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Effect of water stress on photosynthetic light phase in leaves of two ecotypes of *Platanus orientalis* L. plants

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ABSTRACT

Platanus orientalis is considered endangered species that is almost extinct in the natural ecosystems of Western Europe and, because of its hydrophilic habitat, may also be strongly affected by increasing water limitations in Eastern Europe. The effects of drought stress were studied and compared in young plane trees of two ecotypes from Bulgarian and Italian regions. The dynamics of drying and following re-watering was monitored *in vivo* by changes in activity of photosynthetic light phase reactions in leaves of 4-5 month old seedlings subject to controlled moderate water limiting regime for 12 days and subsequent recovery by controlled gradual irrigation for 10 days. The physiological state of photosynthetic machinery was estimated by analysis of signals of prompt and delayed chlorophyll *a* fluorescence measured in attached plane leaves by the Multifunctional Plant Efficiency Analyzer (Hansatech Instruments Ltd., UK). Prompt chlorophyll *a* fluorescence directly correlates to the redox state of the electron carriers in photosystem II at light conditions, while delayed fluorescence indicates rate constants of direct and back electron transport reactions within the same structure. The fluorometer allows simultaneous measurement of both prompt and delayed chlorophyll fluorescence signals that provide complementary information concerning the reactions in the photosynthetic light stage. We show that both donor and acceptor sides of photosystem II as well as intersystem electron transport are inhibited during drought stress. Moreover, the intersystem electron carriers appear to be the most sensitive part, indicated by strongly reduced size of their pool in the thylakoid membrane. The stress reaction of Bulgarian ecotype is expressed clearly. However, fluorescent parameters undergo partial recovery during re-watering. On the other hand, parameters in Italian ecotype exhibit weak changes during drought stress but the effect proceeds throughout the whole monitoring period.

Key words: *Platanus orientalis* ecotypes, water stress, stress monitoring *in vivo*, chlorophyll *a* fluorescence

Introduction

Plane (*Platanus orientalis*) is widely planted in parks and along streets to improve the microclimate of cities. However, it is considered almost extinct in the natural ecosystems of Western Europe (Rosati et al., 2015). The main reasons proposed are the changing water courses for irrigation purposes and the increased expansion of agriculture (WCMC,

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1998). Because of the hydrophilic habitat plane occupy it may also be strongly affected by increasing water limitations in Eastern Europe. So monitoring of water stress in *P. orientalis* may turn out to be essential for understanding the underlying processes behind diminishing of its populations and to estimate the risks of their total extinction. A powerful approach to stress monitoring would be such in which the dynamics of the stress response is studied *in vivo*.

The overall physiological state of plants can easily be indicated by activity of main metabolic processes such as photosynthesis (Blankenship, 2014). It is well known that photosynthetic light reactions are strongly affected by different environmental factors (Brestic & Zivcak, 2013) including drought (Oukarroum et al., 2009). To investigate the changes in photosynthetic light phase different fluorescent methods have been developed. They are nondestructive, fast and inexpensive compared to other physiological and biochemical methods.

A widely used approach to stress monitoring is the recording of chlorophyll *a* fluorescence signals during illumination with highly intensive (above 2000 μmol photons m^{-2} s^{-1}) photosynthetic active (actinic) light of samples previously held in dark (Bussotti et al., 2010). A transition from dark- to light- adapted state of the photosynthetic machinery is induced (Stirbet & Govindjee, 2011). That is the reason why the fluorescent transients are also called induction curves (IC). Those show characteristic rise corresponding to reduction of electron carriers (EC) in the electron transport chain (ETC). Right after illumination photochemistry in both photosystems occurs and primary electron acceptors are being reduced. Their accumulation starts to prevent photochemistry and part of the excited energy states of the antenna are deactivated in form of prompt fluorescence (PF). It is emitted by chlorophyll a molecules of the antenna complexes mainly of photosystem II (PSII) while photosystem I (PSI) contribution is small. Since that the prompt fluorescence rise is positively and directly related to the reduction of primary quinone (Q_A). PF IC (Figure 1) consists of four main levels: F_O – initial (at 20th μs after illumination) fluorescence when all Q_A molecules are oxidized, F_J (at 3rd ms) – level at which Q_A is reduced, F_I (at 30th ms) – level at which secondary quinone acceptor (Q_B) is reduced and F_P (at around 300th ms) – maximum level at which plastoquinone (PQ) pool in the thylakoid membrane is fully reduced. Two secondary levels in the PF transients are F_K (at 300 μs) and F_L (at 150 μs). Normally they are not present and are only visible in limiting stress conditions. After F_P the signal starts to decrease because of the Rubisco enzyme activation and the utilization of NADPH in the dark phase of photosynthesis. NADP oxidizes reduced end electron acceptors of PSI which in turn cause re-oxidation of all carriers in the ETC to the level when the rates of reduction and oxidation are equal.

