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Photosynthetic efficiency of two *Platanus orientalis* L. ecotypes exposed to moderately high temperature – JIP-test analysis

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Abstract

Plane (*Platanus orientalis*) is a popular park tree in Europe but is almost extinct in natural ecosystems, because of climate changes. In our study, two ecotypes of plane tree (Bulgarian and Italian) were submitted to moderately elevated temperature that occurs in summer. Our aim was to compare stress reactions, tolerance, and adaptability of these plants. Leaf age had a significant impact on the stress effects. For correct interpretation of the results, we investigated leaves at different positions from the apical bud, *i.e.*, leaves of different age. We assessed their photosynthetic efficiency at room temperature and after treatment at moderately high temperature by simultaneous measurement of prompt and delayed chlorophyll fluorescence, as well as light reflection at 820 nm. For more precise interpretation of the obtained results we did principal component analysis. The two studied plane ecotypes showed different tolerance to the elevated temperature. Plants of Italian ecotype showed better adaptivity and developed advantageous photosynthetic characteristics, while Bulgarian ecotype was more affected. Plants of both ecotypes recovered from the heat stress.

Additional key words: difference curves; electron transport chain; JIP-test; photosynthesis; photosystem I; photosystem II.

Introduction

Global climate changes force plants to overcome a number of environmental challenges and find a way to adapt to the new conditions (Bucharova *et al.* 2016). To avoid species extinctions, some researchers and conservation managers have been advocating for the intentional translocation of species or populations, which is termed 'assisted migration' (Hewitt *et al.* 2011). The strategy of assisted migration is based on the assumption that plants are primarily adapted to their specific local climate (Black *et al.* 2011). If there is a shift in the climate zones, then local populations will be more vulnerable, and ecotypes from other locations with a climate similar to the novel conditions in the target area are expected to perform better than the local populations. There are not many *in vivo* experiments to verify if the assisted migration method works in real conditions, and most of the supporting data come from climate envelope models. Bucharova *et al.* (2016) performed one of the few experiments of comparing different ecotypes of plant species and their tolerance to heat stress by measuring biomass. Although their results indicated no significant differences, it is possible the used methods were not sensitive enough. Plants subjected to rather usual stress, such as warmer environment during summer, respond by small, but significant changes (Strasser and Tsimilli-Michael 2001). Tests, used to study high temperature-induced changes, need to be both very sensitive and informative. Very efficient approach is measuring Chl *a* fluorescence, which gives detailed data

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Abbreviations: BG – Bulgarian ecotype; DC – difference curves; DF – delayed chlorophyll *a* fluorescence; F_V/F_M – maximum quantum yield of PSII photochemistry; IT – Italian ecotype; MR820 – modulated light reflection at 820 nm; OEC – oxygen-evolving complex; PF – prompt chlorophyll *a* fluorescence; PC – plastocyanin; PCA – principal component analysis; PSA – photosynthetic apparatus; PQ – plastoquinone.

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This article honors Prof. Reto J. Strasser at his 75th birthday for his outstanding and unique contributions in the use and analysis of chlorophyll *a* fluorescence towards understanding photosynthesis.

with high sensitivity (Allakhverdiev *et al.* 2008, Kalaji *et al.* 2014a). Elevation in temperature affects the excitation energy transfer (spillover) between the two photosystems as well as the rate constants of the photochemical processes (Zaharieva *et al.* 2004). It is shown that PSII is more sensitive to temperatures above 35°C, and structural changes occur in the thylakoid membrane – granal structures are influenced, ion permeability rises, the proton gradient decreases (Velitchkova *et al.* 1989, Bukhov *et al.* 1999). One of the key components of the photosynthetic machinery, the oxygen-evolving complex, is highly susceptible to heat damage even at moderate temperatures, above 32°C (Kouřil *et al.* 2004). On the other hand, cyclic electron transfer around PSII (Havaux 1998) and the electron donation to P_{680}^+ by Y_D tyrosine (Ducruet 1999) are activated with increasing temperature as a protective mechanism, but these two processes have limited contribution during moderate heat treatments (Pospíšil and Tyystjärvi 1999). Moderate elevation in temperatures resulted in dissociation of the LHCII complex (Goltsev *et al.* 2010). Heat-induced structural changes in LHC can lead to misbalance in trapped energy between the two photosystems (Zhang and Sharkey 2009, Mathur *et al.* 2011).

Plane tree (*P. orientalis*) is one of the most desirable park trees in Europe, but it is almost extinct in natural ecosystems due to abnormally high temperatures, drought, and excessive light (Velikova *et al.* 2018). Studying plane tree reactions and tolerance to different levels of stress is important in order to preserve and grow the species habitats. In our study, we compared the impact of moderately high temperatures (naturally occurring) on two ecotypes of plane tree – Bulgarian and Italian, thus assessing the differences in the acclimation and adaptive mechanisms established for these two ecotypes during the evolution. Deciduous plants like *P. orientalis* undergo a cyclic process where leaves develop, mature, grow old, and finally fall (Woolhouse 1987, Lichtenthaler and Babani 2004). During this process their physiological state changes. When subjected to a stress factor, plants tend to stop growing due to misbalanced sink–source relationships (White *et al.* 2016). Source activity refers to the rate at which essential external resources are acquired by the plant and become available internally, while sink activity refers to the internal utilization of these resources (Lawlor and Paul 2014). Usually these relationships are well balanced between leaves of different ages, but changes in the growing conditions may disturb the balance and provoke more pronounced effects with young leaves acting more effectively as a sink, withdrawing metabolites from older leaves. As a result the young leaves continue to grow and develop but the older ones enter senescence faster (Yordanov *et al.* 2007). Evidence for such behavior has been found in maize plants under water deficiency (Huang *et al.* 2018). This indicates that the overall plant status depends on the state of both young and old leaves.

This research aimed to monitor the *in vivo* reaction of photosynthetic apparatus (PSA) in *P. orientalis* plants of two ecotypes (Bulgarian and Italian) to moderate temperature stress and to analyze how leaves of different ages (young, mature, and old) react. We analyzed different aspects of light emitted from PSA – light emitted after direct illumination (PF), light emitted by processes of recombination (DF) and scattered light (MR820) – all correlated directly with specific characteristics of the electron transport chain. Combined together, these signals give a large set of information about the conditions of the plant objects *in vivo*. Regardless of the fact that the PF and DF are emitted by the same population of PSII antenna chlorophylls (Chl), they carry different, complementary information about the quantitative characteristics of the photosynthetic process (Goltsev *et al.* 2009a). Thus, we were able to broaden the JIP-test applicability and to demonstrate that even comparatively weak changes in the physiological state of the leaves could be monitored, and even initial stages of stress can be evaluated. For better understanding of the results and further validation of the conclusions, we performed principal component analysis (PCA) which provided visualization and better assessment of the generalized PSA data at these experimental conditions.

Materials and methods

Plant growth and sample preparation: *P. orientalis* plants of two different ecotypes – Bulgarian (from Kresna gorge) and Italian (from Sicily), were grown from seeds in a growth chamber with 12/12-h day/night periods and incident light intensity of 150 μmol(photon) m^{-2} s^{-1} PAR. The plants were analyzed in the second vegetative season (1.5 years old) after fully developing of at least six leaves. The leaves were denoted as young, matured, and old according to their position on the tree floors along the stem: young – at the top, matured – in the middle, and old – at the bottom floor. The leaves were marked so the measurements were carried out on specific spots every day throughout the experiment.

