RESEARCH ARTICLE

Dark drops of prompt chlorophyll fluorescence as a novel approach for evaluation of the photosynthetic machinery state

Stella Dimitrova [1]
Kolyo Dankov [1]
Momchil Paunov [1]
Bojidara Pavlova [1]
Vasilij Goltsev [1]
Violeta Velikova [2]
Tsenko Tsonev [2]
Hazem Kalaji [3]
Reto Strasser [4]

Authors' addresses:
[1] Department of Biophysics and Radiobiology, Faculty of Biology, St. Kl. Ohridski University of Sofia, Sofia, Bulgaria.
[2] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria.
[3] Department of Plant Physiology, Warsaw University of Life Sciences SGGW, Warsaw, Poland.
[4] Bioenergetics Laboratory, University of Geneva, Geneva, Switzerland.

Correspondence:
Stella Dimitrova
Department of Biophysics and Radiobiology, Faculty of Biology, St. Kl. Ohridski University of Sofia, 8 Dragan Tzankov Blvd., Sofia, 1164, Bulgaria.
e-mail:dimitrova.stella76@gmail.com

ABSTRACT

Analyzing the changes that occur in the photosynthetic machinery of plants is one of the most informative approaches for evaluation of the plant physiological state, their stress reactions, productivity and the adaptive mechanisms that develop in order to protect the plant in a changing environment. An informative method for detection and analyzing of the photosynthetic process is measuring the chlorophyll *a* fluorescence emitted from leaves. Illumination of a plant sample induces a rise in the chlorophyll *a* fluorescence that draws characteristic induction curves which carry broad spectrum of data about every step of the photosynthetic process. Chlorophyll *a* fluorescence gives two types of signals – prompt (PF) and delayed chlorophyll fluorescence (DF). The Multifunctional Plant Efficiency Analyzer (MPEA), developed by Hansatech is constructed to measure both simultaneously in only one measurement, but as the initial steps of their kinetics overlap they cannot be recorded together while the sample is illuminated. Thus the apparatus is set to switch between light (recording of PF) and dark (recording of DF decay kinetics) conditions at specific periods of time. When the light is switched off during the dark period oxidation processes of the Photosystem II (PSII) reaction center occur which lowers the level of chlorophyll fluorescence. Thus when the light is put on again, the PF signal continues from a lower level. This phenomenon is called Dark Drops (DD) of chlorophyll fluorescence and it can be a valuable source of information. The aim of our study is to develop a new approach to collect and analyze the information from the Dark Drops and use it to investigate the changes in the photosynthetic process in leave samples of the plant *Phaseolus vulgaris* in normal conditions and in conditions of heat stress.

Abbreviations: AL – Actinic light, DD – Dark drops, DF – Delayed fluorescence, F – Fluorescence, MPEA – Multifunctional Plant Efficiency Analyzer, MR820 – Modulated reflection at 820 nm, PAM – Pulse Amplitude Modulation, PF – Prompt fluorescence, PSI – Photosystem I, PSII – Photosystem II, Q_A – Quinone A, Q_B – Quinone B, RC – Reaction Center

Key words: chlorophyll fluorescence, dark drops, plant physiology, plant stress, heat stress

RESEARCH ARTICLE

Introduction

The Photosynthetic machinery of plants is very sensitive to changes in their overall state (Blankenship, 2013). Changes in the environmental conditions trigger stress reactions and different adaptive mechanisms in the plants organism (Strasser, 1988). Evaluation of the photosynthetic process can give information about the presence of stress factors, the plants response to them, their tolerance and protective mechanisms, about their vitality and productivity during different conditions.

There are different approaches for measuring the photosynthetic activity of plants, given by the tools of biochemistry, plant physiology, molecular biology and biophysics. One of the most perspective approaches is measuring of the chlorophyll *a* fluorescence emitted by plants when illuminated with actinic (photosynthetic active) light (Schansker et al., 2014). Among all other methods, this is the fastest, most informative method that can be used *in vivo* in native conditions and it doesn't need any expensive materials.

There are two basic ways to measure chlorophyll *a* fluorescence – the Pulse Amplitude Modulation (PAM) (Schreiber, 2004) and direct measuring in micro and millisecond time ranges of the induction kinetics of prompt (PF) and delayed chlorophyll fluorescence (DF) as well as modulated reflection at 820 nm signals. The last approach could be realized by the Multifunctional Plant Efficiency Analyzer (MPEA), developed by Hansatech (Kalaji et al., 2012). Both approaches give very detailed information about the overall physiological state of the plant, its vitality and the condition of every single step in the photosynthetic electron transport chain. Data, collected from both methods contributes to form a full picture of the photosynthetic process. MPEA can provide more parameters compared to PAM and it has a better time definition which allows a more precise observation of the photosynthetic processes. On the other hand, PAM has the ability to measure some important parameters that give information about the quenching of chlorophyll fluorescence. Finding a parallel between the two approaches can help establishing a better picture of the entire process (Goltsev et al., 2014).

A more profound investigation of the fluorescent effects observed is very important in order to develop new informative approaches for evaluation of plants physiological state while using the potential of the present instruments at its fullest (Murchie et al., 2013).

Chlorophyll *a* fluorescence gives two types of signals – prompt and delayed chlorophyll fluorescence (Kalaji et al., 2014, Salvatori et al., 2014). MPEA is constructed to measure both simultaneously in only one measurement, but as the initial steps of their kinetics overlap they cannot be recorded together while the sample is illuminated. Thus the instrument is designed to switch between light (recording of PF) and dark (recording of DF decay kinetics) conditions at specific periods of time (Strasser et al., 2010).

When a dark adapted plant sample is illuminated with actinic light the chlorophyll *a* fluorescence emitted from the antennae complexes rises (Figure 1) (Strasser et al., 2004).

