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Differential accumulation of photosynthetic proteins regulates diurnal photochemical adjustments of PSII in common fig (*Ficus carica* L.) leaves

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ABSTRACT

Molecular processes involved in photosystem II adaptation of woody species to diurnal changes in light and temperature conditions are still not well understood. Regarding this, here we investigated differences between young and mature leaves of common fig (*Ficus carica* L.) in photosynthetic performance as well as accumulation of the main photosynthetic proteins: light harvesting complex II, D1 protein and Rubisco large subunit. Investigated leaf types revealed different adjustment mechanisms to keep effective photosynthesis. Rather stable diurnal accumulation of light harvesting complex II in mature leaves enabled efficient excitation energy utilization (negative L-band) what triggered faster D1 protein degradation at high light. However, after photoinhibition, greater accumulation of D1 during the night enabled them faster recovery. So, the most photosynthetic parameters, as the maximum quantum yield for primary photochemistry, electron transport and overall photosynthetic efficiency in mature leaves successfully restored to their initial values at 1 a.m. Reduced connectivity of light harvesting complexes II to its reaction centers (positive L-band) in young leaves increased dissipation of excess light causing less pressure to D1 and its slower degradation. Decreased electron transport in young leaves, due to reduced transfer beyond primary acceptor Q_A^- most probably additionally induced degradation of Rubisco large subunit what consequently led to the stronger decrease of overall photosynthetic efficiency in young leaves at noon.

1. Introduction

The combination of increased irradiance and elevated temperature is among the most commonly experienced stresses under field conditions. In such conditions, plants mostly receive more sunlight than can be used for photosynthesis. Photosynthetic apparatus acclimation to diurnal changes in light and temperature conditions includes short-term adjustments to reduce the excess of excitation (Desotgiu et al., 2012). Photosystem II (PSII) was recognized for a long time as one of the major regulatory components of the photosynthetic apparatus. Effect of high light stress on PSII reaction centers (RCs) is often manifested as disturbed photosynthetic electron transport and reduced PSII efficiency known as photoinhibition (Sharma et al., 2015). When photoinhibition occurs, utilization of electrons by light-independent reactions is inadequate, excess excitation leads to plastoquinone (PQ) pool reduction and thus changes the accessibility of electron acceptors (Vass, 2012). Photoprotection and repair of photosynthetic apparatus after photoinhibition occurs at different stages of energy conversion. Light induced damage of PSII in low and moderate light conditions can be repaired fast enough to maintain photo-

Abbreviations: Chl, chlorophyll; DTT, dithiothreitol; ECL, enhanced chemoluminescence; HRP, horseradish peroxidase; LHCII, light harvesting complex II; ML, mature leaves; PEA, plant efficiency analyzer; PPFD, photosynthetic photon flux density; PQ, plastoquinone; PSII, photosystem II; RC, reaction center; Rubisco LSU, 1,5-bisphosphate carboxylase/oxygenase large subunit; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; YL, young leaves.

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synthetic activity. Under exposure to high irradiation, extent of photodamage exceeds repair processes (Minagawa and Takahashi, 2004; Tikkanen and Aro, 2012). However, the damage of PSII is generally directed to a RC core subunit, the D1 protein. The D1 is involved in primary charge separation and its Q_B-binding site is crucial for electron transport through PSII (Tomek et al., 2003; Faraloni and Torzillo, 2010). When exposed to light, D1 protein is always subjected to photodamage thus leading to photoinhibition (Aro et al., 1993; Tikkanen and Aro, 2012). Rapid degradation of damaged protein and incorporation of newly synthesized one into PSII is an important step in recovering from photoinhibition. However, light harvesting chlorophyll *a/b* complex (LHCII) of PSII antennae are involved in light absorption processes and in transferring excitation energy to RC. In the condition of excess light, LHCII proteins participate in photoprotection by catalyzing thermal energy dissipation (Ballottari et al., 2012). Both, LHCII and D1 protein content gradually increase during the leaf development (Aro et al., 1993; Takeuchi et al., 2002). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the crucial enzyme of the Calvin cycle responsible for carbon fixation. Young leaves possess lower levels of this enzyme than mature ones. The increase of photosynthetic activity during leaf development consequently rises Rubisco levels (Premkumar et al., 2001; Maayan et al., 2008; Lepeduš et al., 2011). Rubisco accumulation is usually at the highest levels in the morning (Schaffer et al., 2001). However, the increase of light intensity and temperature stimulate its degradation (Desimone et al., 1996; Hrstka et al., 2007).

Photosynthetic capacity and the composition of photosynthetic pigments is closely related to leaf age. Young leaves generally show lower amounts of photosynthetic pigments and lower efficiency of PSII (Jiang et al., 2006; Lepeduš et al., 2011). In most cases, young leaves have incompletely developed PSII what may limit light harvesting and electron transport processes consequently causing photoinhibition (Cai et al., 2005; Drozak and Romanowska, 2006; Jiang et al., 2006). Growing on top of branches all over the canopy, young leaves develop various adaptations to cope with harsh environmental conditions. Red coloration originating from anthocyanins (Manetas et al., 2002; Liakopoulos et al., 2006) and a higher pool of xanthophyll cycle components in young leaves usually have a photoprotective function (Krause et al., 1995; Thiele et al., 1996). However, the way how lightly green leaves exposed to excess light develop different mechanisms to protect vulnerable photosynthetic apparatus remains to be elucidated.

Therefore, measurement of chlorophyll *a* fluorescence can give the information about the tolerance to light and temperature stress as well as on the utilization of light in photosynthetic apparatus (Müller et al., 2001; Strasser et al., 2004). Under natural conditions, typical diurnal course pattern of photosynthetic performance with two peaks exists. One is in the late morning and another in the late afternoon with the midday depression around noon. Although midday depression is mostly influenced by strong irradiation, photoinhibition is usually intensified when increased irradiance is combined with elevated temperature or some other environmental factor (Guo et al., 2009; Panda, 2011).