Temperature treatments were monitored automatically by *MRL-351* versatile environmental test chamber (*Sanyo*, Osaka, Japan). Plants were grown normally at 23°C during night and 28°C during the day. Temperature treatment started with switching the light on (beginning of the day). The growing temperature rose to 41°C in six steps of 3°C, each lasting 45 min. After that the plants were subjected to 41°C for 4 h. This period of exposure to the elevated temperature was long enough to trigger changes in the photosynthetic machinery. All the measurements, including control ones, were done at room temperature.

The experiment lasted for seven days: a control day with normal conditions (0 d), 3 d (1st, 2nd, and 3rd d) of moderate temperature treatment, as described above, and 3 d (4th, 5th, and 6th d) of recovery at control conditions.

Chl *a* fluorescence and modulated light reflection at 820 nm measurements: Chl *a* fluorescence measurements were performed with *Multifunctional Plant Efficiency Analyzer* (*M-PEA*) device (*Hansatech Instrument*, King's Lynn, UK). The protocols for the experiment were set using specialized software – *M-PEA Plus*. They were constructed in order to give informative qualitative and quantitative data by simultaneous measurement of PF induction curve (IC), DF decay kinetics, and changes in the modulated light reflection at 820 nm, related to P_{700}^{+} PC (plastocyanin) oxidation and rereduction kinetics. Measurements were carried out in a dark room in order not to compromise the steady dark-adapted state of the plants, achieved by darkening them for at least 1 h. The duration of a single measurement was 1 s with saturating light intensity of 4,000 μmol(photon) m^{-2} s^{-1} PAR. The DF decay kinetics were recorded after this 1-s illumination with saturating light.

Data processing: The obtained data were processed through specialized software – *M-PEA Data Analyzer*, developed at the Department of Biophysics and Radiobiology, Faculty of Biology, Sofia University.

Difference curves were calculated and L, K, H, and G bands (described below) were obtained. Nine important JIP parameters, described in the Appendix, were selected to monitor the changes, occurring in the PSA after temperature treatment: φ_{P0} (F_V/F_M), φ_{D0}, ψ_{E0}, δ_{R0}, PI_{ABS}, PI_{total}, N, RC/CS_0, ABS/RC. Characteristic life times, τ_i, of DF decay curves were mathematically extracted by nonlinear 7-parameter regression. Rates of oxidation and rereduction of P_{700}^{+} + PC^{+} were calculated as absolute values of slopes of linear regression of the MR820 signal in two particular time ranges.

Statistics: The experiment was performed for three individual plants per ecotype with 36 repetitions for each group per day – young, mature, and old (three plants × two leaves of each group per plant per day × six measurements per leaf). The data are presented as mean ± SE. One-way analysis of variance (*ANOVA*) followed by *Holm-Sidak*'s test with α=0.05 was performed with *Sigma Plot 11.0* to find statistically significant differences between the particular value at each day and the control (at Day 0). Statistically significant differences were found for ψ_{E0}, δ_{R0}, PI_{ABS}, PI_{total}, N, RC/CS_0, and rereduction rate of P_{700} parameters for Bulgarian (BG) and PI_{total} and RC/CS_0 parameters for Italian (IT) ecotype. In order to assume the overall temperature induced response of the leaves from each age and from each ecotype, the data set of all the measured parameters was processed by principal component analysis (PCA) in *R 3.3.3* using *prcomp* package after logarithmic transformation. PCA plots were constructed by *ggbiplot* package with minor custom improvements.

Results and discussion

Difference curves at specific steps of the PF induction curve: Many different reactions influence the rise of the Chl fluorescence intensity measured in leaves – both in the photochemical phase (photoinduced Q_A reduction) and thermal phase (redox reactions from the PSII acceptors to PSI end acceptors) of the induction transient. The impact of each process is complex and it is hard to be analyzed only by the induction curves derived from direct measurement of PF. The ICs were normalized for visualization of the OJIP transitions. These transitions occur at different times, which allows us to calculate difference curves (DC) and to obtain detailed information about the changes in the PSA. DC of specific parts of the induction curve were calculated by subtracting the values of the IC for the control plants from the IC values of the stressed plants (Strasser *et al.* 2004). The resulting curves are known as bands, and their positive or negative values determine the efficiency and rate of different reactions at every stage of the process (Fig. 1S, *supplement*). There are four bands, described by Strasser *et al.* (2004) and Stirbet and Govindjee (2012): L band (between O and 300 μs), K band (between O and J), H band (between J and I), and G band (between I and P). The L band is associated with the arrangement of PSII units in the thylakoid grana membrane – 'grouping', and with the 'connectivity' of LHCII and PSII reaction centers (Strasser and Stirbet 2001, Stirbet 2013). The functionality of PSII (*i.e.*, antenna size, connectivity between PSII RCs) depends on the protein packing in the PSA (Stirbet 2013). K band gives information about the changes in the fraction of operating oxygen-evolving complex, respectively, about the state of the donor side of PSII (Srivastava and Strasser 1996, Srivastava *et al.* 1997). It is a specific rise with maximum at about 300 μs on the OJIP induction curve, which is mostly hidden between O and J in normal conditions but is often observed under heat or drought stress (Guissé *et al.* 1995, Srivastava *et al.* 1995, Srivastava and Strasser 1996, Lazár *et al.* 1997). In previous review on the JIP-test applicability, Strasser *et al.* (2004) suggested the calculation of H and G bands, which have not been well studied so far. We used ΔW_{JI} and ΔW_{IP} normalization (used by Samborska *et al.* 2019) to study these phases in detail, and we labelled them H and G band, respectively. H band gives information about the electron flow to the end acceptors in PSII, strongly correlated with the PQ pool size and the degree of its reduction. G band (between I and P) is related to the efficiency of electron flow to the end acceptors in PSI (phylloquinone – FeS cluster – ferredoxin – FNR – NADP) (Strasser *et al.* 2004) and to the relative PSI content (Ceppi *et al.* 2012). Several pathways compete for the reduced Fd, which donates electrons to various destinations, such as FNR (one of several assimilatory pathways), molecular O_2, cytochrome b_6f, or the redox-regulatory system *via* FTR (Fd-thioredoxin reductase) (Scheibe *et al.* 2005). At dark-adapted conditions, the majority of FNR is membrane-bound and mostly is not active for oxidation of reduced Fd, although the partial involvement of the membrane-bound FNR pool in photosynthetic electron transport cannot be ruled out completely (Benz *et al.* 2010).

The original OP curve did not show significant differences between the analyzed plant ecotypes (data not shown). We constructed difference curves as difference between double normalized relative variable fluorescence curves in treated and control samples at OP time region as well as at four specific intervals: OK, OJ, JI, and IP. DC are calculated as in Kalaji *et al.* (2018). L band (ΔW_{OK}), K band (ΔW_{OJ}), H band (ΔW_{JI}), and G band (ΔW_{IP}) are presented in Fig. 1 for both ecotypes – BG and IT, and for the leaves of the three different ages – young, mature, and old. Each value represents the mean of the maximal amplitudes of difference curves (the highest deviation of the values of relative variable fluorescence at Day X from the values at Day 0, not a difference at a certain induction time point).