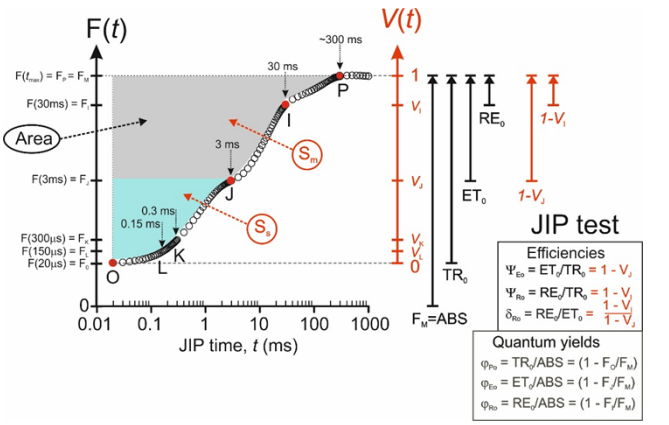

Figure 1. *Induction Curve of the PF signal, measured with MPEA. The OJIP characteristic points are presented. Some of the parameters that can be directly derived are shown.*

This is illustrated by the induction curve of PF, which shows 4 significant characteristic points – F_O (20 µs after the beginning of illumination, this is the level of PF, when all reaction centers of Photosystem II (PSII) are oxidized), F_J (2 ms, level of PF, when the quinone molecule Q_A is being reduced – the rise of the chlorophyll fluorescence between F_O and F_J represents the kinetics of the electron transport to Q_A), F_I (measured 20-30 ms after the beginning of the protocol, it corresponds to the level of PF, when the rest of the quinone molecules are being reduced) and F_P (300 ms to 1 s from the beginning, level of PF when the entire plastoquinone pool is reduced). The index names of these characteristic points give the name of the chlorophyll fluorescence test, used to evaluate the photosynthetic process quantitive – OJIP test. When the Rubisco enzyme is activated, Photosystem I (PSI) starts to pull electrons to satisfy the needs of Rubisco for reduced NADPH molecules and thus the electron carriers of

RESEARCH ARTICLE

PSII, PSI and between them are being oxidized. The level of chlorophyll fluorescence becomes lower. This process is known as photochemical quenching (qP) (Vredenberg et al., 2009). Also a non-photochemical quenching (NPQ) occurs, which is composed of different components such as the xanthophyll cycle and migration of the light harvesting complexes, known as State transitions (Muller et al., 2001).

There are more than 30 different parameters that can be derived from the described induction curve of PF (Stirbet et al., 2011). Each gives information about the condition of a given electron carrier alone and in relation to all other electron carriers or about a different process that occurs in the chloroplast.

One of the least studied phenomenon with potential significant application are the DD of PF, observed after brief periods (0.1-100 ms) of darkening of the plant sample during PF induction.

In our study we created protocols which can measure the changes in the DD during the induction period and we developed an algorithm to analyze them and use the collected data as a new valuable source of information about the photosynthetic machinery. In order to test the new approach we used *Phaseolus vulgaris* plants and we measured the dynamics of DD of PF in normal conditions and during heat stress.

Materials and Methods

Plant growing

The primary leaves from 20-25 days old bean plants *Phaseolus vulgaris*, cv. "Cheren Starozagorski" are used. The plants were grown in pots with turf-soil mixture and placed in a phytostatic box at room temperature and day-night regime light/dark – 12/12 hours. Luminescent illumination was of 200 μmol photons·m^{-2}·s^{-1}. All plants were decapitated accordingly (Yordanov et al., 2008).

Measuring chlorophyll a fluorescence

For measuring of chlorophyll *a* fluorescence we used the fluorometer Multifunctional Plant Efficiency Analyzer – MPEA, developed by Hansatech Instruments, Ltd. (King's Lynn, UK) described earlier (Strasser et al., 2010). The measurement conditions are previously designed in a specific script (protocol), managed in the MPEA Plus program. The experimental protocol is than uploaded in the measuring device of the instrument and recording may begin. The experiments are held in a dark room in order to provide dark adaptation for the studied plants.

Primary data processing and analyzes of the collected data are carried out using the software "MPEA Data Analyzer", developed by Petko Chernev from the Department of Biophysics and Radiology, Faculty of Biology, Sofia University. The software provides simultaneous visualization and analyzes of the three signals, measured by the MPEA – PF, DF and MR820 – modulated light reflection (Figure 2).

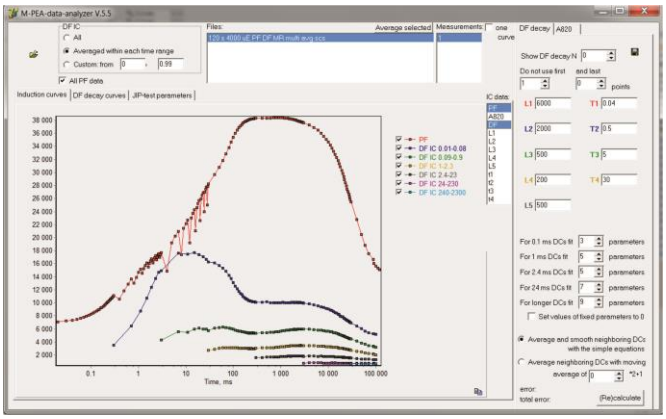

Figure 2. *Dialog window of the PF analyzer MPEA, developed by Petko Chernev from the Department of Biophysics and Radiology, Faculty of Biology, Sofia University.*

Experimental protocols for measuring the DD dynamics

In order to measure PF and DF simultaneously, the MPEA instrument is designed to measure PF while the sample is being illuminated and to record the DF decay kinetics at specific moments when the light is switched off (Goltsev et al., 2013). This regime is set to alternate between three light periods and a dark period. 300 μs after the beginning of illumination of a dark adapted plant sample the light is being switched off for 100 μs for DF recording. Seven cycles of 100 μs darkness and 300 μs illumination are counted within the first 3 ms. After the third ms another seven cycle regime is started which alternates between light and dark in the same 3:1 proportion, but the duration of the dark interval is 1 ms. During the dark intervals re-oxidation processes occur, which leads to lowering of the level of chlorophyll fluorescence. Thus, when the light is switched back on, the recording of the induction curve of PF continues from a little lower level and a characteristic dip is observed – Dark Drop (DD) of the PF (Goltsev et al., 2013). After 30 ms there are no DD observed, because the recording of PF is not

RESEARCH ARTICLE

carried immediately after the dark interval but a little bit afterwards, thus giving time for the reaction centers to get reduced to the level they have reached before the dark period (Figure 3).