For comparison of fluorescence transients it is easier to show them in relative values not in the recorded units – F(t). When the PF signal is double normalized to the minimum (Fo) and maximum (Fm) level of the IC the relative variable fluorescence – V(t), is derived (Figure 1).

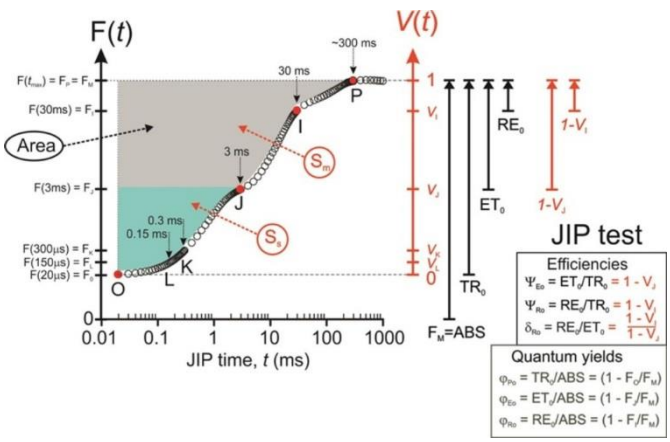

Figure 1. *Typical induction curve of prompt fluorescence. Characteristic points of the transients are labeled with letters O, L, K, J, I and P and the moment of their appearance in the induction (or JIP) time is noted. Fluorescence levels are presented in two scales: F(t) for recorded values and V(t) for relative values. In boxes derivation of the efficiencies and quantum yields of electron transport are shown. Those parameters are calculated from the fluxes of: total light energy absorbed by the antenna chlorophylls (ABS), energy trapped in photochemical process of PSII (TR), energy used for reduction of the intersystem electron carriers (ET) and energy used for reduction of the end electron acceptors of PSI (RE). In turn those energy fluxes correlate directly to different fluorescence levels. Area is the total complementary area between fluorescence induction curve and F = FM. For further explanation see the text and Table 1.*

The analysis of the PF transients, called JIP test, is developed by Strasser et al. and gives many structural and functional parameters of the ETC (Strasser et al., 2004). The parameters used in this study are listed and explained in Table 1. The way some parameters are directly calculated from the characteristic points of the IC is shown in Figure 1.

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Table 1. *Description and calculation of fluorescence (JIP test) parameters.*

Fluorescence parameter	Description
$\varphi_{P_O} = (1 - F_O)/F_M$	Maximum quantum yield of primary photochemistry (at t = 0)
$\varphi_{E_O} = (1 - F_J/F_M)(1 - V_J)$	Efficiency/probability that an electron moves further than Q_A
$\varphi_{R_O} = (1 - F_I/F_M)(1 - V_I)$	Quantum yield for reduction of end electron acceptors at the PSI acceptor side (RE)
$t(F_M)$	Moment in the induction time at which F_M is reached
$S_m = (Area)/(F_M - F_O)$	Normalized total complementary area above the O-J-I-P transient (reflecting multiple turnover Q_A reduction events)
$N = S_m M_0 (1/V_j)$	Turnover number: number of Q_A reduction events between time 0 and t(F_M)
$\gamma_{RC} = Chl_{RC}/Chl_{total}$	Probability that a PSII Chl molecule functions as RC
$M_0 = 4(F_{300\mu s} - F_0)/(F_M - F_0)$	Approximated initial slope (in ms^{-1}) of the fluorescence transient V = f(t)
$TRo/RC = M_0(1/V_J)$	Absorption flux (of antenna chlorophylls) per RC
	Trapping flux (leading to Q_A reduction) per RC
$PI_{ABS} = \frac{\gamma_{RC}}{1-\gamma_{RC}} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_{Eo}}{1-\psi_{Eo}}$	Performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors
$PI_{total} = PI_{ABS} \frac{\delta_{Ro}}{1-\delta_{Ro}}$	Performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors

Delayed fluorescence (DF) has a different mechanism of origin from PF. DF is the result of back electron transport reactions (Goltsev et al., 2005b, Goltsev et al., 2009) since the probability of back electron transfer in the ETC is never zero. When the energy turns back to the reaction center (RC) chlorophyll it can be transferred to the antenna and converted back to light. DF quanta are radiated by the same chlorophyll a molecules the PF is emitted by so both signals cannot be separated spectrally. Moreover intensity of DF as compare to PF is more than 2 orders of magnitude less. Those two facts make simultaneous recording of DF and PF impossible. DF can be measured only in dark when decay of its signal is observed. Modern fluorometers like Multifunctional Plant Efficiency Analyzer (M-PEA) allows for construction of induction curves from DF decay curves (DC) (Strasser et al., 2010). Their acquisition during the induction time is possible because the actinic light is switched off for short periods. Those dark periods lasts from to 100 μs to 1, 2.4, 24, 240 ms and reach 2.4 s in different induction time intervals. The dark:light period ratio is always 1:3. DCs are best fitted with poliexponential functions, consisting mainly of 4 components. The fastest DF component (in the micro- and sub-millisecond time range) are explained by the decrease of the concentration of charge couples because of redox reactions in which one of the separated charges takes part. Averaging points from particular dark decay period (when one component is predominant) at different moments in the induction time gives induction curves of DF. The simultaneously measured induction curves of PF and DF are presented in Figure 2.

During the induction period DF transients show characteristic shape with several maximal and minimal levels (Figure 2). The maxima designated with I and minima with D (Goltsev et al., 2005a) correspond to different processes. They reflect redox reactions on both the donor and acceptor side of PSIIand depend on the formation of the electrical and proton transmembrane gradient on thylakoid membrane (Goltsev et al., 2009). DF provides valuable information about the reactions in thylakoid membranes and its analysis complement that of PF IC (Oukarroum et al., 2013).

The aim of our study is to understand the effect of water stress on photosynthetic light reactions in *Platanus orientalis* leaves. To comprehend the whole picture of stress development close to that in nature the dynamics of the response to drying and re-watering is investigated. Thus the monitoring of plant stress has to be conducted

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nondestructively, for many days and each individual measurement to be fast and easily reproducible. To meet these objectives we harness the powerful methods of chlorophyll a fluorescence measurements *in vivo*. A further goal is to evaluate the similarities and differences in the behaviour of photosynthetic machinery undergoing water stress in Bulgarian and Italian ecotypes of *Platanus orientalis*. The information acquired can help for taking adequate conservation measures in corresponding areas.

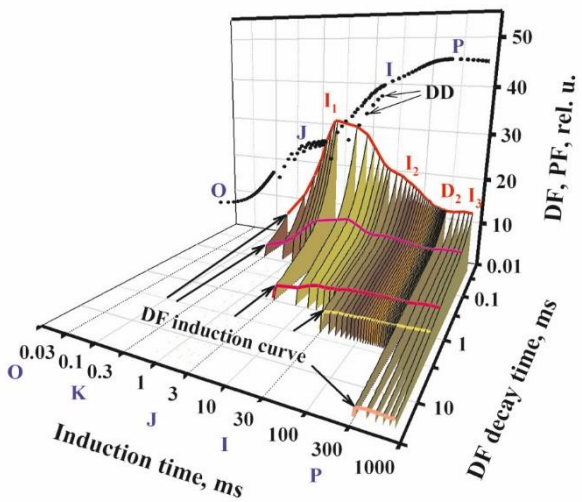

Figure 2. *3D presentation of the dynamics of prompt and delayed chlorophyll fluorescence signals simultaneously measured by M-PEA system. The scheme illustrates the construction of the induction curves of DF collected for different dark decay intervals. The characteristic points of both signals are designated. For DF those are peaks I1, I2, D2 and I3. For details see the text.*