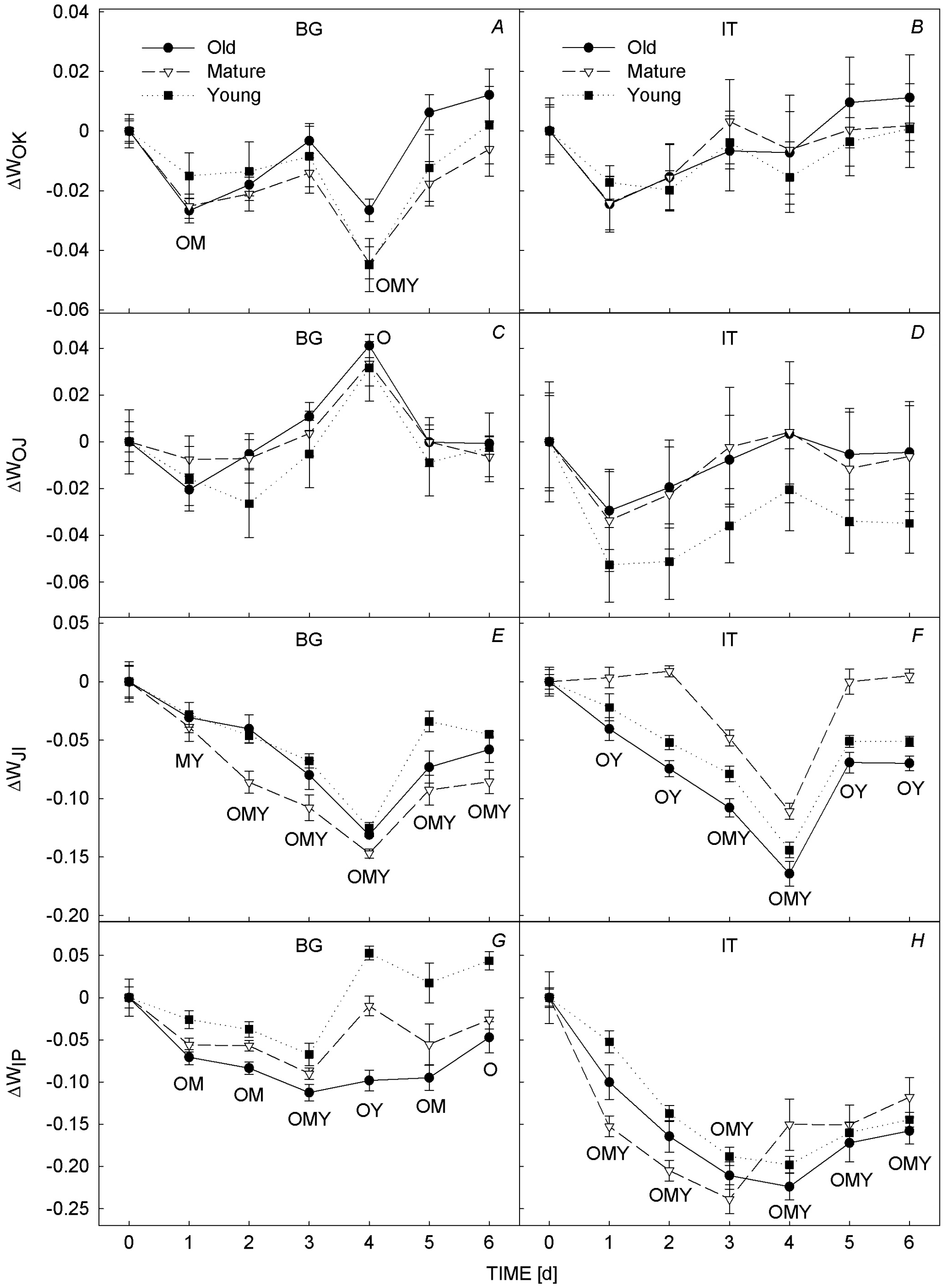

Fig. 1. Maximal amplitudes of the difference curves, *i.e.*, the maximal differences between the values of relative variable fluorescence for respective day of the experiment and the control values (Day 0), for the characteristic bands in OJIP induction curves for leaves of different age: young, mature. and old, from Bulgarian (BG, *left side*) and Italian (IT, *right side*) ecotypes *Platanus orientalis*. The days of the experiment, represented on the *x*-axes, are: Day 0 – control day, Days 1–3 – days of the temperature treatment, Days 4–6 – recovery period. Variable fluorescence ΔW for each band was calculated by double normalization of the raw PF signal at specific steps as follow: for OK phase, L band (*A*,*B*): $W_{t(OK)} = (F_t - F_O)/(F_K - F_O)$; $\Delta W_{OK} = W_{t(OK)(\text{day of the experiment})} - W_{t(OK)(\text{Day }0)}$; for O-J phase, K band (*C*,*D*): $W_{t(OJ)} = (F_t - F_O)/(F_J - F_O)$; $\Delta W_{OJ} = W_{t(OJ)(\text{day of the experiment})} - W_{t(OJ)(\text{Day }0)}$; for JI phase, H band (*E*,*F*): $W_{t(JI)} = (F_t - F_J)/(F_I - F_J)$; $\Delta W_{JI} = W_{t(JI)(\text{day of the experiment})} - W_{t(JI)(\text{Day }0)}$; for IP phase, G band (*G*,*H*): $W_{t(IP)} = (F_t - F_I)/(F_P - F_I)$; $\Delta W_{IP} = W_{t(IP)(\text{day of the experiment})} - W_{t(IP)(\text{Day }0)}$. Statistically significant differences (obtained with one-way *ANOVA*, *Holm-Sidak*'s test, α=0.05) between the value on a particular day and the control one (at Day 0) are denoted with *letters*: O for the old, M for the mature and Y for the young leaves.

L band: The L band (ΔW_{OK}) gives information about the grouping, or connectivity – the relative position of antenna complexes of different RCs in relation to each other (Stirbet 2013). It is mathematically derived by double normalization between F_O and $F_{300\mu s}$. A positive L band indicates greater distances between the PSII antennae and thus less efficient energy exchange while a negative L band is a sign of greater grouping and more efficient energy exchange between the neighbouring PSII units (Srivastava *et al.* 1997, Kalaji *et al.* 2018). Plants of the IT ecotype did not show significant change in ΔW_{OK} during the experiment thus the connectivity of their antenna complexes and the PSII units grouping is probably not affected. The L band for the BG ecotype samples was more influenced (Fig. 1*A*,*B*). Young and mature leaves demonstrated higher amplitudes of the L band, which can indicate better connectivity between the PSII units and more efficient energy transfer. Young and mature leaves also managed to keep their grouping strong during the recovery phase. Connectivity of PSII antennae had been reported as a protective mechanism against excess light (Živčák *et al.* 2014). Old leaves did not manage to keep high connectivity between their antenna complexes, which indicates greater sensitivity of their membranes. A weak initial reaction occurred in the old IT leaves on the first day of the treatment only. It seems they adapted very quickly and there were no significant differences compared to the control during the rest of the investigated period.