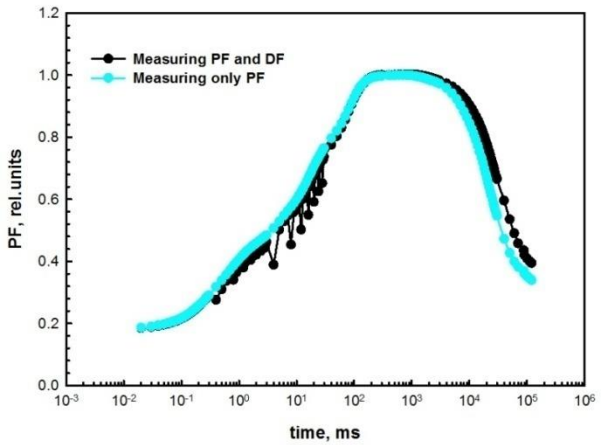

Figure 3. *Induction curves of PF, measured with Protocol 1 (see Materials and methods). The duration of the record is 120 sec. Illumination is saturating – 4000 µmol photons·m-2·s-1. Blue curve presents the form of the curve without application of Dark intervals. Black curve shows the DD dips, formed when the light is switched off for measuring of DF.*

Protocol 1 – Record of the of Prompt and Delayed Chlorophyll fluorescence transients during dark to light adaptation

The duration of the measurement is 120 sec. The leaves are illuminated with light intensity of 4000 µmol photons·m^{-2}·s^{-1}.

We used Protocol 1 in two regimes – at the first one the PF signal alone is measured during 120 s and after switching the actinic light off the decay of the DF signal was recorded during 3 s. At the second regime the PF and DF are measured simultaneously – the sample is illuminated with series of impulses where PF is recorded, alternating with dark intervals when DF decay kinetics signal is recorded.

Protocol 2 – Investigation of the Dark restore of the shape of PF rise

The illumination was interrupted for dark periods with different duration. The sample was illuminated with saturating pulses for 300 ms with Light Intensity of 4000 µmol photons·m^{-2}·s^{-1}. Between the saturating pulses the plant leaf was subjected to dark periods with different duration – 10 ms, 30 ms, 100 ms, 1 s, 3 s, 10 s, 30 s, 60 s, 180 s, 600 s.

Protocol 3 – Recording of the Dark Drops of PF

This protocol is a set to twenty of 300-ms records at 4000 µmol photons·m^{-2}·s^{-1}. The saturating 300 ms pulse, is followed by 30-ms pulse or intermittent light for recording of the DD of PF. Between successive DD measurements the sample is subjected to 30 s periods of dark or of illumination with low intensive light of 50, 100, 150 and 200 µmol photons·m^{-2}·s^{-1} (Figure 4).

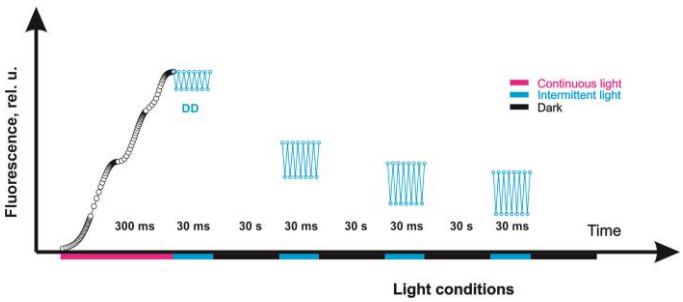

Figure 4. *Schematic representation of the structure of Protocol 3. After a 300 ms saturating pulse of 4000 µmol photons·m-2·s-1, a sequence of 30 ms pulses starts, interrupted by 30 s dark intervals between each pulse. During the intervals the sample is illuminated with actinic light of 0, 50, 100, 150 and 200 µmol photons·m-2·s-1.*

Protocol 4 – Parallel simultaneous Recording of the Dark Drops of PF and Chlorphyll Fluorescence Quenching analyzes

This protocol is a set of 2 successive measurements of PF (300 ms, 4000 µmol photons·m^{-2}·s^{-1}) and DD (30 ms of intermittent light) records repeated 21 times. The period between each measurement is 30 s with or without illumination by actinic light with intensity of 50, 100, 150 and 200 µmol photons·m^{-2}·s^{-1} (Figure 5).

The two measurements are:

- 0.3 s of illumination with 4000 µmol photons·m^{-2}·s^{-1}. A non-interrupted induction curve of PF is recorded with no interruptions for DF measuring.
- 0.03 s of illumination with 4000 µmol photons·m^{-2}·s^{-1} with switched on DF registration, the kinetics of the fluorescent signal with dark induced dark drops is observed.

RESEARCH ARTICLE

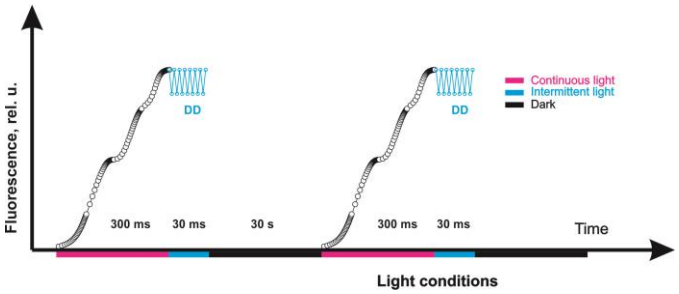

Figure 5. *Schematic representation of the structure of Protocol 4. Two measurements – one of 300 ms and the other of 30 ms with saturating light of 4000 µmol photons·m-2·s-1 are repeated 21 times. Between every measurement a dark interval of no illumination or illumination with 50, 100, 150 or 200 µmol photons·m^{-2}·s^{-1} is applied.*

Temperature treatment

Leaves of *Phaseolus vulgaris* plants are subjected to heat stress at 5 different temperatures: 20°C (control, room temperature), 35°C, 40°C, 45°C and 50°C. Heating is managed with a thermostatic plate. Measurements are carried on leaves, directly placed on the thermostatic plate at the start of the record.