Materials and Methods

Plants and growth conditions

The experimental object of this study is *Platanus orientalis* L. Seeds from Bulgarian and Italian ecotypes were collected from their natural habitats and sown in containers at Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences. The 1 month old seedlings were replanted in small pots at Department of Biophysics and Radiobiology, Faculty of Biology, University of Sofia, and put in phytostatic box. A mixture of TS 3 peat substrate, Klasmann-Deilmann (Geeste, Germany), river sand, quartz sand and perlite was used in mass ratio of 140:15:5:3. When the seedlings turned 2-2.5 months a second replanting in big pots was applied. The plants were grow at photosynthetic photon flux density (PPFD) of 200 μmol photons m^{-2} s^{-1}, 12/12 h day/night photoperiod and temperature of 20-25 °C. The water regime of a single plant was 50 ml/week at the beginning, then increased to 100 and finally to 500 ml/week reflecting the growing water needs of the plants.

Fluorescent measurements and experimental design

The dynamics of the drought stress and re-watering of 4-5 month old *Platanus orientalis* trees are investigated by obtaining the signals of PF and DF in attached leaves. The fluorometer used is Multifunctional Plant Efficiency Analyzer (M-PEA), developed by Hansatech Instruments Ltd. (King's Lynn, UK). Special computer software allows for control of the M-PEA apparatus, downloading of the recorded data and primary visualization as well as for composition of wide range measuring protocols which can easily be uploaded to the measuring block. A well-developed leaf from the middle level of every seedling is chosen to be investigated throughout the whole experiment and is measured consequently by two protocols:

1) Recording PF for 1 s at light intensity of 4000 μmol photons m^{-2} s^{-1} and then single DF decay for 3 s in dark;

2) Recording PF and DF signals simultaneously for 1 min at light intensity of 2000 μmol photons m^{-2} s^{-1}.

In this work the PF induction data and single DF decay data from the first protocol and DF induction data from the second are shown. The plants are held in dark for an hour to ensure their full dark adapted state before the beginning of the fluorescence measurements. They proceed in a dark room in order to keep the samples in this physiological state. The measurements with each protocol are repeated at least three times and carried out every other day.

The drying process lasts 12 days and the re-watering – 10 days. Both of them are done gradually. The parameter used to monitor the water content in the pots is the Fraction of the Transpirable Soil Water (FTSW). FTSW is calculated every day from the weight of the pots: at the time, when poured with water to extend so they cannot take up more (maximum) and when they are completely dry (minimum). The FTSW at the day right before the begging of the drying (0^{th} day) was 0.8, on the driest (12^{th}) day – around 0.25 and then returned to 0.76 at the last (22^{nd}) day. The daily change in FTSW was about 0.05.

Data analysis

Primary analysis of the collected fluorescence data is carried out with the software M-PEA Data Analyzer 5.4,

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developed by Petko Chernev at the Department of Biophysics and Radiology, Faculty of Biology, University of Sofia. The software provides simultaneous visualization of the PF and DF signals. Secondary analysis and the processing of the raw data are performed in Microsoft Excel as well as the calculation of the PF (JIP) and DF parameters. The mean values +/– standard error of mean (SEM) are presented in the figures. Since calculation of SEM is impossible for some parameters the Bonferroni t-test at significance level 0.05 is applied.

Results

Prompt fluorescence. JIP test

The water stress response of the photosynthetic light phase in plane leaves was monitored by means measuring of prompt fluorescence induction curves (Figure 3). The shape of the curves changed profoundly in Bulgarian (BG) ecotype. FJ level increased at drought and partially decreased after re-watering. FI followed similar but weaker trend. Higher FJ indicated slower rate of electron transport from QA to QB and FI – slower rate of electron transport from QB to PQ. On the other hand the differences in the Italian (IT) ecotype while still statistically significant were much weakly expressed.