K band: The K band (ΔW_{OJ}) is an indicator for the operation of the PSII donor side and the stability of the oxygen-evolving complex (OEC) (Guissé *et al.* 1995, Strasser *et al.* 1996). We introduced the parameter W_{OJ} which gives almost the same information as the parameter F_K/F_J introduced by Srivastava *et al.* (1997). The correlation between these two parameters is presented in the Fig. 2S (*supplement*). Positive K band corresponds to slower electron donation thus revealing damage in the OEC and impaired balance between electron donation and further transfer of electrons to Q_A (Chernev *et al.* 2003, Kalaji *et al.* 2014b). OEC is very sensitive to temperature changes (Havaux 1993, Yamane *et al.* 1998). BG ecotype leaves showed negative K band during the first day of the treatment, which was more pronounced in old leaves, while young leaves experienced the same effect on the second day (Fig. 1*C*). On the last day of the temperature treatment (Day 3) and especially on the first day of the recovery period (Day 4) all of the BG samples showed a positive K band. The positive K band could also be a result of accelerated electron withdrawal from Q_A^- toward Q_B and following electron carriers (Zhang and Sharkey 2009), but according to ψ_{E0} values (Fig. 2), the electron flow was actually delayed; thus we concluded that the positive K band is mainly a result of impaired OEC. All BG leaves recovered during the last 2 d. IT leaves did not reduce their OEC activity during the whole experiment – there were no positive differences in K band values (Fig. 1*D*).

H band: The H band (ΔW_{JI}) gives information about reactions taking place further along the electron transport chain to the PQ molecules (Strasser and Tsimilli-Michael 1998, Strasser *et al.* 1999). A negative band corresponds to a slower reduction of the PQ pool in the stressed plants (compare to the control), and as a result – less reduced PQ pool at the maximum (Kalaji *et al.* 2017). Older leaves from both BG and IT ecotypes showed negative H bands. In the BG leaves, this effect is probably a result of reduced donor side capacity of the PSII reaction centers as a whole, as it is already shown by the positive K band. In IT samples, the negative H band is not accompanied by positive K band, thus it can be caused mostly by increased size of PQ pool. This assumption corresponds to the higher values of the JIP parameters N and ψ_{E0} (Fig. 2).

G band: The G band (ΔW_{IP}) gives information about the electron flow to the end electron acceptors of PSI (Strasser *et al.* 2004). BG leaves of all ages showed positive G bands, while in IT samples G bands were negative. The temperature treatment probably induced different alterations in the size of PSI end acceptor pool in BG and IT plant ecotypes during the latter stages of the photosynthetic light phase. They stayed modified until the 3rd d – the end of the temperature treatment period, and start to go back to the control levels in the recovery period but IT ecotype did not reach the control levels until the end of the experiment. Young and mature leaves recovered faster than old ones, especially for BG ecotype.

JIP-test analysis: Although Chl *a* fluorescence is a very small fraction of the dissipated energy from the photosynthetic apparatus, its contribution to the understanding of PSA structure and function is widely accepted (Strasser *et al.* 2004, Tsimilli-Michael and Strasser 2013). Illumination of dark-adapted samples with actinic light results in Chl *a* fluorescence rise first demonstrated by Kautsky (Kautsky and Hirsch 1931). When plotted on logarithmic time scale, the rise of the Chl *a* fluorescence reveals an induction curve with many phases, rich in information about the state of the system *in vivo* (Strasser and Govindjee 1992). The kinetics of the fluorescence rise from O (Fo) to P (F_M) shows two inflection points at about 2 ms and 30 ms, labelled as J and I steps respectively, giving the name of the JIP-test. The JIP-test is an approach to calculate important parameters, describing energy fluxes of absorption and trapping the light energy, quantum yields of primary photochemistry, efficiency of the electron transport between acceptors, 'performance indexes', and driving forces for each process and many others (Kalaji *et al.* 2014c, 2017). The most commonly used parameters, derived from OJIP curve, reflect conservative characteristics which do not change significantly over weak native stress situations. An important part of understanding the intrinsic mechanisms determining plant structure and functionality is a selection of the most adequate parameters to trace fine changes in the PSA electron transport chain. We selected nine fluorescent parameters (explained in Appendix), defined by Strasser and Strasser (1995) and Strasser and Tsimilli-Michael