Results

Investigation of the dynamics of re-oxidation of the RCs of PSII

In order to study the dark relaxation kinetics of PSII we used Protocol 2 which allows observation of the changes that occur in the PF induction curve during the transition from dark adaptation to light adaptation. The PF rises during illumination with 300 ms saturating pulses of 4000 µmol photons·m^{-2}·s^{-1}, followed by dark periods with different duration – 10 ms, 30 ms, 100 ms, 300 ms, 1 s, 3 s, 10 s, 30 s, 60 s, 180 s, 600 s (Figure 6).

The shape of the PF induction curve tightly corresponded to the duration of the dark interval. Shorter dark adaptation periods for relaxation resulted in different induction curves, compared to the dark adapted induction curve. After 600 s of darkness, the induction curve of PF almost returned to its initial state (Figure 6 A).

The prolonged dark periods lowered the F_O level faster than the other parameters of PF, because the process of Q_A re-oxidation is relatively faster than the oxidation of Q_B and the plastoquinone pool (Figure 6 B).

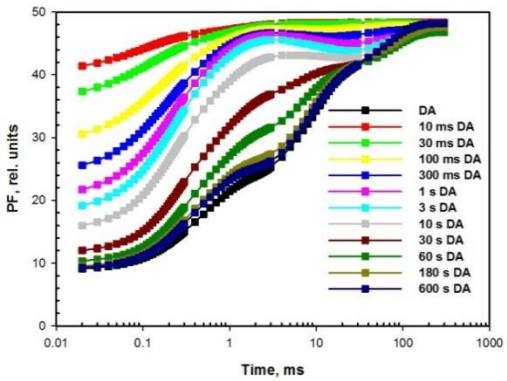

Figure 6A. *Induction curves of PF, showing the dynamics of the re-oxidation of PS II electron carriers.*

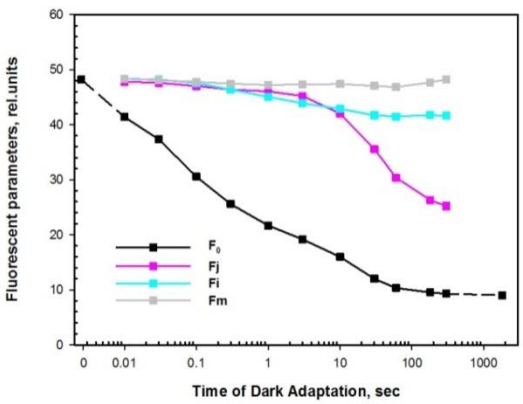

Figure 6B. *Changes in important photosynthetic parameters, measured with Protocol 2. Light intensity of the 300 ms saturating pulses is 4000 µmol photons·m^{-2}·s^{-1}.*

Analyzes of Dark Drops of PF during different light intensity of the background illumination and in conditions of heat stress

The induction curve of PF changes during the dark periods because of re-oxidation processes and dark adaptation. We studied the changes of the DD of PF during dark relaxation and during illumination with low intensity light of 50, 100, 150, 200 µmol photons·m^{-2}·s^{-1}, which are photosynthetic active and are typical for growing plants. For this experiment we used Protocol 3.

A 1 ms dark interval led to 2% lower level of F_P, thus there was no full reduction of the RC of PSII (Figure 7).

RESEARCH ARTICLE

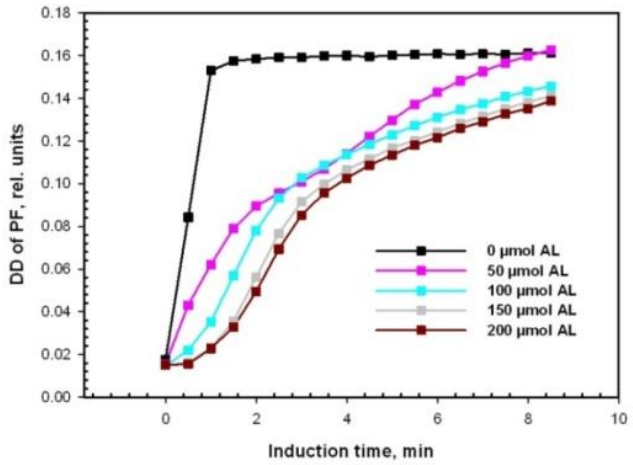

Figure 7. *Changes in the DD of PF, measured with Protocol 3 during different light intensity of the background illumination.*

\Samples, which were not subjected to low intensity illumination, but experienced dark intervals after the first saturating light pulse showed fast rise of the DD amplitude after only 30 s. Probes, illuminated with 50, 100, 150 and 200 µmol photons·m^{-2}·s^{-1} during the intervals demonstrated different dynamics – the amplitude of the DD grew slower.

We analyzed the DD dynamics after the leaf sample was subjected to heat stress (Figure 8 and 9).

We measured changes in DD amplitudes in samples with and without background illumination of 200 µmol photons·m^{-2}·s^{-1}. At zero intensity of the actinic light at room temperature the DD values rapidly increased with the transition from light to dark adaptation (Figure 8). After approximately 2 min of recording the DD amplitudes stabilized. When the temperature was slightly increased at 35ºC there was decay in the DD amplitudes. Increasing of the temperature led to lowering of the level of relative DD. At 45ºC full inactivation was observed.

When there was background illumination during the intervals the values of the DD increased at higher temperatures compared to their values at room temperature (Figure 9). Nevertheless at 45ºC inactivation of the PSII was observed, which led to decay in the DD amplitudes.