Common fig (*Ficus carica* L.) is widely cultivated, Mediterranean deciduous tree, characterized by remarkable vegetative growth and well adapted to different environmental factors and climates. Fig leaf development begins in early spring, and production of young leaves continues until midsummer. The lifespan of common fig leaves is from six to three months depending on sprouting time. Generally, in woody plants young leaves initiate inside the canopy if they are formed in the middle of the summer and are shaded with mature leaves. Contrary, young fig leaves are developing at the top of the branches during the whole summer. Therefore, both leaf types are directly exposed to high irradiation and increased temperature. In this work, we aimed to investigate short-term defense strategies in two distinct developmental fig leaf stages (young and mature) under field conditions and to follow responses of PSII to diurnal changes in light and temperature conditions. It is well known that stressful conditions provoke effective strategies for photoprotection and repair of the photosynthetic apparatus that could be seen at different levels of light conversion along electron transport chain (Melis, 1999; Živcak et al., 2014). Our previous research (Mlinarić et al., 2016) revealed that young fig leaves have fully functional PSII apparatus and that downregulation of photosynthetic activity at midday was an efficient photoprotective strategy that enabled them to cope with unfavorable environmental conditions. Mature leaves, on the other hand, continuously maintained fully functional photosynthetic efficiency at the level of primary PSII photochemistry during the midday. Based on that, we hypothesized that photochemical adaptation of PSII in young and mature leaves depends on their differential diurnal accumulation of the main photosynthetic proteins (D1, LHCII and Rubisco LSU).

To investigate these adaptations, we measured chlorophylls *a* and *b* content, *in vivo* chlorophyll *a* fast fluorescence transients and accumulation of LHCII, D1 protein and Rubisco LSU, during the day course.

2. Materials and methods

2.1. Plant material

Common fig (*Ficus carica* L.) trees were sampled in Osijek, Croatia (45°33′29.4″S, 18°43′2.7″I) during June 2011. The investigation was performed on fig trees of the same clone to exclude possible variations due different genotype growing on the same soil type plot. Two types of leaves were used: young (YL, 5–6 cm long, ~7 days old) and mature (ML, 20–25 cm long, ~30 days old). Each type of leaves was sampled four times during the day course, at 1 a.m., 7 a.m., 1 p.m. and 7 p.m. Each leaf type sample consisted of five randomly selected leaves making the composite sample. After removal of main veins, leaf tissue was macerated into a fine powder in liquid nitrogen.

For measurements of light intensity and atmospheric temperature in the field (Table 1), Quantitherm QRT1 light meter (Hansatech, UK) sensor was used. It can accurately measure across the normal measuring temperature (10–40 °C) and photosynthetic active radiation range (0–5000 $\mu mol\ m^{-2}\ s^{-1}$ as well as extreme saturating light intensities up to 50 000 $\mu mol\ m^{-2}\ s^{-1}$). The sensor was placed on the surface of every leaf used for analyses. Measurements of light intensity and temperature around entire canopy did not differ between positions of sampled YL and ML.

2.1.1. Photosynthetic pigment determination

Powdered plant material was extracted with ice-cold absolute acetone and then re-extracted several times until it was completely uncolored. The concentrations of chlorophylls (Chl *a* and Chl *b*) were determined spectrophotometrically (Specord 40, Analytik Jena, Germany) according to Lichtenthaler (1987).

2.1.2. Fast chlorophyll a fluorescence kinetics

Diurnal changes in photosynthetic activity were measured every 6 h during 24 h, on ten randomly selected leaves of each

Table 1
Diurnal changes of photosynthetic photon flux density (PPFD; $\mu mol\ m^{-2}\ s^{-1}$) and atmospheric temperature (°C) measured on the surface of each leaf used for analyses (n = 30).

	1 a.m.	7 a.m.	1 p.m.	7 p.m.
Temperature (°C)	17.5 ± 0	17 ± 1	35 ± 2	26 ± 0
PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	0	150 ± 20	1300 ± 100	150 ± 20

type using Handy-PEA fluorimeter (Hansatech, UK). All measurements were performed on dark-adapted leaves in the field. After 30 min of dark adaptation, the leaves were exposed to a pulse of saturating red light (3200 μmol m^{-2} s^{-1}, peak at 650 nm). The OJIP transients were measured by recording data from 50 μs to 1 s that were used by JIP-test to calculate flux ratios (TR_0/ABS, ET_0/TR_0 and ET_0/ABS), energy distribution through PSII per reaction centers (ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC) as well as performance index (PI_{ABS}) together with three related parameters (RC/ABS, TR_0/DI_0 and $ET_0/(TR_0\text{-}ET_0)$) (Strasser et al., 2000). Calculations are shown in Table S1, Supplementary infromation.

The OJIP transients was double normalized between O (50 μs) and P steps and presented as relative variable fluorescence $W_{OP}=(F_t\text{-}F_0)/(F_P\text{-}F_0)$. Fluorescence data were plotted on the logarithmic time scale, and the O, J, I and P steps were marked in plots. Normalization between O and K (300 μs) steps revealed L-band (150 μs) and presented as variable fluorescence $W_{OK}=(F_t\text{-}F_0)/(F_K\text{-}F_0)$ and plotted with difference kinetics $\Delta W_{OK}=W_{OK}-(W_{OK})_{ref}$. Normalization between O and J (2 ms) steps revealed K-band, presented as variable fluorescence $W_{OJ}=(F_t-F_0)/(F_J-F_0)$ and plotted with difference kinetics $\Delta W_{OJ}=W_{OJ}-(W_{OJ})_{ref}$. Values measured at 1 a.m. for YL and ML were used as referent value $(W_{OK})_{ref}$ (Strasser et al., 2004; Yusuf et al., 2010).