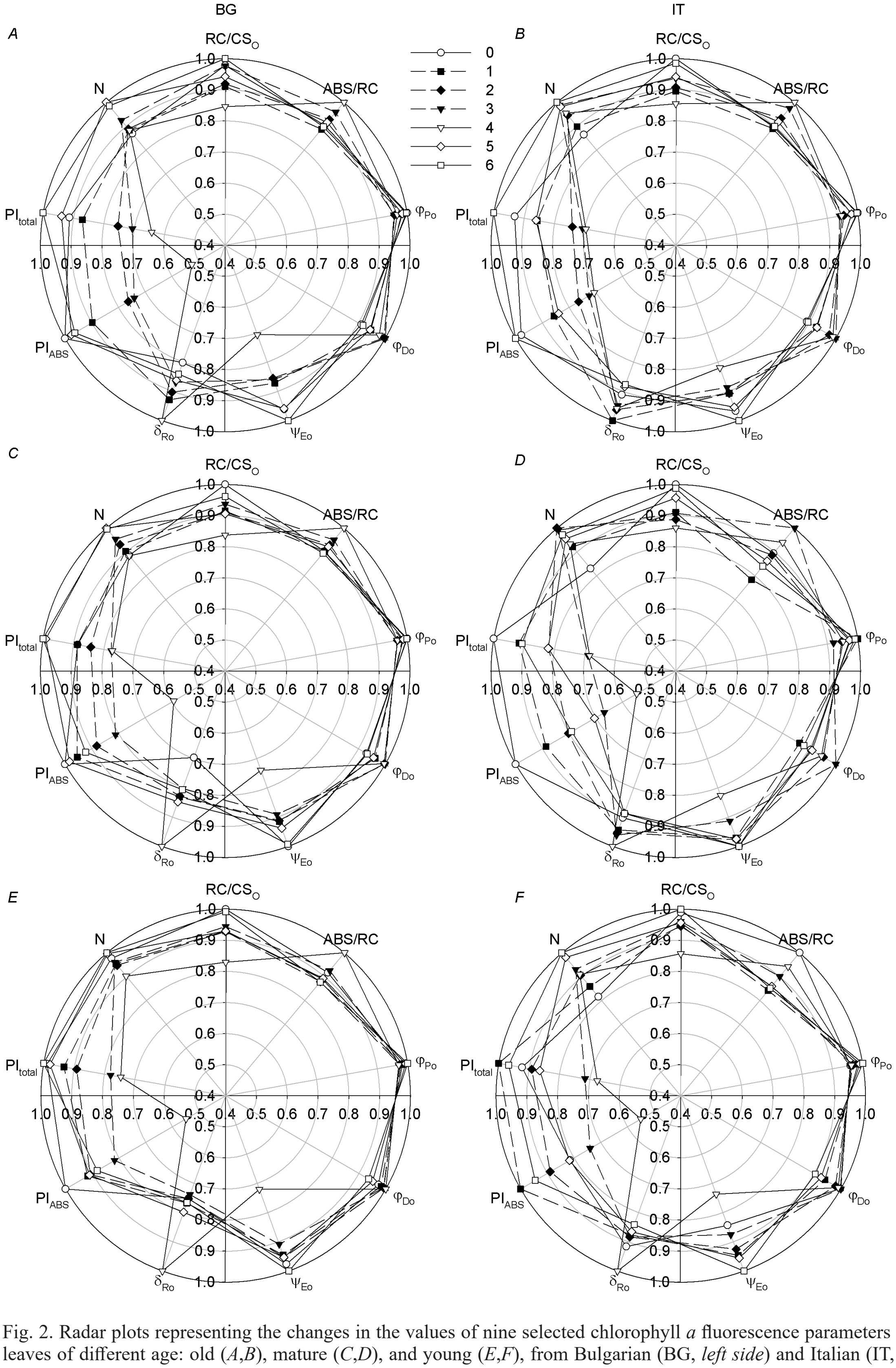

Fig. 2. Radar plots representing the changes in the values of nine selected chlorophyll *a* fluorescence parameters from the JIP-test for leaves of different age: old (*A*,*B*), mature (*C*,*D*), and young (*E*,*F*), from Bulgarian (BG, *left side*) and Italian (IT, *right side*) ecotypes *Platanus orientalis* during the experimental time: Day 0 – control day, Days 1–3 – days of the temperature treatment, Days 4–6 – recovery period. The fluorescence parameters are calculated according to The Energy Flux Theory (Tsimilli-Michael and Strasser 2013) and described in the Appendix.

(2001). Temperature-induced changes of these nine parameters in the two investigated ecotypes of *P. orientalis* are presented in radar plots (Fig. 2). For each day of the treatment the values of each parameter were normalized to the respective maximum.

The maximum quantum yield for primary photochemical reactions (φ_{P0}) is one of the most stable parameters which is hardly influenced by moderate stress (Bolhàr-Nordenkampf *et al.* 1989, Schreiber *et al.* 1995, Stirbet and Govindjee 2011). The moderately high temperature did not induce significant changes in φ_{P0} in both *P. orientalis* ecotypes.

The number of active RC per cross section (RC/CS_0) (Tsimilli-Michael and Strasser 2013, Kalaji *et al.* 2014a) was reduced by stress in mature and old leaves of both investigated plane ecotypes. This parameter was not affected in young leaves probably due to sink–source relations. The lowest values of this parameter were observed on the 4th d (first day of the recovery) for both ecotypes and were less pronounced in the leaves of IT plants. These changes correspond well with the higher values of ABS/RC measured on the same day. ABS/RC exhibited reciprocal dynamics probably because when the number of inactive RCs increases, as a compensation the absorption flux per active RCs is higher (Cao and Govindjee 1990, Yamane *et al.* 1997, Kalaji *et al.* 2016).

'Turnover number' N shows how many times Q_A is reduced from time 0 to t_{FM} (Tsimilli-Michael and Strasser 2013, Kalaji *et al.* 2017, Samborska *et al.* 2018). In most of the leaves from both ecotypes, N had higher values after the treatment which corresponds to bigger PQ pool. Donation of electrons in BG ecotype leaves was slightly slowed down, while IT plants (especially young leaves) appear to have accelerated electron flow from PSII to PSI. The biggest effect was noticed again on the 4th d, and it was only slightly reduced in the next days, especially in IT leaves, which tend to keep the effects. The acceleration of the electron flow in IT samples was also indicated by the rise in ψ_{E0} (especially in young leaves) while in the BG ecotype it was a bit lower than that of the control. Based on the observed δ_{R0} values, we speculate that it is possible that electron flow between the two photosystems and to the PSI end acceptors was slightly accelerated in all samples maybe due to the higher stability of PSI to temperature stress (Sane *et al.* 1984, Havaux *et al.* 1991, Blankenship 2014). Other possible contribution to the changes in δ_{Ro} values can be the increased fraction of damaged PSII donor sides.

Performance indexes are extremely sensitive to changes in plants physiological state (Živčák *et al.* 2008, Tsimilli-Michael and Strasser 2013). In all samples both PI_{total} and PI_{ABS} showed significant reduction in comparison to the control.

DF decay kinetics: Analysis of delayed fluorescence is one of the most informative tools for investigation of the PSA state. As Goltsev and Zaharieva (Goltsev *et al.* 2009b) point out in a very detailed review on the matter, although both PF and DF signals are emitted by the same molecules (Chl *a* molecules from the PSII antenna complexes), the mechanism for the formation of the excited states is different. While PF signal is generated by energy emission of Chls after direct absorption of light or fast energy transfer between antenna molecules, DF is a result of backward electron flow and charge recombination in the RC of PSII which leads to a new excitation of Chl *a* molecules (Zaharieva and Goltsev 2003, Goltsev *et al.* 2004, 2009b). Decay kinetics is presented as a decay curve of multiple overlapping components (Fig. 3S, *supplement*). Each component is a result of electron recombination in specific PSII state (Marković *et al.* 2001).

All decay curves can be presented as a three exponential function:

$$DF(t) = L_1 e^{-t/\tau_1} + L_2 e^{-t/\tau_2} + L_3 e^{-t/\tau_3} + C$$

The constant C introduces all slow components as well as the background electric signal (noise). L_1, L_2, and L_3 are proportional to the concentrations of the PSII states responsible for DF and their quantum yield. The characteristic lifetime for each component (τ_1, τ_2, and τ_3) decreases with $1/e$, and is inversely proportional to the rate constant of the reaction, leading to the disappearance of each state (Lavorel 1975, Goltsev *et al.* 2004, 2009b; Kalaji *et al.* 2014b).

Temperature stress induced changes in the characteristic life times τ_i of DF decay components for leaves of different age in both BG and IT plants are presented in Fig. 3.

τ_1: The 25–35 µs lifetime, τ_1 (Fig. 3*A*,*B*), is an indicator for the electron donation efficiency (Shuvalov and Litvin 1969, Jursinic 1986, Goltsev *et al.* 2009b). The dark decay of DF, emitted in this time region, is mostly a result of electron donation from Z to P_{680}^+ during the initial stages of the photosynthetic process (Goltsev and Zaharieva 2003). The changes in the rate of electron transfer between the catalytic metal cluster and P_{680}^+ could be influenced by long-range electrostatic interactions of the positively charged Y_Z^+ with protonated peripheral groups (most likely carboxylate sidechains) which result in deprotonation and proton release into the aqueous bulk. So, the DF kinetics depends on different factors affecting OEC, as temperature and luminal pH (Zaharieva and Dau 2019).