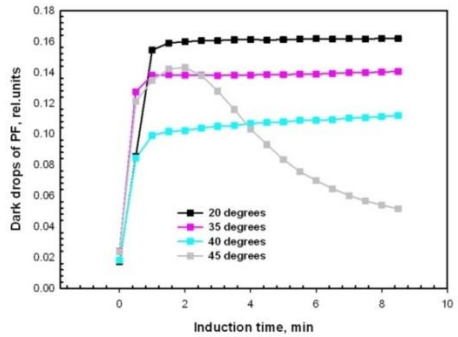

Figure 8. *Changes in the DD of PF, measured with Protocol 3 in samples, treated with different temperatures (20, 35, 40 and 45ºC) during the measurement. The intervals between the light pulses are with no background illumination.*

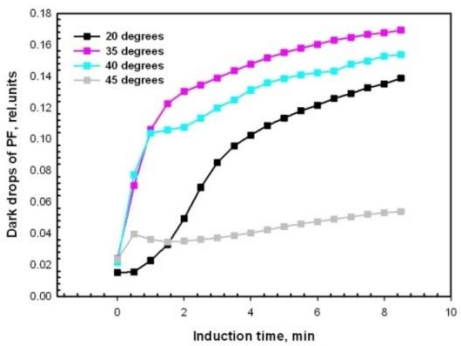

Figure 9. *Changes in the DD of PF, measured with Protocol 3 in samples, treated with different temperatures (20, 35, 40 and 45ºC) during the measurement. Background illumination during the dark intervals is of 200 µmol photons·m^{-2}·s^{-1}.*

Analyzes of the photo induced dynamics of the Photochemical and Non-Photochemical quenching of chlorophyll fluorescence at normal conditions and under heat stress conditions

In order to study the dynamical changes of the Photosynthetic machinery that influence the fluorescent signals, it is important to evaluate these changes during the transition between dark adaptation to light adaptation of the leaves. Such investigation is usually carried on with Pulse Amplitude Modulation (PAM), because the MPEA is based on direct measurement of the fluorescent signal without distinguishing between measuring and actinic light, but such protocols can be constructed experimentally so the analogical results can be achived. In order to execute this task Protocol 4 was developed. The resulting curves allowed calculation of

RESEARCH ARTICLE

different parameters that are typically defined with a PAM fluorimeter – qP and NPQ. We calculated these two parameters at different intensities of the actinic light and analyzed their dynamics for a period of 10 min (Figures 10 and 13).

We also investigated the qP and NPQ changes when the plant sample is subjected to treatment with different temperatures (Figures 11 and 12 for qP and 14 and 15 for NPQ).

Initial decay of the qP was observed in all samples (Figure 10). When there was no background illumination this reduction was very small (about 10%). Increasing of the intensity of the actinic light led to reduction of the PSII acceptors – the relative part of closed RC grew as following – at 50 µmol photons·m^{-2}·s^{-1} it reached around 50%, at 100 µmol photons·m^{-2}·s^{-1} – around 55%, and at 200 µmol photons·m^{-2}·s^{-1} it grew to 75%. A minute after the beginning of the record there was a rise in the values of qP.

A slight increase of the temperatures (35 and 40ºC) did not influence the qP levels (Figure 11).

Inactivation was observed after the third minute of measuring at 45ºC. At higher temperatures (50ºC) inactivation occurred earlier – only after the first minute. All samples exhibited an initial decay of the qP. When the samples were exposed to background illumination of 200 µmol photons·m^{-2}·s^{-1} the initial decay was more pronounced (Figure 12).

In the sample, subjected to actinic light of 200 µmol photons·m^{-2}·s^{-1} the 45ºC temperature became as inactivating as 50ºC for samples with no background illumination.

NPQ reflects the ability of the antennae complexes of PS II to dissipate the absorbed light energy as heat. It is generated by two processes (Govindjee et al., 2002). The fast mechanism of NPQ is managed by reactions of the xanthophyll cycle (Gilmore et al., 1998). When the plant leave is illuminated with saturating light the violaxanthin turns to zeaxanthin – a reaction, catalyzed by the enzyme violaxanthin deepoxidase. The reaction is induced by increased transmembrane proton gradient in the thilakoid membrane. The second, slower mechanism is a result of migration of the PSII light harvesting complex to PSI when PSII is exposed to higher illumination than the photochemical processes can utilize (Wollman, 2001). This disturbs the balance between the two photosystems. The process is called State Transitions and requires the action of specific protein kinases.

After the first minute there was a rise in the NPQ (Figure 13). Neither of the samples reached the maximum level of fluorescence F_p. This was observed least in samples that were not subjected to low light intensity illumination. The impact of NPQ grew with increasing of the intensity of the actinic light. After the first minute, a decay of the NPQ curve was observed. A little bit after the second minute the NPQ raised again.

At room temperature and at slightly elevated temperatures (20, 35 and 40ºC) there was no change of the NPQ in samples without background illumination (Figure 14).

At 45 and 50 degrees different dynamics were observed.

At background illumination, samples didn't exhibit significant change in their NPQ when treated with mild heat stress compared to room temperature (Figure 15).

At 50ºC inactivation of the PS II was observed.

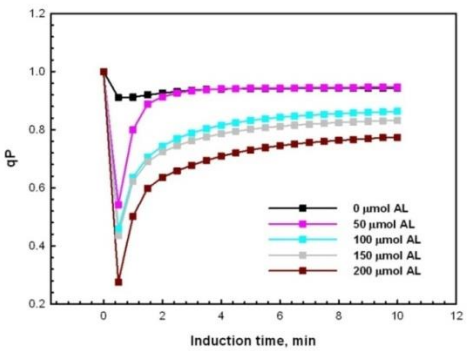

Figure 10. *Changes in the dynamics of qP, measured with Protocol 4 during different light intensity of the background illumination.*

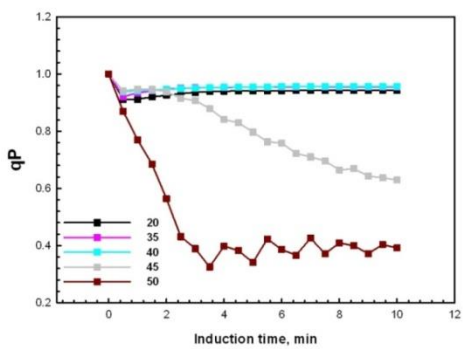

Figure 11. *Changes in the dynamics of qP, measured with Protocol 4 in samples, treated with different temperatures (20, 35, 40, 45 and 50ºC) during the measurement. The intervals between the light pulses are with no background illumination.*

