. ABS/RC, DI_0/RC, TR_0/RC, ET_0/RC, RE_0/RC, and RC/CS_0 can be used as reliable estimates for the optimal time of adaptation of sunflower leaves to dark conditions because the results are consistent (Figure 4a–f). Namely, for all the mentioned parameters, any tested time to darkness leads to correct data. According to ABS/RC, DI_0/RC, TR_0/RC, RE_0/RC and RC/CS_0, the lowest values were recorded in the flowering stage. In contrast, ET_0/RC values were the highest in flowering. Parameters ABS/RC, DI_0/RC, TR_0/RC and ET_0/RC in the sunflower leaves dark-adapted in the flowering stage showed similar values to those recorded by Markulj Kulundžić et al. [11] in the morning conditions.

Adaptation of leaf to dark conditions was also necessary according to the density of RC on chlorophyll *a* basis (RC/ABS), flux ratio trapping per dissipation (TR_0/DI_0), electron transport from Q_A^- to intersystem electron acceptors ($ET_0/(TR_0 - ET_0)$), performance index on absorption basis (PIABS), electron transport from PQH_2 to final photosystem I (PSI) acceptors ($\delta R_0/(1 - \delta R_0)$) and performance index for energy conservation from exciton to the reduction of PSI terminal acceptors (PItotal) as their values without dark adaptation were the lowest compared to all other values determined in dark-adapted sunflower leaves (Figure 5). The values for all of the mentioned parameters increased after 15 min of adaptation compared to values without dark adaptation, after which they decreased with the elongation of dark adaptation time for all parameters except $ET_0/(TR_0 - ET_0)$. Figure 5d shows differences between the PI_{ABS} values by developmental stages, which was previously confirmed in the papers of Markulj Kulundžić et al. [12]. PI_{ABS} values increase with the aging of sunflower plants [12].

According to the parameters RC/ABS, TR_0/DI_0, $ET_0/(TR_0 - ET_0)$ and PI_{ABS}, the optimal time of dark adaptation was from 15 min onwards because values were similar in the required time of adaptation to darkness. On the other hand, the more sensitive parameters PI_{total} and its sensitive component $\delta R_0/(1 - \delta R_0)$ can serve as guidelines in determining the dark adaptation length of sunflower leaves. According to PI_{total} and $\delta R_0/(1 - \delta R_0)$, sunflower leaves should be dark-adapted for 15–30 min as the values of both parameters were statistically different after the 45 to 90 min range of dark adaptation (Figure 5d,e). Markulj Kulundžić et al. [12] and Pavlović et al. [41] proved that $\delta R_0/(1 - \delta R_0)$ is the most sensitive component of PI_{total}, which can also be seen from the results of this research. Given the uniform results for each developmental stage, the conclusions above can be applied to all three developmental stages (vegetation–V6, budding and flowering).

Studying the ChlF parameters by stages of development, it can be concluded that the flowering stage differs the most from the remaining two tested stages regarding the values and behavior of individual parameters. The most likely reason for this is that the sunflower plants develop all the leaf mass, characteristic for the genotype in the flowering stage; that is, the plants reach their maximum number of leaves on the sunflower plant [35]. The leaf area of sunflowers depends on the shape and size of the leaves, their positioning on the plant stem, the genotype and the plant development stage (Figure S1 in the Supplementary Materials). After flowering, the number of leaves decreases, which is a consequence of the maturing of the plant, so the leaves dry and fall off. Some of the functions of the leaves are the formation of grain size and filling, grain yield and oil content, but not all leaves have the same influence on them [42]. However, the main function of the leaves is photosynthesis, which depends on the leaves' position and age in sunflowers.