On the first day of the recovery (4th d of the experiment), τ_1 significantly decreased which indicates higher rates of the electron donation from OEC. This was very evenly pronounced in the leaves of all studied ages of the BG plants, and less expressed in IT ecotype, which had no significant changes in τ_1 during the first 3 d of the experiment. The observed effects could be a result of dynamic changes in the structure of the complexes. The changes in the L band of PF in BG plants are also a result of structural changes, which reveal increased connectivity of antenna complexes, as explained by Li *et al.* (2018). Higher connectivity leads to increased energy flow to the open RCs, *i.e.*, increased electron flow (Stirbet 2013). However, the K bands (presented in Fig. 1) indicated for delay in the electron donation site. This effect can be explained by presenting the entire process in two steps: $OEC \rightarrow Z \rightarrow P_{680}^+$. While the overall effect of the increased temperature was a delay in the donor side, the donation of

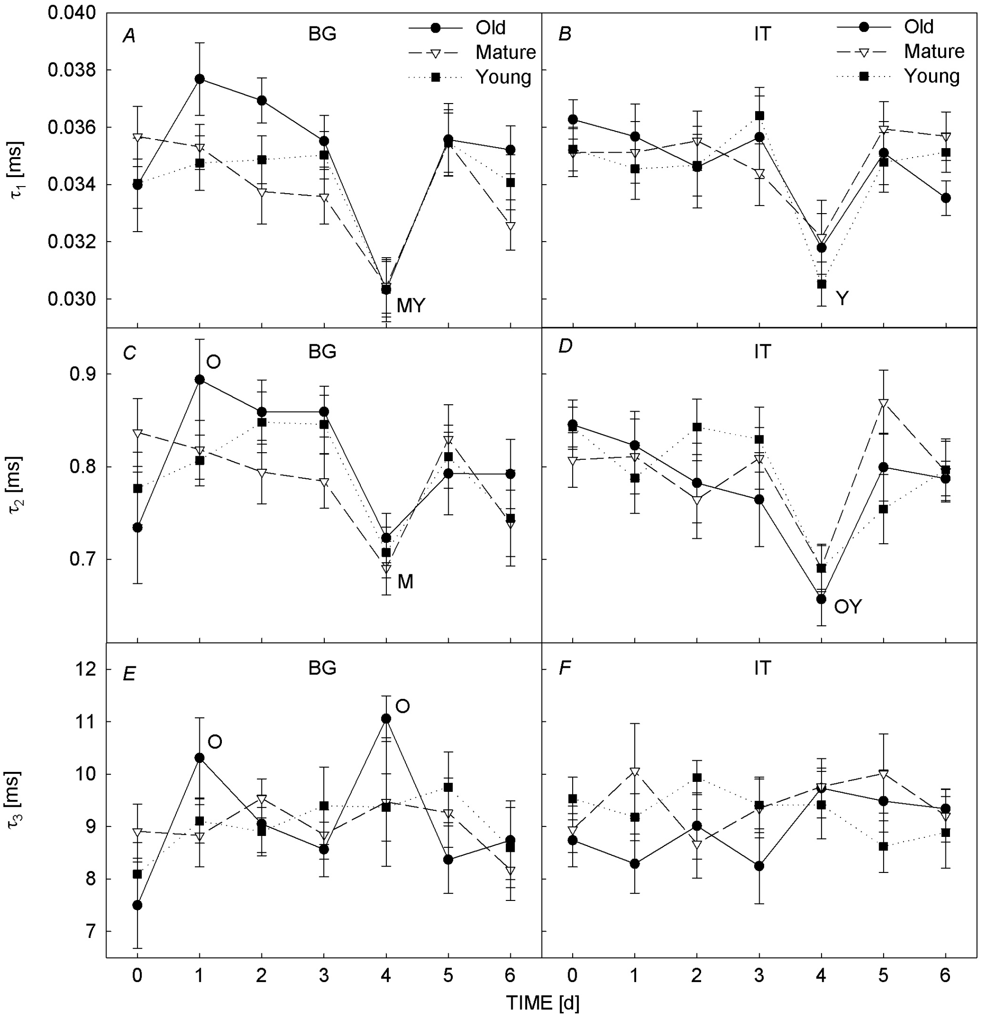

Fig. 3. Temperature induced changes in the characteristic lifetimes of DF decay components (PSII states emitting DF light with their disappearance) with different intensity in leaves of different age: young, mature, and old, from Bulgarian (BG, *left side*) and Italian (IT, *right side*) ecotypes of *Platanus orientalis* during the experiment: Day 0 – control day, Days 1–3 – days of the temperature treatment, Days 4–6 – recovery period. (*A*,*B*) τ_1 (25-35 μs lifetime), (*C*,*D*) τ_2 (sub-millisecond lifetime), and (*E*,*F*) τ_3 (millisecond lifetime). Lifetime values were obtained by nonlinear regression of the DF decay kinetic curves by a 7-parameter fitting equation, performed by *M-PEA Data Analyzer* software. The derivation of the different DF decay components from the initial DF decay curves is explained in Fig. 3S. Statistically significant differences (obtained with one-way *ANOVA*, *Holm-Sidak*'s test, α=0.05) between value at particular day and the control (at Day 0) are denoted with *letters*: O for the old, M for the mature, and Y for the young leaves.

electrons between Z and P_{680}^+ could be accelerated, so the recombination rate was reduced and τ_1 had lower values.

τ_2: The τ_2 lifetime (Fig. 3*C*,*D*) is related to the efficiency of the electron transport between Q_A^- and Q_B (Goltsev *et al.* 2009a, Oukarroum *et al.* 2013). It is strongly dependent on Q_B redox state, which has three reduction states: Q_B (no electron occupation), Q_B^- (semi-reduced, only one of the two electron sites is occupied), and $Q_B^=$ (fully reduced Q_B) (Goltsev *et al.* 1980, Lazár 1999). Since the measurement lasts for a very short period of time and the PAR is of high intensity, the number of Q_B and Q_B^- is very small in comparison to the number of $Q_B^=$. The initial state of the electron carriers in PSII acceptor side immediately after the illumination turning off is $Q_A^-Q_BH_2$. Small part of the PSII reaction centers will be with oxidized state of donors $Z^+P_{680}^+$. Thus the DF emitting state is ($Z^+P_{680}^+Q_A^-Q_BH_2$), and P_{680}* generated by recombination between P_{680}^+ and Q_A^- has a lifespan of about 100 μs (Haveman and Lavorel 1975). On the first day of the treatment, τ_2 rose mostly in old leaves probably due to structural changes, induced by elevated temperature, but on the 4th d a recovery was observed. IT leaves did not experience changes during the first days of the experiment, but there was also a decrease in τ_2 on day 4. The overall reaction was weaker in IT trees.

τ_3: The τ_3 lifetime (Fig. 3*E*,*F*) correlates with recombination mostly between $Q_B^=$ and S_3 state of OEC ($S_3^+ZP_{680}Q_AQ_B^=$) (Zaharieva and Goltsev 2003, Goltsev *et al.* 2005). These PSII states are relatively stable and decay in seconds, so their concentration did not change during the 1-s measurement, and it can be assumed as a constant. The intensity of the DF, however, is determined not only by the concentration of PSII light emitting states, but also by the energization state of the thylakoid membrane. DF decay in 10–20-ms time range is mainly a result of thylakoid membrane de-energization (Schreiber and Neubauer 1990). We did not observe significant changes in τ_3 in this experiment.

Modulated light reflection at 820 nm (MR820) provides information about the electron flow through PSI (Harbinson and Woodward 1987, Oukarroum *et al.* 2013). Due to comparatively slow rereduction of P_{700}^+ + PC^+, a pool of oxidized P_{700} + PC molecules is formed during illumination. The oxidized form does not absorb light at 700 nm but absorbs at 820 nm, which leads to disappearance of the absorption band at 700 nm and increased absorption (decreased reflection) at 820 nm (Oja *et al.* 2003, Goltsev *et al.* 2010). It is important to analyze the slopes of MR820 decay (rate of oxidation of P_{700} + PC) and rise (rate of rereduction of P_{700}^+ + PC^+ by reduced PQ pool) (Strasser *et al.* 2010). A detailed view of MR820 raw curves and the derivation of V_{OX} and V_{RED} is presented in Fig. 4S (*supplement*).