RESEARCH ARTICLE

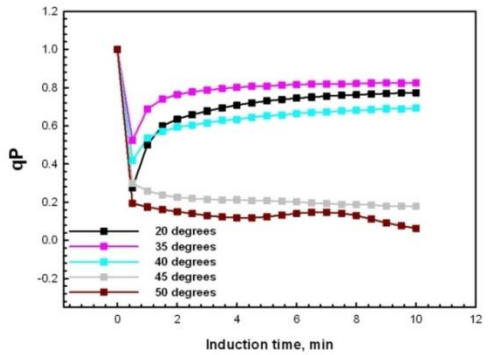

Figure 12. *Changes in the dynamics of qP, measured with Protocol 4 in samples, treated with different temperatures (20, 35, 40, 45 and 50ºC) during the measurement.*

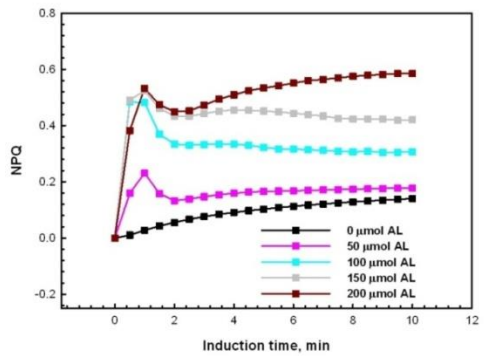

Figure 13. *Changes in the dynamics of NPQ, measured with Protocol 4 during different light intensity of the background illumination.*

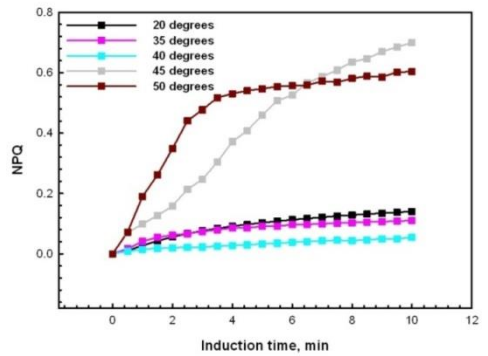

Figure 14. *Changes in the dynamics of NPQ, measured with Protocol 4 in samples, treated with different temperatures (20, 35, 40, 45 and 50ºC) during the measurement. The intervals between the light pulses are with no background illumination.*

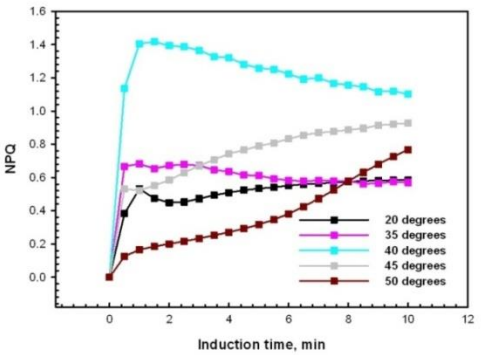

Figure 15. *Changes in the dynamics of NPQ, measured with Protocol 4 in samples, treated with different temperatures (20, 35, 40, 45 and 50ºC) during the measurement. Background illumination during the dark intervals is of 200 µmol photons·m^{-2}·s^{-1}.*

Analyzes of the photoinduced dynamics of the Dark Drops of PF and their change in conditions of heat stress

In order to test the ability of the electron transport chain to oxidize Q_A during the transition between light and dark adaptation we used Protocol 4 for DD analyzes, which is set to repeat a sequential 300 ms of continuous light, 30 ms intermittent light pulses and 30 s dark periods.

There were no changes in DD amplitudes in samples that were not illuminated by actinic light (Figure 16).

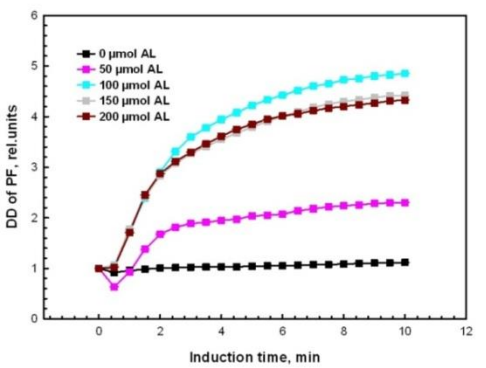

Figure 16. *Changes in the photo-induced dynamics of DD of PF, measured with Protocol 4 during different light intensity of the background illumination.*

RESEARCH ARTICLE

Plants that were exposed to low light with different intensity demonstrated a small initial decay, followed by a steady rise. At 50 µmol photons·m^{-2}·s^{-1} there was a rise in the DD amplitudes that is probably due to Rubisco activation, induced by the light stimulus. At 100, 150 and 200 µmol photons·m^{-2}·s^{-1} the rise was even more pronounced.

At 20 and 35ºC the characteristic small dip after the first minute was observed. At higher temperatures this decay disappeared (Figure 17).

At higher temperatures the amplitude of the relative DD raised. At 50ºC thermal inactivation occurred which lowered the level of the DD. When there was background illumination of 200 µmol photons·m^{-2}·s^{-1} increasing of the temperature led to lowering of the DD (Figure 18). At high temperatures (45 and 50ºC) thermal inactivation occurred.