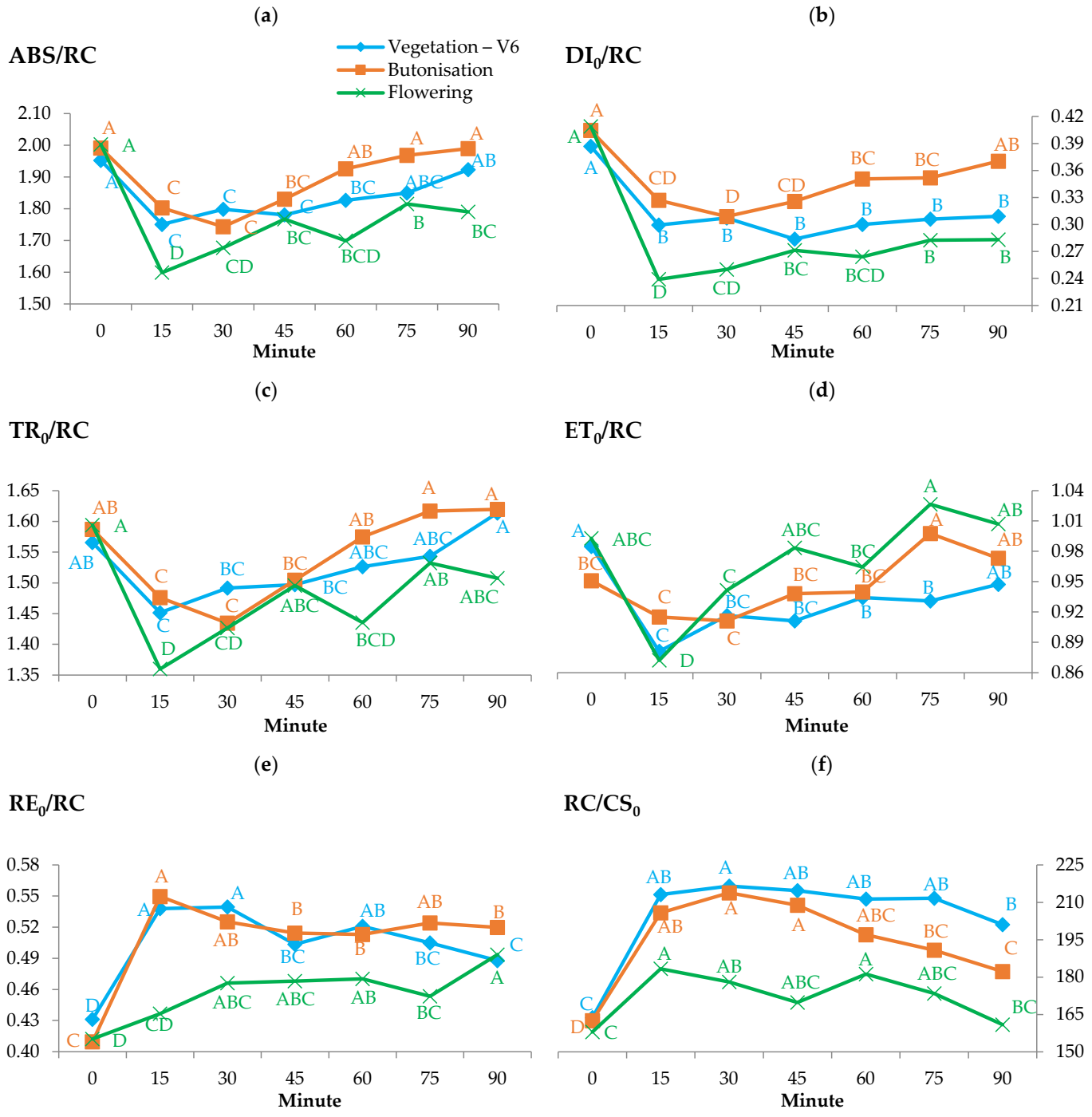

Figure 4. Chlorophyll *a* fluorescence parameters: (**a**) absorption flux per active reaction center (RC) (ABS/RC); (**b**) dissipation flux per active RC (DI_0/RC); (**c**) trapping flux per active RC (TR_0/RC); (**d**) electron transport flux per active RC (ET_0/RC); (**e**) electron flux reducing terminal electron acceptors at the PSI acceptor side per RC (RE_0/RC); (**f**) density of active photosystem II reaction centers (RCs) per cross-section (RC/CS_0) in sunflower vegetation—V6, budding and flowering stages after exposure to 0, 15, 30, 45, 60, 75 and 90 min of dark adaptation. The same letters do not show statistical differences within the developmental stage.