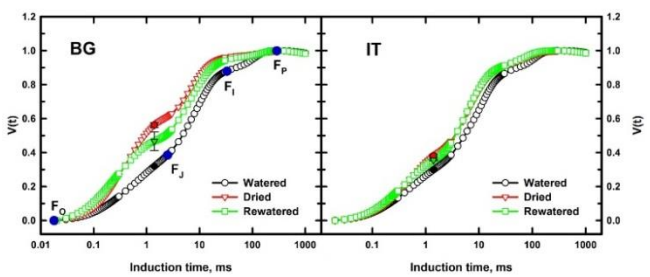

Figure 3. *Induction curves of prompt fluorescence recorded for 1 s in leaves of Bulgarian (left) and Italian (right) ecotype of Platanus orientalis measured at three different treatment periods: on the day before the start of drying (black curve), on the "driest" day (red) and on the last day of re-watering (green). The PF values given are for relative variable fluorescence. For clarity the characteristic fluorescence levels (blue dots) are designated only for one curve and SEM is shown only for one point (black edged) of each curve.*

A wide set of JIP test parameters were used to estimate the whole state of the light reactions before, during and after drying. A multiparametric image of the stress response for both ecotypes was constructed as a Radar Plot (Figure 4). Photosynthetic machinery of the Bulgarian ecotype undergoes drastic changes. Limited potential of energy storage in the light photosynthetic phase was observed – both performance indexes (PIABS and PItotal) diminished under stress and showed weak recovery. The reason was found to be the reduced values of the quantum efficiencies of the electron transport after QA (φEo and φRo) since the quantum efficiency of photochemistry (φPo) stayed the same throughout the whole monitoring period. Moreover the observation was in correlation with higher VJ values and lowered energy fluxes ETO/RC and REO/RC, total area above the IC (Sm) and turnover number (N). Interestingly both trapped (TRO/RC) and dissipated energy flux (DIO/RC) increased along with the absorption flux (ABS/RC). Almost all parameters underwent recovery at least to some extent.

Italian seedlings showed very different stress response. The PIABS was extremely stable parameter and the overall photosynthetic potential PItotal was affected weakly. φPo was reduced while φRo increased. Only φEo, ETO/RC, REO/RC and N showed similar trend as in the BG ecotype. No significant changes were observed for the remaining parameters. However virtually all the parameters did not recovered.

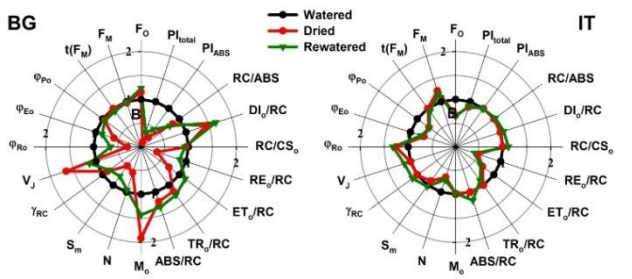

Figure 4. *JIP test parameters for Bulgarian (left) and Italian (right) ecotype of Platanus orientalis. The parameters are calculated from the ICs presented on Figure 3. All other experimental conditions are the same. Each parameter value is normalized to that derived at the day before the drying began.*

For pinpointing the drought sensitive steps in the ETC the relatively new approach of differential curves (Oukarroum et al., 2007) was applied (Figure 5). Multiple transient maxima and minima aroused showing the magnitude of the deviation between the signals from non- and water stressed samples at different steps of the V(t) ICs. The main peak appeared to be around fluorescence level J in Bulgarian plants. It illustrated

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well the limitations at the acceptor side of PS II occurring during stress. The main peak in curves from Italian seedlings was between level J and I and again much lower. A good distinction was established between the behaviour of both ecotypes after re-watering: BG curves after step K recover somewhat while IT showed no recovery except at J.

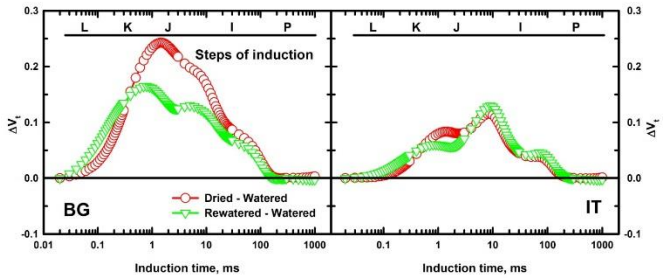

Figure 5. *Differential curves of the relative variable fluorescence calculated by subtraction of the V(t) values acquired from not yet stressed plants from the values recorded during drought and re-watering at the same moments in the induction time. The conditions are the same as in Figure 3.*