Rates of oxidation (V_{OX}) and rereduction (V_{RED}) of P_{700} + PC for both ecotypes of plane tree for the three ages are presented in Fig. 4. Both BG and IT plants showed small changes in the oxidation rates. The rate of rereduction was higher in BG samples, especially on the 4th d of the experiment. The higher values of δ_{R0} measured on this day, support this observation as an increase of this parameter indicates for accelerated electron flow between PSII and PSI thus affecting the balance between electron transport to P_{700} and withdrawal of electrons to the further carriers (Kalaji *et al.* 2014c). IT plants did not experience any temperature effect on P_{700} rereduction rates.

Principal component analysis: For further assessment of the results, we performed a PCA based on all collected data from the PF, DF, and MR820 signals for each individual leaf measurement in the process of temperature treatment. The results from PSA are presented in a two-dimensional plot, thus reducing the multidimensional data set into a plot with two uncorrelated variables – Principal component 1 (PC1) and Principal component 2 (PC2) (Fig. 5). This transformation is mathematically performed in a way that PC1 has the largest variance thus accounting for as much of the variability in the data as possible, and PC2 is the second most varying component (Dąbrowski *et al.* 2016, Zhou *et al.* 2016, Kalaji *et al.* 2018).

PCA plots of both BG and IT ecotypes *P. orientalis* showed similar pattern of grouping of the data in relation to temperature treatment with ellipsoids corresponding to 4 d of the experiment – 0 (control), 3 (3rd d of elevated temperatures), 4 (the 1st d of recovery), and 6 (3rd d of

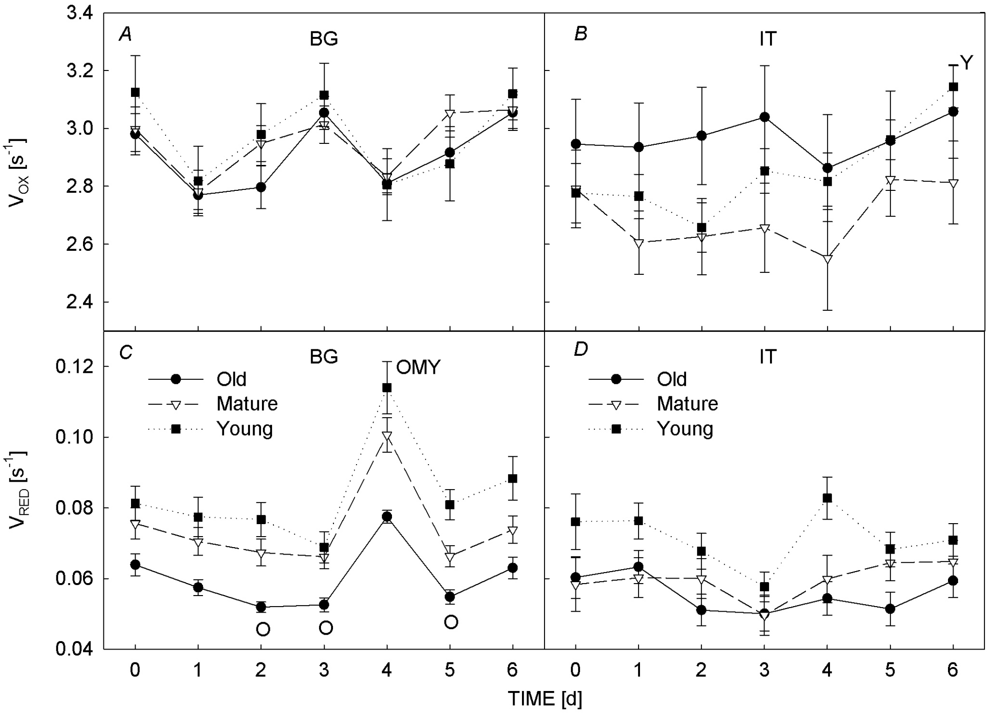

Fig. 4. Rates of oxidation (*A*,*B*) and rereduction (*C*,*D*) of P700 + PC, measured by MR820 (modulated light reflection at 820 nm) for leaves of different age: young, mature, and old, from Bulgarian (BG, *left side*) and Italian (IT, *right side*) ecotypes of *Platanus orientalis* during the experiment: Day 0 – control day, Days 1–3 – days of the temperature treatment, Days 4–6 – recovery period. The rates were calculated from MR820 curves as demonstrated in Fig. 4S. Statistically significant differences (obtained with one-way *ANOVA*, *Holm-Sidak*'s test, α=0.05) between the value at particular day and the control one (at Day 0) are denoted with *letters*: O for the old, M for the mature, and Y for the young leaf.