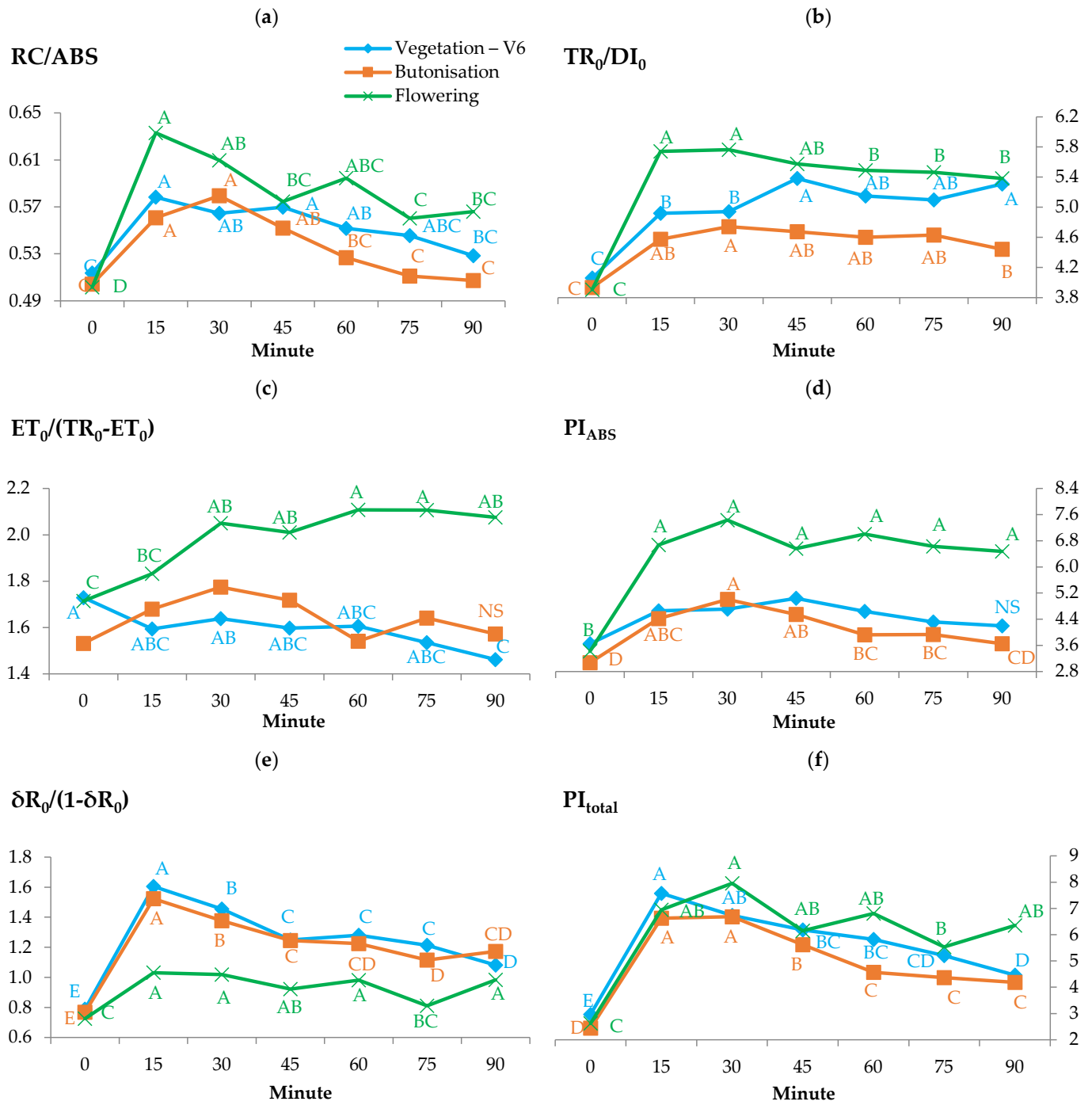

Figure 5. Chlorophyll *a* fluorescence parameters: (**a**) density of RC on chlorophyll a basis (RC/ABS); (**b**) flux ratio trapping per dissipation (TR_0/DI_0); (**c**) electron transport from Q_A^- to intersystem electron acceptors ($ET_0/(TR_0 - ET_0)$); (**d**) performance index on absorption basis (PI_{ABS}); (**e**) electron transport from PQH_2 to final photosystem I (PSI) acceptors ($\delta R_0/(1 - \delta R_0)$; (**f**) performance index for energy conservation from exciton to the reduction of PSI terminal acceptors (PI_{total}) in sunflower vegetation—V6, budding and flowering stages after exposure to 0, 15, 30, 45, 60, 75 and 90 min of dark adaptation. The same letters do not show statistical differences within the developmental stage; NS—not significant.

4. Conclusions

Inadequate handling and inexperience during the measurement of chlorophyll *a* fluorescence (ChlF) could affect the measurement result. The presented transient curves

and ChlF parameters results gave evidence that sunflower leaf adaptation is necessary before ChlF measurement. Studying the OJIP curves and their individual bands clearly shows the difference between the developmental stages, which most likely depends on the leaf mass's activity per sunflower development's vegetative stage. At the same time, results show that the lowest required sunflower leaf dark adaptation of 15 min is enough to achieve the adaptation of the photosynthetic apparatus to obtain correct data. Predominantly in the majority parameters, there were no significant differences between measurements from 15 to 90 min, indicating the clips for dark adaptation could be left for a longer time on the sunflower leaf tissue without compromising the results. On the other side, the parameters $\delta R_0/(1-\delta R_0)$ and PI_{total} stood out as more sensitive parameters that need to be determined up to 30 min after adaptation.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14050954/s1, Figure S1: Scheme of sunflower developmental stages. Table S1. Mean values and standard deviations of chlorophyll *a* fluorescence parameters.

Author Contributions: Conceptualisation, A.M.K.; methodology, A.M.K. and M.V.V.; software, I.V.; formal analysis, A.M.K.; investigation, A.M.K., T.D. and M.M.K.; resources, A.S.; writing—original draft preparation, A.M.K.; writing—review and editing, M.V.V., M.M.K., A.S., T.D., A.M. and I.L.; supervision, A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in the study are included in the article and Supplementary Materials, further inquiries can be directed to the corresponding authors.

Acknowledgments: We want to thank everyone from the Department for Breeding and Genetics of Industrial Plants from Agricultural Institute Osijek who participated in the research on the effect of abiotic factors on sunflower plants.

Conflicts of Interest: The authors declare no conflicts of interest.

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