2.1.3. SDS-PAGE and immunodetection

The SDS-PAGE was made according to (Laemmli, 1970). Plant material was extracted with hot (80 °C) sodium dodecyl sulfate (SDS) buffer (0.13 M Tris/HCl (pH = 6.8), 4.6% SDS, 16% glycerol and 0.01 M DTT) for 10 min at 80 °C (Lepeduš et al., 2005). Protein content was determined using the bovine serum albumin as a standard (Bradford, 1976). The volume of each loaded homogenate was estimated to contain 20 μg of total cell proteins. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Towbin et al., 1979). Membranes were incubated with primary antibodies against D1 (dilution 1:5000, Agrisera), LHCII (dilution 1:1500, polyclonal antibody that recognizes several proteins of LHCII complex, obtained from prof. Karin Krupinska, Kiel, Germany) and Rubisco LSU (dilution 1:5000, Agrisera) and then with HRP anti-rabbit IgG secondary antibody (Santa Cruz, dilution 1:10000). Membranes were incubated with Lumi-Light Western Blotting substrate (Roche). SDS-PAGE and immunodetection was repeated three times. Protein bands were detected on ECL films (AGFA) with standard procedure and quantified using Kodak 1D Image analysis.

2.1.4. Data analysis

Statistical differences between measurements in YL and ML fig leaves during day course were analyzed using one-way analysis of variance (ANOVA) followed by *post-hoc* Fisher's Least Significant Difference (LSD) test. Results are presented as means ± standard deviation of five replicates for pigment analysis and ten replicates for fluorescence measurements. Differences were considered significant at $p < 0.05$. For statistical analyses, Statistica 8.0 software (StatSoft, Inc. 2007) was used.

3. Results

Fluorescence transients (Fig. 1) in young (YL) and mature (ML) fig leaves were measured during the day course. For both leaf types, transients measured at 1 p.m. showed the biggest deviation compared to the ones measured at 1 a.m. (Fig. 1A and B). O-P normalized curves in both, YL and ML (Fig. 1C and D) revealed the most deviated shapes at 1 p.m. compared to measurements at 1 a.m. Initial fluorescence rise was speeded up from O to J in all normalized curves for ML (Fig. 1D) compared to YL (Fig. 1C). The J step in YL showed the highest increase at 1 p.m. while the I step decreased compared to 1 a.m. In ML, I step revealed the most intense decrease measured at 1 p.m.

Concentrations of Chl *a* and Chl *b* (Table 2) were significantly higher in ML than in YL during the day course. Concentrations of Chl *a* and Chl *b* in YL increased only at 1 p.m. with respect to night values (1 a.m.). ML revealed a significant increase in Chl *a* concentration during day course compared to 1 a.m. while the Chl *b* values showed diurnal variations with a significant increase at 7 a.m. followed by a decrease at 1 p.m.

Fluorescence intensity at 50 μs (F_0) (Table 2) showed highest values at 1 p.m. in both leaf types. Maximal fluorescence intensity (F_m) showed the lowest values at 1 p.m. in both leaf types. Variable fluorescence at 2 μs (V_J) in YL started to increase at 7 a.m. and reached a maximum value at 1 p.m. and then, at 7 p.m. decreased to the values measured at 1 a.m. Oppositely, the variable fluorescence at 30 μs (V_I) in YL showed no significant changes during the day course. While, ML revealed lower values at 1 p.m. compared to 1 a.m. for both parameters.

Maximum quantum yield (TR_0/ABS) in both leaf types significantly decreased at 1 p.m. compared to 1 a.m. The probability that absorbed photon moves an electron further than Q_A^- (ET_0/ABS) in both YL and ML showed a substantial decrease at 7 a.m. and 1 p.m. compared to values measured at 1 a.m. At 7 p.m., ET_0/ABS values started to increase. The probability that trapped exciton moves an electron further than Q_A^- (ET_0/TR_0) revealed substantial decrease at 7 a.m. and 1 p.m. in both, YL and ML leaves compared to 1 a.m. Both leaf types showed full recovery of this parameter to the levels measured at 1 a.m.

There was the apparent difference in the L-band between YL (Fig. 2A) and ML (Fig. 2B). YL revealed positive and ML negative trend. Both leaf types showed the highest amplitude of curves measured at 1 p.m. compared to 1 a.m. The K-band showed for both leaf types positive curves, as well as higher amplitude measured at 1 p.m. compared to 1 a.m. (Fig. 2C and D).

Absorption (ABS/RC, Fig. 3A), trapping (TR_0/RC, Fig. 3B) and dissipation (DI_0/RC, Fig. 3D) in both leaf types increased significantly at 1 p.m. compared to 1 a.m. The highest increase was measured in ML for TR_0/RC and in YL for DI_0/RC at 1 p.m. Electron transport (ET_0/RC, Fig. 3C) showed a substantial decrease in YL at 7 a.m. and 1 p.m. compared to values at 1 a.m., while ML revealed no significant difference.

YL showed significantly lower values for performance index (PI_{ABS}, Fig. 4A) and its components, the density of reaction centers on chlorophyll *a* basis (RC/ABS, Fig. 4B), flux ratio trapping per dissipation, (TR_0/DI_0, Fig. 4C) and electron transport beyond Q_A^- ($ET_0/(TR_0\text{-}ET_0)$, Fig. 4D) compared to ML during the day. However, the PI_{ABS} in both leaf types showed a substantial decrease at 7 a.m. and at 1 p.m. compared to 1 a.m. At 7 p.m. PI_{ABS} values significantly increased compared to 1 a.m. Three accompanying parameters showed in both leaf types similar pattern. The exception was decreased $ET_0/(TR_0\text{-}ET_0)$ (Fig. 4D) in ML at 7 a.m. compared to 1 a.m. At 1 p.m. the value of this parameter remained at the same level as at 7 a.m.