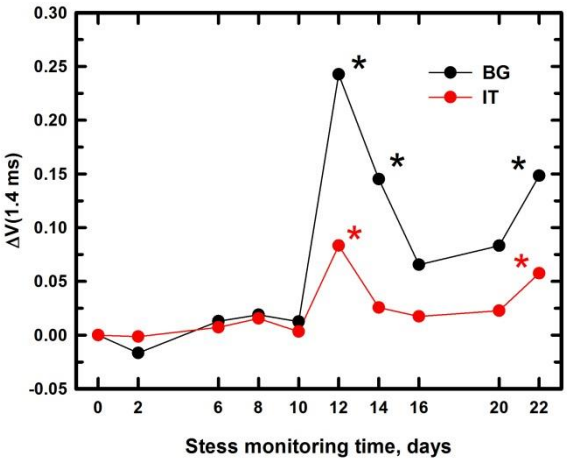

Figure 6. *Water stress dynamics of the amplitude at 1.4 ms of differential curves of V(t). Asterisks indicate if the Bonferroni t-test for the corresponding points in the V(t) IC was passed at significance level 0.05. Other conditions are the same as in Figure 5.*

The dynamics of the amplitude of the peak at 1.4 ms for the whole monitoring period was constructed (Figure 6). The stress response in both ecotypes turned out to develop fast, just for 2 days – between 10th and 12th after the begging of the experiment. Strangely the effect at the last (22nd) day was significant even after a recovery to insignificant values was observed. BG recovered for 4 days and IT just for 2.

The additional fluorescence levels K and L cannot be easily spotted on the PF IC and the construction of differential curves was used to estimate the water stress response of the underlying processes. Moreover, they cannot be directly studied by JIP test. The point FK correlated with electron transport activity in the donor side of PSII namely oxygen evolving complex (OEC). When the structure of OEC is disrupted it cannot donate electrons to the reaction center of PSII and oxidized RC chlorophyll (P680+) which are non-photochemical quenchers of fluorescence are accumulated. FK becomes a visible peak in the IC at 0.3 ms, even the dominant phase in severe stress conditions (Strasser et al., 2004). K peak was illustrated with differential curves of variable fluorescence normalized from O to J (Figure 7). Both ecotypes showed maximum level at times typical for the K peak only after the re-watering. The peak was higher again in favor of BG. On the driest day such a maximum was not present.

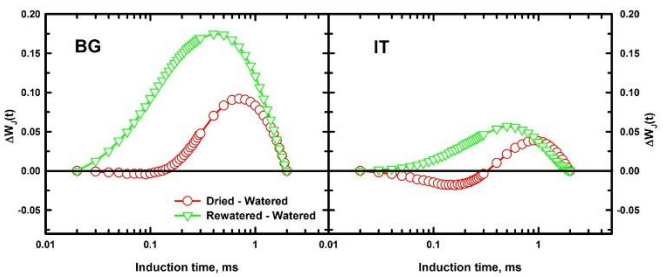

Figure 7. *Differential curves of variable fluorescence normalized from O to J. Each value is calculated as difference between normalized W(t) for stressed and non-stressed samples: ΔW = Wstressed – Wcontrol. Other conditions are the same as in Figure 5.*

The development of the K peak was visualized as the dynamics of the amplitude at 0.5 ms during the experimental period (Figure 8). The magnitude of the peak was increasing throughout the whole period almost monotonously. However the statistically significant changes occurred after 10-12 days of drying depending on the ecotype. Thus the OEC disturbance appeared to be stress induced and irreparable during investigated period.

The fluorescence level at L relates to the possibility for migration of the exited state energy between the antenna complexes of PSII. When there is none connectivity the

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initial fluorescent rise is in direct proportional correlation to the non-photochemically active RC (those containing reduced QA).