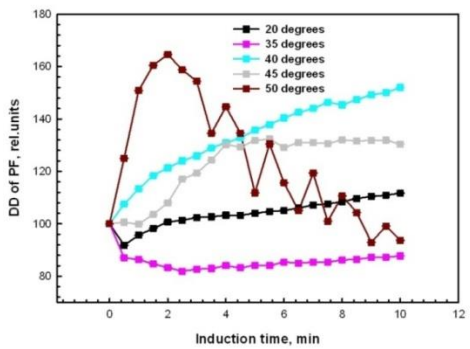

Figure 17. *Changes in the photo-induced dynamics of DD of PF, measured with Protocol 4 in samples, treated with different temperatures (20, 35, 40, 45 and 50ºC) during the measurement. The intervals between the light pulses are with no background illumination.*

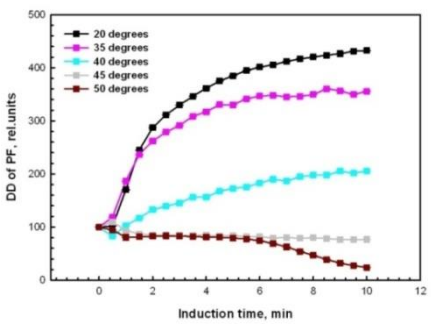

Figure 18. *Changes in the photo-induced dynamics of DD of PF, measured with Protocol 4 in samples, treated with different temperatures (20, 35, 40, 45 and 50ºC) during the measurement. Background illumination during the dark intervals is of 200 µmol photons·m^{-2}·s^{-1}.*

Discussion

The analyzes of DD during different light intensity of the background illumination show slower rise of the DD amplitudes in plants, exposed to actinic light during the intervals between measuring compared to those samples that remain in the dark (Figure 7). The PSII RC of these samples are re-oxidized for a longer time, because they are always exposed to some photosynthetic active light which prohibits full oxidation and sustain a certain level of reduction (Goltsev et al., 2003). The rate of re-oxidation keeps on growing, because the low intensity is in fact strong enough to activate the enzyme Rubisco, which is activated by light and it starts to pull electrons trough PSI speeding up the re-oxidation of the plastoquinone pool and Q_A (Salvucci et al., 2004). The plastoquinone pool is oxidized faster, but it does not reach the maximum level of oxidation.

Samples without background illumination, subjected to heat stress show that increasing of the temperature before the point of inactivation leads to lower DD amplitudes (Figure 8). This is probably due to acceleration of the forward and reverse reactions in the electron transport chain thus the equilibrium shifts (Kukushkin et al., 1999). Temperatures of 45ºC and more inhibit the electron transport due to conformational changes of the PSII complex thus changing the speed of the electron transfer to the plastoquinone (Allakhverdiev et al., 2008). At the initial points of the record there is no significant inactivation, because the temperature effects have not yet influenced the plant sample.

When there is background illumination with low intensity, the pattern changes and there is a rise in the DD amplitudes in plants, treated with higher non-inactivating temperatures (Figure 9). The reason is probably the combination between light and thermal induction of State transitions – migration of the Light Harvesting Complexes of PSII to PSI, which leads to higher activity of PSI and faster absorption of electrons from PSII (Haldrup et al., 2001). At 45ºC heat stress damages the photosynthetic structures and inhibits the electron transport thus promoting the fluorescence emitting and lowering the levels of the relative DD (Salvucci et al., 2004).

Analyzes of the photoinduced photochemical quenching (qP) show an initial decay, observed mostly in samples, treated with background illumination, because the low, but active light leads to reduction of the reaction centers of PSII

RESEARCH ARTICLE

(Figure 10). The qP quickly reaches an equilibrium in samples that do not subjected to low light. The rise of qP in samples, exposed to 50, 100, 150 and 200 μmol photons·m^{-2}·s^{-1} is due to activation of the Rubisco enzyme, which pulls electrons trough PSI thus accelerating the electron transport between the two photosystems (Vredenberg et al., 2009). When the samples are exposed to higher temperatures the initial decay becomes smaller, because non-inactivating temperatures accelerate the electron-transport reactions (Takahashi et al., 2008). Temperatures of 50ºC damage the PSII complex (Figure 11). Plant leaves, subjected to background illumination are being inactivated with temperatures of 45ºC (Figure 12).

Analyzes of the photoinduced NPQ show a rise in its values due to activation of the xanthophyll cycle in the first minute of recording (Demmig-Adams, 2005) (Figure 13). Samples that were subjected to background illumination show a little decay after the first minute, which is probably due to activation of Rubisco that pulls electrons trough PSI and photophosphorilation which partially lowers the proton gradient and the lumen's acidity (Salvucci et al., 2004). A little after the second minute of recording the NPQ rises again due to activation of the State transitions mechanism of protection. The quantum yield of fluorescence from the PSI antennae complexes is significantly smaller compared to the fluorescent yield from PSII, thus migration of the LHCII from PSII to PSI leads to less fluorescence emission (Ivanov et al., 2008). The changes in the NPQ become smaller and smaller until it reaches stationary level around the tenth minute. Samples exposed to heat stress exhibit different dynamics of the NPQ only at high temperatures of 45ºC and 50ºC (Figure 14). At 45ºC the change of the NPQ is due to activation of the State transitions mechanism. When the samples experience background illumination at such temperatures the NPQ rises, but the entire role of the xanthophyll cycle disappears (Figure 15).

Analyzes of the photoinduced dynamics of the relative DD show changes in the DD values only when there is actinic background illumination during the intervals (Figure 16). There is a small initial dip, which corresponds to the additional reduction of the plastoquinone pool. During the first 2 minutes the DD amplitudes rise due to Rubisco activation initiated by the light stimulus (Salvucci et al., 2004). The curve shows two phases: 1. Photochemical phase, illustrating the pulling of electrons trough PSI and oxidation of PSII reaction centers (Vredenberg et al., 2009); 2. Non-photochemical phase, showing the State transitions mechanism – thus a more pronounced activity of the PSI reaction center and a more active absorption of electrons from PSII (Muller et al., 2001).