The LHCII abundance (Fig. 5A) in YL at 1 a.m. was about 40% lower compared to ML. There were no apparent changes in relative abundance of LHCII protein during the day course in ML, while YL showed an obvious increase at 7 a.m. and 1 p.m. followed with a decrease at 7 p.m. The abundance of D1 protein (Fig. 5B) at 1 a.m. was about 60% lower in YL compared to ML. Both leaf types showed an obviously higher accumulation of D1 protein at 7 a.m. compared to 1 a.m. followed by a gradual decrease in accumulation at 1 p.m. and 7 p.m. The abundance of Rubisco LSU (Fig. 5C) in YL at 1 a.m. was about 54% lower compared to ML. ML showed stable accumulation of Rubisco LSU during the day course while, YL revealed substantial increase at 7 a.m. and after that gradual decrease at 1 p.m. and 7 p.m.

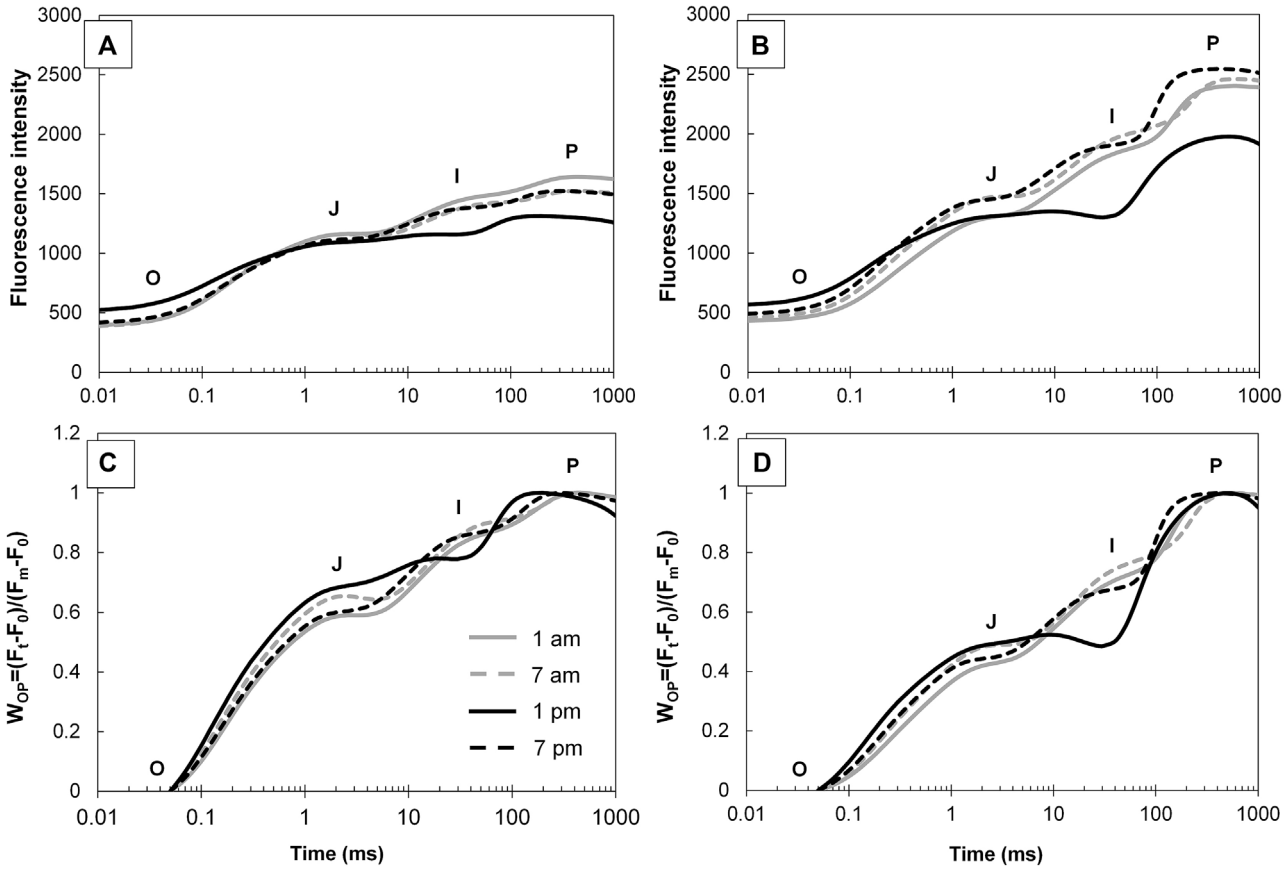

Fig. 1. Chlorophyll *a* fluorescence kinetics (OJIP transients) of dark adapted young (A and C) and mature (B and D) leaves of common fig. The O, J, I and P steps are marked in both, normal, direct transients (A and B) as well as in O-P normalized transients ($W_{OP}=(F_t-F_0)/(F_P-F_0)$, C and D). Each curve represents average kinetics of ten replicates.

Table 2
Diurnal changes in concentrations of photosynthetic pigments as well as chlorophyll *a* fluorescence parameters and flux ratios measured in young (YL) and mature (ML) leaves of common fig (*Ficus carica* L.). Results are presented as the mean of ten independent measurements ±SD. Different letters represent significant difference at p(F) < 0.05 (ANOVA LSD).