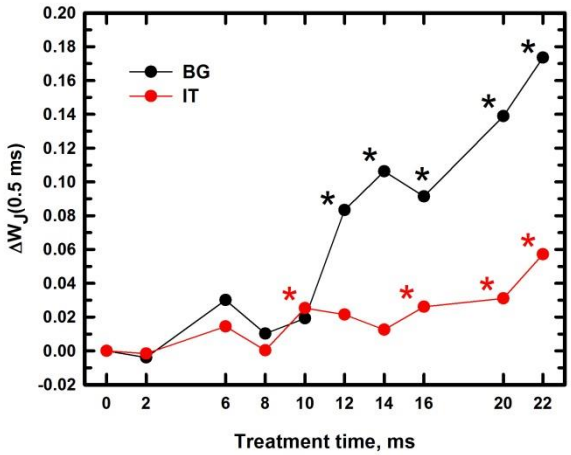

Figure 8. *Water stress dynamics of the amplitude at 0.5 ms of differential curves of the variable fluorescence normalized from O to J. Asterisks indicate if the Bonferroni t-test for the corresponding points in the variable fluorescence curves was passed at significance level 0.05.Other conditions are the same as in Figure 5.*

If connectivity appears the correlation becomes hyperbolic because (at least) part of the excess energy can go to photochemically active RC. Then the FL level will be lowered. Assessment of L peak was done similarly to K peak (Figure 9).

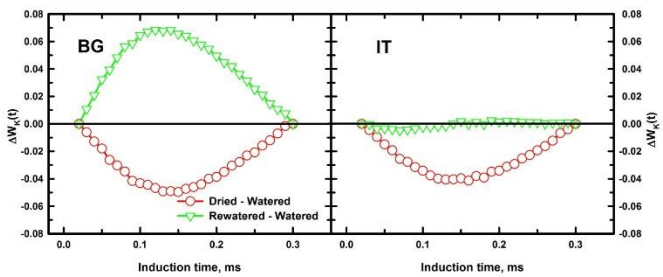

Figure 9. *Differential curves of variable fluorescence normalized from O to K. Other conditions are the same as in Figure 5.*

During drought both ecotypes expressed negative L peaks – the antennae became more connected. During re-watering connection between antennae was lost. However the magnitude of the process was different: BG showed positive L peak even bigger in amplitude than the negative one and IT – nearly straight line. In the first case antenna connectivity was weaker in comparison to the pre-stress levels and in the second – it returned to those values.

The negative L peak developed just for 2 days after the 10th day of the experiment (Figure 10). The different course during re-watering in both ecotypes explained above was well observed.

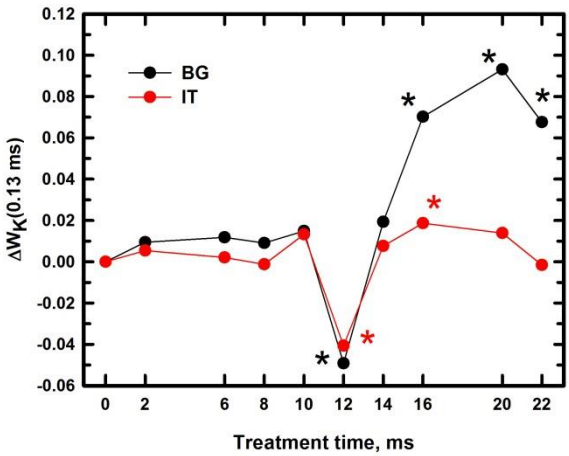

Figure 10. *Water stress dynamics of the amplitude at 0.13 ms of differential curves of the variable fluorescence normalized from O to K. Asterisks indicate if the Bonferroni t-test for the corresponding points in the variable fluorescence curves was passed at significance level 0.05. Other conditions are the same as in Figure 5.*

Delayed fluorescence

The induction curves of DF were measured for better analysis of the water stress response (Figure 11). After illumination separate charge pairs start to accumulate and DF intensity increase to maximum level I1. The second peak I2 corresponds to the moment when ET rate throught PQ pool is accelerated by active PSI reaction center (Goltsev et al., 2009). The decrease of DF intensity after I1 coincides with the reduction of PSII electron acceptors and closure of PSII reaction centers and they are maximally closed when the point D2 is reached. The followed DF rise to D4 reflects the accumulation of transmembrane proton gradient (Goltsev et al., 2005b, Goltsev et al., 2009).