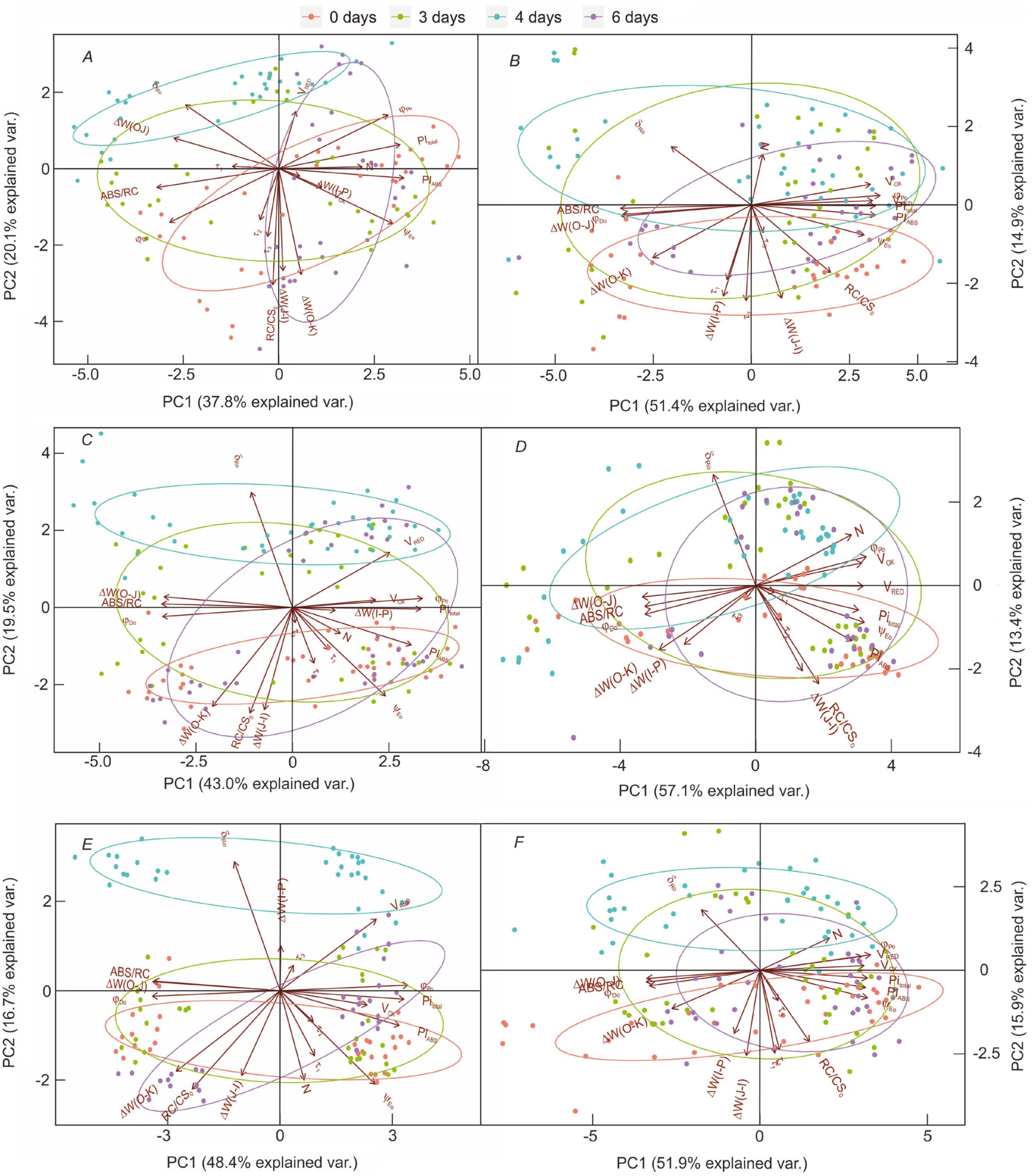

Fig. 5. Principal component analysis creating a 2D plot with two uncorrelated variables PC1 and PC2 for all the PF, DF and MR820 parameters presented so far in the article for leaves of different age: old (*A*,*B*), mature (*C*,*D*), and young (*E*,*F*), from Bulgarian (BG, *left side*) and Italian (IT, *right side*) ecotypes of *Platanus orientalis* for four days of the experiment: Day 0 – control day; Day 3 – 3rd d of the temperature treatment; Day 4 – 1st d of recovery; and Day 6 – 3rd d of recovery. PCA was carried out in *R* programming language and free software environment *prcomp* package for computation and *ggbiplot* for visualization.

recovery). This was expected as the plants of one species have similar behavior during moderate stress. Nevertheless, there are some significant differences between the two ecotypes in the volumes of the reactions. BG plants showed higher variance in the photosynthetic characteristics thus creating higher deviations between the studied groups. On the 3rd d of the treatment, the results showed big initial dispersion, while on the 4th d, most of the leaves were already affected. The stress reaction was reflected mostly by PC2. This was well pronounced for young leaves and less prominent in old and mature ones. The last two exhibited similar photosynthetic characteristics although mature leaves showed wider spread of variance along the *y*-axis (PC2) on the 6th d than that of old leaves. This indicates that some of the individuals were already fully recovered, and some remained altered. Young BG leaves did not express significant reaction on the 3rd d, but sharply changed their Chl fluorescence patterns on the 4th d and showed predominant recovery of most of the leaves on the 6th d. PC1 dispersion for control group corresponds to φ_{P0} variance, however, φ_{P0} was not significantly influenced by the elevated temperature. The stress reaction in mature and old leaves, presented by PC2, was revealed mostly by variance in the rates of rereduction of P_{700}, number of active RC per cross section, destabilization of OEC, and changes in the connectivity between antenna units. In the young leaves, the electron flow between PSII and PSI was also significantly influenced. PCA of the results from IT ecotype plants showed ellipsoids with much higher overlapping areas, indicating a less pronounced stress reaction, demonstrated by PC2.

Conclusion: Our results indicate that plane tree leaves responded to the temperature changes not only during the treatment but adaptive reactions continued to occur in the next days until the leaves adjusted their structures and functionality to the normal conditions. The maximum effect of elevated temperature on photosynthesis for both ecotypes was observed on the 4th d of the experiment, which was the first day of recovery. This might be a result of reorganization during the initial recovery phase of the same structures which have been affected by the temperature stress. During the following 2 d, IT plane trees recovered fully and even developed advantageous alterations, while BG ecotype leaves were more affected by the elevated temperature. Although the BG ecotype leaves also recovered during the last 2 d, they were not positively influenced by the stress. Effects were more pronounced in the old leaves. The young leaves showed rapid changes during the temperature stress, but also full recovery afterwards.

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Appendix. JIP parameters and their meaning. Minimum fluorescence, recorded in dark-adapted samples is labelled F_O. Maximum fluorescence is labelled as F_M and F_t is the fluorescence signal, recorded at time t after the beginning of illumination with PAR. Parameters, whose relative change during the temperature treatment was monitored and presented in Fig. 2, are in *grey table cells*.

JIP-test parameters	Meaning
ABS	Photon flux absorbed by PSII
TR	Part of ABS trapped by the active PSII, leading to Q_A reduction
$F_V = F_t - F_O$	Variable fluorescence
$V_t = (F_t - F_O)/(F_M - F_O)$	Relative PF at moment t
$M_0 = 4\ (F_{300\mu s} - F_{50\mu s})/(F_M - F_O)$	Maximal turnover rate of Q_A

$S_m = (A_m)/(F_M - F_O)$	Normalized total complementary area between the O-J-I-P transient and $F = F_M$ (reflecting multiple turnover Q_A reduction events)
$S_s = V_J/M_0$	Normalized total complementary area corresponding only to OJ phase (reflecting single turnover Q_A reduction events)
$N = S_m/S_s$	Turnover number; number of Q_A reduction events between time 0 and t_{FM}
$ABS/RC = M_0\ (1/V_J)(1/\varphi_{P0})$	Absorption flux per reaction center
$\varphi_{P0} = F_V/F_M = (F_M - F_O)/F_M = (1 - F_O/F_M)$	Maximum quantum yield of primary photochemistry which defines the probability of absorbed energy to reach and to be trapped in PSII RCs
$\varphi_{D0} = 1 - \varphi_{P0} = (F_O/F_M)$	Quantum yield (at t = 0) of energy dissipation
$\psi_{E0} = ET_0/TR_0 = (1 - V_J)$, where $V_J = (F_J - F_O)/(F_M - F_O)$	Probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-
$\delta_{R0} = RE_0/ET_0 = (1 - V_I)/(1 - V_J)$	Efficiency for an electron to move from reduced carriers between the two photosystems to the end acceptors of PSI
$RC/CS_0 = \varphi_{P0}\ (V_J/M_0)(ABS/CS_0)$	Number of active (Q_A-reducing) reaction centers per cross section
$PI_{ABS} = \frac{RC}{ABS} \times \frac{\varphi_{Po}}{1 - \varphi_{Po}} \times \frac{\psi_0}{1 - \psi_0}$ $= \frac{\gamma}{1 - \gamma} \times \frac{\varphi_{Po}}{1 - \varphi_{Po}} \times \frac{\psi_0}{1 - \psi_0}$	Performance index on absorption basis; indicates the PSII functional activity in relation to the absorbed energy
$PI_{total} = PI_{ABS} \times \frac{\delta_{Ro}}{1 - \delta_{Ro}}$	Total performance index, which corresponds to the activity of PSII, PSI and the energy flow between them