		1 a.m.	7 a.m.	1 p.m.	7 p.m.
Chl *a*	YL	0.517 ± 0.039 **e**	0.529 ± 0.049 **e**	0.793 ± 0.026 **d**	0.669 ± 0.024 **de**
	ML	1.103 ± 0.046 **c**	1.806 ± 0.147 **a**	1.329 ± 0.142 **b**	1.509 ± 0.254 **b**
Chl *b*	YL	0.233 ± 0.037 **e**	0.220 ± 0.024 **e**	0.361 ± 0.017 **d**	0.271 ± 0.007 **e**
	ML	1.029 ± 0.035 **b**	1.139 ± 0.035 **a**	1.087 ± 0.034 **b**	0.824 ± 0.075 **c**
F_0	YL	365.82 ± 30.19 **d**	364.24 ± 17.73 **d**	543.36 ± 33.14 **a**	400.31 ± 32.09 **c**
	ML	398.73 ± 14.17 **c**	413.64 ± 24.48 **c**	553.50 ± 54.92 **a**	444.29 ± 18.71 **b**
F_m	YL	1642.98 ± 175.39 **c**	1524.37 ± 122.85 **c**	1312.82 ± 126.04 **d**	1523.95 ± 121.76 **c**
	ML	2401.58 ± 123.79 **a**	2459.85 ± 192.59 **a**	1972.00 ± 207.42 **b**	2544.34 ± 144.16 **a**
V_J	YL	0.585 ± 0.023 **c**	0.651 ± 0.024 **b**	0.682 ± 0.021 **a**	0.599 ± 0.025 **c**
	ML	0.419 ± 0.019 **e**	0.482 ± 0.029 **d**	0.487 ± 0.081 **d**	0.443 ± 0.019 **e**
V_I	YL	0.825 ± 0.014 **ab**	0.868 ± 0.009 **a**	0.779 ± 0.027 **b**	0.852 ± 0.020 **a**
	ML	0.685 ± 0.017 **cd**	0.721 ± 0.022 **c**	0.485 ± 0.114 **e**	0.670 ± 0.016 **d**
TR_0/ABS	YL	0.777 ± 0.008 **b**	0.759 ± 0.024 **b**	0.566 ± 0.038 **d**	0.737 ± 0.013 **c**
	ML	0.835 ± 0.010 **a**	0.832 ± 0.006 **a**	0.715 ± 0.057 **c**	0.825 ± 0.006 **a**
ET_0/ABS	YL	0.293 ± 0.020 **e**	0.238 ± 0.021 **g**	0.153 ± 0.020 **h**	0.268 ± 0.021 **f**
	ML	0.460 ± 0.017 **a**	0.407 ± 0.024 **c**	0.341 ± 0.058 **d**	0.431 ± 0.019 **b**
ET_0/TR_0	YL	0.377 ± 0.023 **c**	0.313 ± 0.024 **d**	0.269 ± 0.021 **e**	0.363 ± 0.025 **c**
	ML	0.552 ± 0.019 **a**	0.490 ± 0.029 **b**	0.478 ± 0.081 **b**	0.522 ± 0.019 **a**

Chl *a* – chlorophyll *a* (mg/g FW), Chl *b* – chlorophyll *b* (mg/g FW), F_0 – fluorescence intensity at 50 μs, F_m – maximal fluorescence intensity, V_I – variable fluorescence at 2 μs – I step, V_J – variable fluorescence at 30 μs – J step, TR_0/ABS – maximum quantum yield, ET_0/TR_0 – probability that trapped exciton moves an electron further than Q_A^- and ET_0/ABS – probability that absorbed photon moves an electron further than Q_A^-.

4. Discussion

In vivo chlorophyll *a* fluorescence measurements were used to determine the efficiency of the photosynthetic apparatus in young and mature leaves, with special reference to PSII photochemistry. The OJIP transients reflect the successive reduction of the electron acceptor pool of PSII (Strasser et al., 2004). In normal physiological conditions, fluorescence transient goes from initial O (F_0) to maximal P (F_m) fluorescence intensity and contains two intermediate steps, J step at 2 ms and I step at 30 ms (Strauss et al., 2006). The O-J reveals additional information, namely L (at about 150 μs) and K-band (at about 300 μs) (Yusuf et al., 2010). The shape of the