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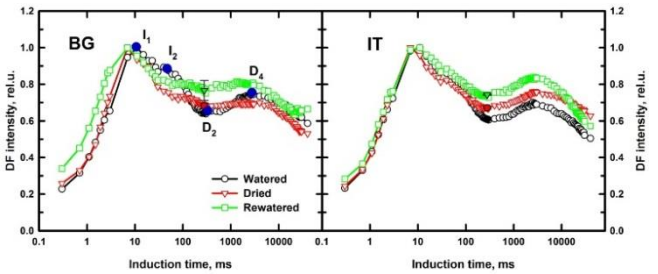

Figure 11. *Induction curves of delayed fluorescence recorded for 1 min in leaves of Bulgarian (left) and Italian (right) ecotype of Platanus orientalis measured at the same treatment periods as PF curves in Figure 3. All DF values are normalized to the maximum level I1. For clarity the characteristic fluorescence levels (blue dots) are designated only for one curve and SEM is shown only for one point (black edged) of each curve.*

The dynamics of the I2/I1 ratio was plotted on the left panel of Figure 12. That parameter reflects the ratio of the electron transport flux between the two photosystems to the flux of the excitations trapped in RC of PSII (Zaharieva et al., 1999). A decrease in I2/I1 after 8-10 days of drought stress for both ecotypes was observed. However BG was affected more hardly and recovered more weakly than IT did.

The last step in the water stress monitoring was to compare the DF decay curves at the 0th, 12th and 22nd experimental day (right panel of Figure 12). Drought stress effect on the micro and sub-millisecond components only in BG plants was demonstrated. The fluorescence levels during those time intervals were lowered compared to the values before stress application and after re-watering.

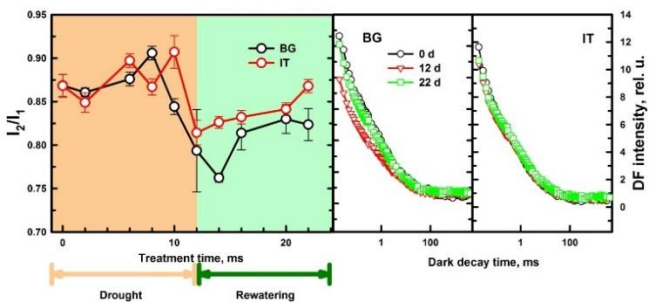

Figure 12. *Left panel – Dynamics of the DF parameter I2/I1 during the whole experimental period (left). Conditions are the same as in Figure 11. Right panel – courses of DF decay curves in plane leaves measured before (black), during (red) and after (green) drought stress. For clarity the SEM for dark decays are not shown.*

Discussion

The response of the photosynthetic light phase to water stress have been studied in detail by recording prompt and delayed fluorescence in *Platanus orientalis* leaves and by different data manipulation approaches of those signals. Both donor and acceptor sides of photosystem II are inhibited during drought. However, when analyzing the PF ICs the strongest negative stress effect is on the electron transport between the photosystems. It is easily noticed by higher J and I levels – the rate of the process gets slower. The quantum efficiencies of ET and RE processes are lowered as well. We speculate the reason behind those observations is the smaller size of the PQ pool in the thylakoid membrane at drought stress. The alterations in other JIP parametes support that finding.

Another sensitive part of the ETC chain when comes to limiting water conditions is the donor side of the PSII, namely OEC. Disturbances in OEC structure reveal to be strong and develop even after re-watering. The high sensitivity of OEC to water stress is in correspondence to that found in other plant species (Oukarroum et al., 2007).

We also observed significant thylakoid membrane reorganizations by looking at the antennae connectivity levels. We assume that when the free water in the chloroplast decreases under drought antenna complexes come closer physically and the connection between them rises. After re-watering the previously gained connection is lost. In Bulgarian ecotype during re-watering the thylakoid structures cannot recover fully, water is not incorporated properly into them, antenna are displaced even further, thus the connection is weaker than it was before the stress application.

The stress reaction of Bulgarian ecotype is expressed clearly. However, almost all fluorescence parameters undergo partial recovery during re-watering. On the other hand, parameters in Italian ecotype exhibit weak changes during drought stress but the effect proceeds throughout the whole monitoring period.

Acknowledgement

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