When the plant samples are subjected to heat stress the initial dip disappears in plants, treated with temperatures over 35ºC (Figure 17). At 35ºC the rate of the electron transport is higher thus the reduction of the plastoquinone is becoming more effective, which leads to smaller DD amplitudes compared to the samples, measured at room temperature. Samples, measured at 40ºC show thermal activation of the State transition process which leads to more effective oxidation of the PSII electron carriers. Plants, treated with higher temperatures experience thermal inactivation and damage of the photosynthetic structures. Plant samples illuminated with low intensity background light show smaller DD amplitudes when subjected to increased temperature in comparison to room temperature (Figure 18). This is due to the constant sustaining of a certain level of reduction combined with the accelerated electron transport to the plastoquinone caused by the temperature treatment. Higher temperatures of 45 and 50ºC caused thermal inactivation.

Acknowledgement

Authors are grateful to Bulgarian National Scientific Fund (Project № DFNI B02/8) for financial support.

References

Allakhverdiev SI, Kreslavski VD, Klimov VV, Los DA, Carpentier R, Mohanty P. 2008. Heat stress: An overview of molecular responses in photosynthesis. Photosynth. Research, 98(1-3): 541-550.

Blankenship RE. 2013. Molecular mechanisms of photosynthesis. John Wiley & Sons.

Demmig-Adams B. 2005. Linking the xanthophyll cycle with thermal energy dissipation. Discov. Photosynth., 923-930.

Gilmore AM, Shinkarev VP, Hazlett TL and Govindjee G. 1998. Quantitative analysis of the effects of intrathylakoid pH and xanthophyll cycle pigments on chlorophyll a fluorescence lifetime distributions and intensity in thylakoids. Biochem., (39): 13582-13593.

Goltsev V, Zaharieva I, Lambrev P, Yordanov I, Strasser R. 2003. Simultaneous analysis of prompt and delayed chlorophyll a fluorescence in leaves during the induction period of dark to

RESEARCH ARTICLE

light adaptation. J. Theor. Biol. 225(2): 171-183.
Goltsev V, Kalaji H, Kouzmanova M, Allakhverdiev SI. 2014. Variable and delayed chlorophyll a fluorescence – basics and application in plant sciences. [Peremennaja I zamedlennaja fluorescencia chloropfilla a – teoreticheskie osnovy I prakticheskoe prilojenie v issledovanii rastenij]. M.-Izhevsk, Computer Sciences Institute, 220 pp. [In Russian]
Govindjee, Seufferheld MJ. 2002. Non-photochemical quenching of chlorophyll a fluorescence: Early history and characterization of two xanthophyll-cycle mutants of Chlamydomonas reinhardtii. Func. Plant Biol., 29(10):1141-1155.
Haldrup A, Jensen PE, Lunde C, Scheller HV. 2001. Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Plant Sci. 6(7): 301-305.
Ivanov AG, Sane PV, Hurry V, Öquist G, Huner NP. 2008. Photosystem II reaction centre quenching: mechanisms and physiological role. Photosynth. Research, 98(1-3): 565-574.
Kalaji H, Goltsev V, Bosa K, Allakhverdiev SI, Strasser R, Govindjee. 2012. Experimental in vivo measurements of light emission in plants: A perspective dedicated to David Walker. Photosynth. Res., 114: 69-96.
Kalaji HM, Schansker G, Ladle RJ, Goltsev V, Bosa K, Allakhverdiev SI, Brestic M, Bussotti F, Calatayud A, Dąbrowski P. 2014. Frequently asked questions about in vivo chlorophyll fluorescence: practical issues. Photosynthesis Research., 122(2):121-158.
Kukushkin AK, Stepin DV. 1999. Electronic structure of plastoquinones and electron transfer between photosystems in higher plants. Biofizika, 44(1): 102-111.
Muller P, Li XP, Niyogi KK. 2001. Non-photochemical quenching. A response to excess light energy. Plant Physiology. 125:1558-1566.
Murchie EH, Lawson T. 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. J Exp. Bot., 64(13): 3983-3998.
Salvatori E, Fusaro L, Gottardini E, Pollastrini M, Goltsev V, Strasser RJ, Bussotti F. 2014. Plant stress analysis: Application of prompt, delayed chlorophyll fluorescence and 820 nm modulated reflectance. Insights from independent experiments. Plant Physiology and Biochemistry. 85: 105-113.
Salvucci ME and Crafts-Brandner SJ. 2004. Inhibition of photosynthesis by heat stress: the activation state of Rubisco as a limiting factor in photosynthesis. Physiol. Plantarum, 120(2): 179-186.
Schansker G, Tóth SZ, Holzwarth AR and Garab G. 2014. Chlorophyll a fluorescence: beyond the limits of the QA model. Photosynthesis Research. 120(1-2):43-58.
Schreiber U. 2004. Pulse-Amplitude-Modulation (PAM) fluorometry and saturation pulse method: an overview. Chlorophyll a fluorescence: a signature of photosynthesis, p. 279-319.
Stirbet A, Govindjee. 2011. On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: basics and applications of the OJIP fluorescence transient. J. Photochem. Photobiol. B, (1-2): 236-257.
Strasser RJ. 1988. A concept for stress and its application in remote sensing. Applicationf of Chlorophyll Fluorescence. 333-337.
Strasser RJ, Tsimilli-Michael M, Qiang S and Goltsev V. 2010. Simultaneous in vivo recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. Biochim. Biophys. Acta. 1797:1313-1326.
Strasser RJ, Tsimilli-Michael M, Srivastava A. 2004. Analysis of the chlorophyll a fluorescence transient. Advances in Photosynthesis and Respiration. 321-362.
Takahashi S and Murata N. 2008. How do environmental stresses accelerate photoinhibition? Trends in Plant Science. 13(4):178-182.
Vredenberg W, Durchan M, Prasil O. 2009. Photochemical and photoelectrochemical quenching of chlorophyll fluorescence in photosystem II. Biochim Biophys Acta. (12):1468-1478.
Wollman FA. 2001. State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. EMBO J. 20(14):3623-3630.
Yordanov I, Goltsev V, Stefanov D, Chernev P, Zaharieva I, Kirova M, Gecheva V, Strasser RJ. 2008. Preservation of PS II Electron Transport from Senescence-Induced Inactivation in Primary Leaves after Decapitation and Defoliation of Bean Plants. J. Plant Physiol. 165(18):1954-1963.