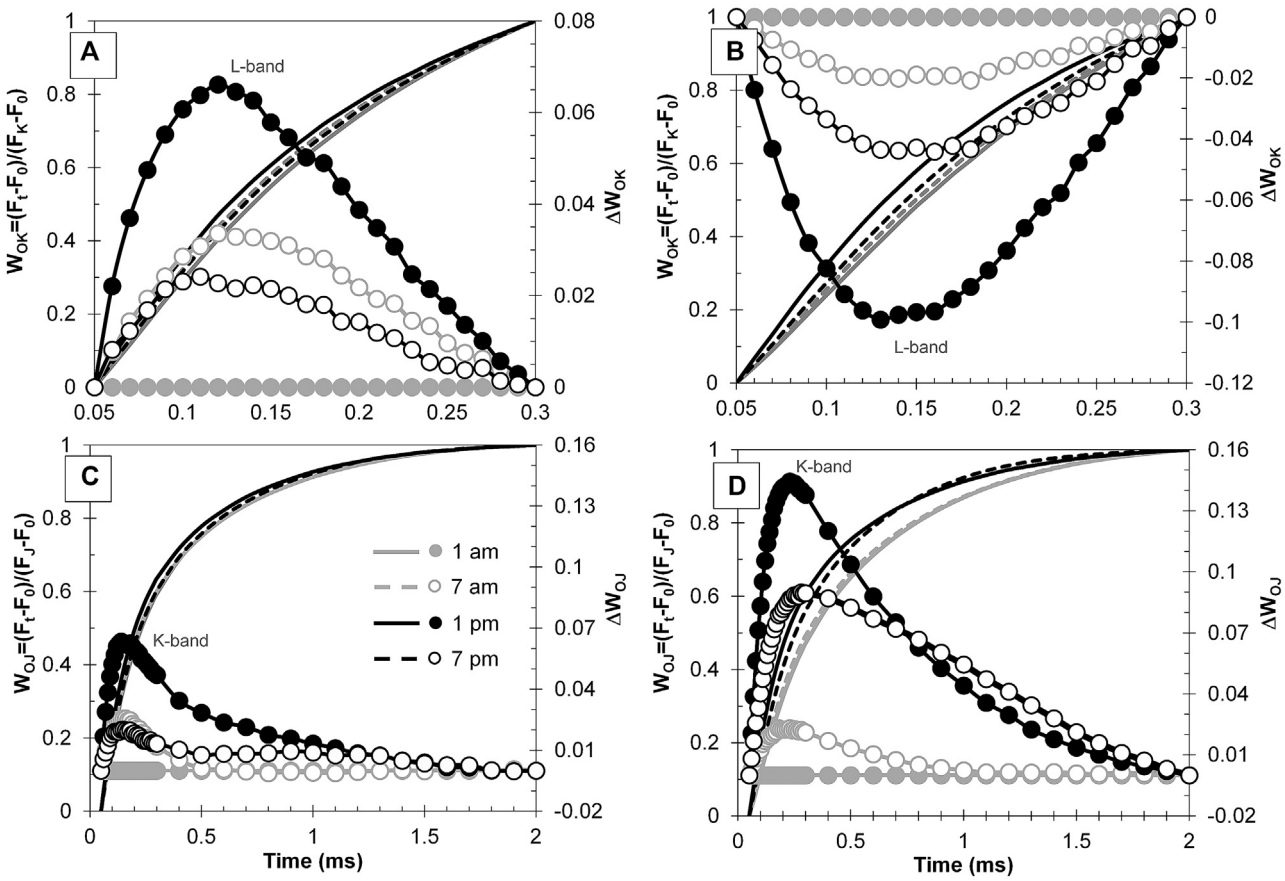

Fig. 2. Changes in the shape of the chlorophyll *a* fluorescence transient curves in young (A and C) and mature (B and D) leaves of common fig. Fluorescence data were normalized between O and K steps (L-band; panels A and B) and expressed as variable fluorescence $W_{OK}=(F_t-F_0)/(F_K-F_0)$. Fluorescence data were plotted with difference kinetics $\Delta W_{OK}=W_{OK}-(W_{OK})_{ref}$ in the 50–300 μs time range. Fluorescence data normalized between O and J steps (K-band; panels C and D) were expressed as variable fluorescence $W_{OJ}=(F_t-F_0)/(F_J-F_0)$ and plotted with difference kinetics $\Delta W_{OJ}=W_{OJ}-(W_{OJ})_{ref}$ in the 50 μs–2 ms time range. Values measured at 1 a.m. for YL and ML were used as referent value $(W_{OK})_{ref}$. Each curve represents average kinetics of ten replicates. Lines (solid and dashed) refer to difference kinetics (ΔW) curves, while solid lines with marks (open and closed) refer to variable kinetics (W) curves.

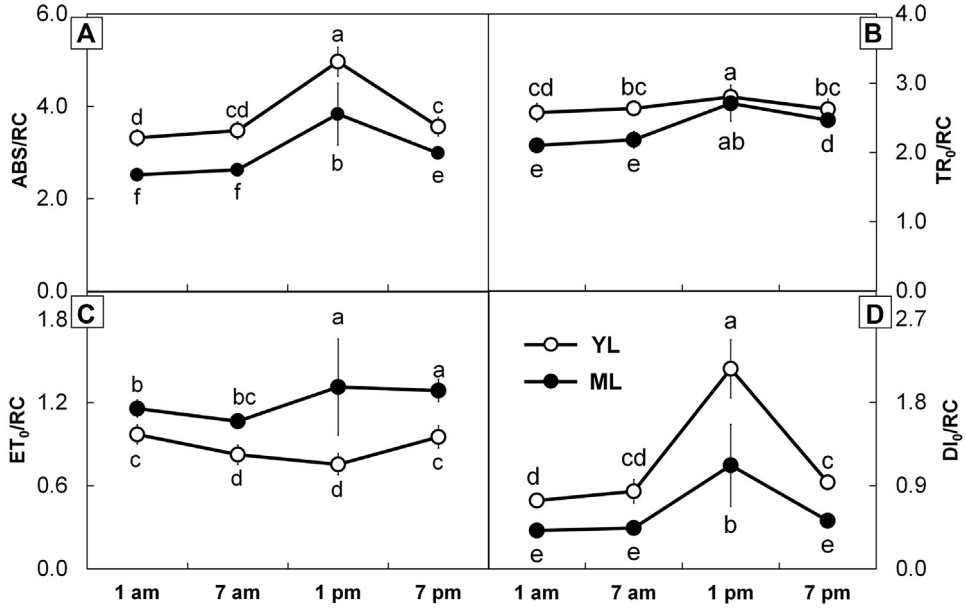

Fig. 3. Specific energy fluxes (absorption (ABS/RC; panel A), trapping (TR_0/RC; panel B), electron transport (ET_0/RC; panel C) and dissipation (DI_0/RC; panel D) per active reaction center) measured in young (YL) and mature (ML) leaves of common fig. Results are presented as the mean of ten independent measurements ±SD. Different letters represent significant difference at $p(F) < 0.05$ (ANOVA LSD).

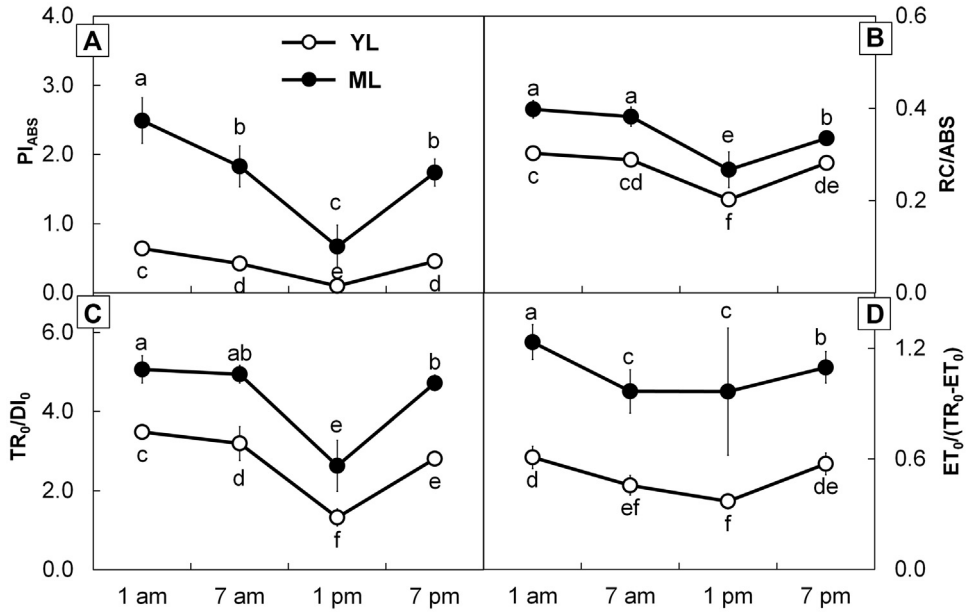

Fig. 4. Performance index (PI_{ABS}; panel A) and its components: RC/ABS (density of reaction centers on chlorophyll *a* basis; panel B), TR_0/DI_0 (flux ration trapping per dissipation; panel C) and $ET_0/(TR_0\text{-}ET_0)$ (electron transport beyond Q_A^-; panel D) measured in young (YL) and mature (ML) leaves of common fig. Results are presented as the mean of ten independent measurements ± SD. Different letters represent significant difference at p(F) < 0.05 (ANOVA LSD).

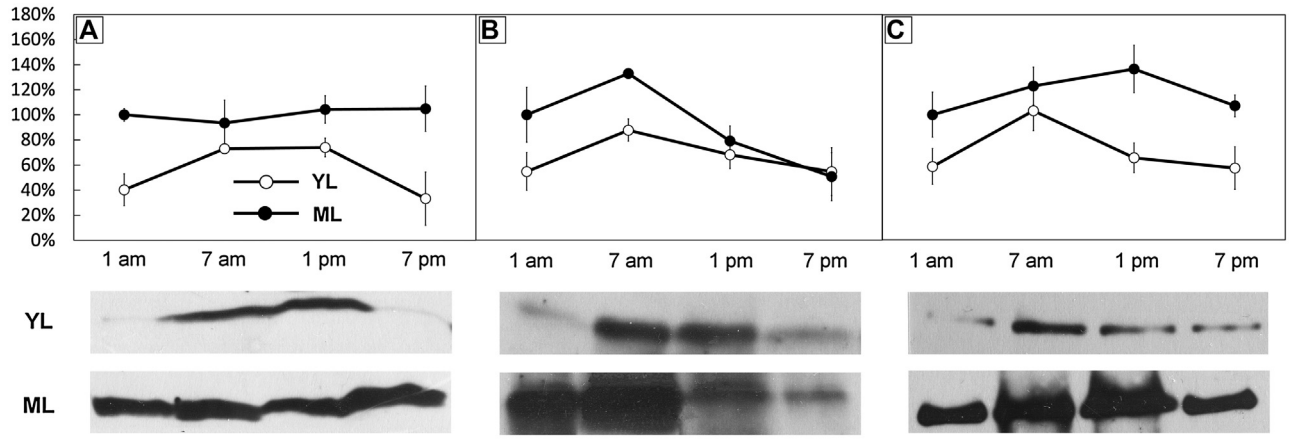

Fig. 5. Diurnal changes in relative abundance of LHCII (A), D1 (B) and Rubisco LSU (C) proteins in young (YL) and mature leaves (ML) of common fig. Upper graphics show average relative band density of three replicates ± standard error of the mean. Values are displayed as % relative to values measured at 1 a.m. (100%) in ML. Photographs show representative immunoblots of investigated photosynthetic proteins in YL and ML. Each loaded homogenate contained 20 μg of total cell proteins.

OJIP curve is very sensitive to environmental stress (Strasser et al., 2004), such as daily changes of light intensity and temperature. As young fig leaves are developing at the top of the branches, both leaf types were exposed to high irradiation and increased temperature. Despite that, young leaves are capable of enduring such daily variations by downregulating photosynthetic activity. However, to ensure competent photosynthesis, young and mature fig leaves activated different protection mechanisms. While young leaves displayed adequate antioxidative system and downregulated photosynthetic activity accompanied with sustained degradation of Rubisco LSU, mature leaves were able to maintain functional PSII activity and steady level of Rubisco LSU at the expense of efficient antioxidative system (Mlinarić et al., 2016). Measuring OJIP transients and parameters during the day course in young and mature fig leaves gave detailed insight into photosynthetic apparatus functioning. OJIP transients in both YL and ML, in particular at 1 p.m. (Figs. 1A and 1B), revealed alterations in the characteristic shapes of the transients due to changes in light intensity and temperature during the day. Although YL showed lower values of F_0 (O step) when compared to ML, both leaf types showed significantly higher F_0 values measured at 1 p.m. what indicated slight instability of photosynthetic apparatus due to increased amount of unconnected chlorophylls to the RC of PSII (Gilmore et al., 1996). Further, F_m values (P step) measured in YL were lower compared to ML. At 1 p.m., both leaf types revealed a significant decrease of F_m values what indicated inactivation of D1 protein (Perales-Vela et al., 2016).

Additional analyses of OJIP transients revealed detailed differences in photosynthetic apparatus functioning between YL and ML. O-P normalized curves in YL and ML revealed different shapes measured at 1 p.m.(Figs. 1C and 1D). In both, YL and ML significant increase at J step (V_J) were detected. Variable fluorescence at J step (V_J), stands for the part of PSII RCs that close during single charge separation (Strasser et al., 2004). Therefore, increase at J step was the result of restricted re-oxidation of Q_A^-. Additionally, YL revealed overall speeding up O-J rise (Fig. 1C) compared to ML what means that YL had a higher fraction of reduced Q_A after single

charge separation. In the same time, values of ET_0/TR_0 parameter in both leaf types decreased (Table 2). This parameter describes the probability that trapped exciton moves an electron further than Q_A^- (Strasser et al., 2000). Lower values of ET_0/TR_0 parameter in YL, as well as a decrease at 1 p.m. in both leaf types (Table 2), imply lower plastoquinone (PQ) exchange capacity, namely at the Q_B site. Gao et al. (2013) reported that young *Malus micromalus* leaves possess either low concentration of PQ or inactive PQ molecules. Due to the size of PQ pool, young *Malus* leaves showed limited PSII activity at acceptor side since the exchange of PQ at the Q_B site depends on electrons from PSII. However, as *Malus* leaves expanded, efficient electron transport was achieved as a result of increased availability of free electron acceptors.

In young fig leaves, the I step (V_I) changed slightly during the day course. On the other hand, ML revealed substantially decreased values at I step measured at 1 p.m. compared to 1 a.m. Also, YL revealed higher overall V_I values regarding ML (Table 2). The I step can give us approximate measure of Q_B-non-reducing PSII RCs that are unable to reduce Q_B (Lebkuecher et al., 1999; Jiang et al., 2006). Schansker and Strasser (2005) reported that older leaves possess a lower fraction of Q_B-non-reducing reaction centers what is in concordance with our results. Lower V_I values in mature elm leaves were also reported earlier by Jiang et al. (2006). They concluded that younger leaves in earlier developmental phases possess higher levels of Q_B-non-reducing centers responsible for blocked electron transport further than Q_A and poor transfer of electron towards PSI. Also, lower values of quantum yield for electron transport beyond Q_A (ET_0/ABS, Table 2) in YL compared to ML and the decrease of the same parameter in both leaf types at 7 a.m. and 1 p.m. corroborated with the presence of Q_B-non-reducing centers.

Our further investigations included shapes of L and K-bands (Fig. 2) that are known to display sensitivity to diurnal changes of light intensity (Desotgiu et al., 2013). The L-band is an indicator of energetic connectivity of antennae to PSII RC units (Yusuf et al., 2010) what implies better excitation energy utilization and system stability (Strasser et al., 2004; Yusuf et al., 2010). Our results revealed different amplitudes of L-band in both leaf types measured during the day. YL showed positive, while ML revealed negative L-band shapes (Figs. 2A and 2B). This indicated that ML had higher connectivity and more efficient utilization of excitation energy even at 1 p.m. compared to YL. Positive L-bands in YL (with the highest peak measured at 1 p.m.) indicated inferior antennae connectivity and might be a sign of disturbed energy transfer at light harvesting level (Srivastava et al., 1997). The K-band analysis also revealed differences between ML and YL (Figs. 2C and 2D). Although both leaf types revealed positive K-bands with the highest values measured at 1 p.m., values were evidently higher in ML compared to YL. The increase (positive) K-band indicates inactivation of OEC and/or increase of functional antenna size, and it is very sensitive to high temperature (Srivastava et al., 1997; Yusuf et al., 2010) and high light (Huang et al., 2016). Therefore, it seems that ML showed greater OEC inactivation that led to a discrepancy between acceptor and donor side of PSII (Oukarroum et al., 2007). Also, ML, unlike YL, showed relatively high K-band still at 7 p.m. suggesting that higher temperature and intense light at noon induced prolonged negative effect on the capacity of PSII donor side in ML (Gomes et al., 2012).

Although significantly lower TR_0/ABS values were measured in YL compared to ML, the PSII in both leaf types might be considered as fully functional (Table 2). The exception was observed at 1 p.m. when TR_0/ABS values in both leaf types dropped below 0.75, what is considered as boundary value of PSII functionality (Bolhar-Nordenkampf et al., 1989). Similar reactions of young developing leaves were reported previously (Lepeduš et al., 2005; Jiang et al., 2006). Although YL revealed much stronger photoinhibition than ML, it was shown to be fully reversible in both leaf types since the TR_0/ABS values were restored above 0.75 during night period (Table 2). It was reported (Force et al., 2003) that more accurate assessment of the photoinhibition process can be obtained through the changes between flux ratios and specific fluxes per RC rather than by a decline in TR_0/ABS. This is because specific fluxes consider only active RCs that can reduce Q_A (Strasser et al., 2004). A significant decrease in TR_0/ABS in both leaf types at 1 p.m. (Table 2) was accompanied by an increase in both ABS/RC and TR_0/RC (Fig. 3A and B). This indicated inactivation of a certain part of RCs most probably due to OEC inactivation as well as the transformation of active RCs to "silent" or Q_A-non-reducing ones. Q_A-non-reducing centers can efficiently absorb excitation energy, but they are unable to reduce Q_A. Instead, excess excitation energy is mostly dissipated as heat (Strasser et al., 2004; Yusuf et al., 2010). This corroborated with increased DI_0/RC at 1 p.m. in both YL and ML (Fig. 3D). However, a much more pronounced increase in DI_0/RC in YL at 1 p.m. than in ML (Fig. 3C), accompanied by a decrease in ET_0/RC and reduced electron transport capacity (ET_0/TR_0, Table 2) indicated their higher susceptibility to photoinhibition.

The performance index (Fig. 4) allowed us to estimate the severity of PSII photoinhibition. PI_{ABS} was shown to be a very sensitive indicator of plant functionality (Christen et al., 2007; Živčak et al., 2008). This is a multi-parametric expression that includes absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET) as well as dissipation of excess excitation energy as heat (DI) (Tsimilli-Michael et al., 2000; van Heerden et al., 2007). Young leaves showed nearly four times lower values than ML, but both leaf types displayed significant decrease measured at 1 p.m. In both leaf types, it was evident that all three parameters (RC/ABS, TR_0/DI_0, $ET_0/(TR_0-ET_0)$) equally contributed to a reduction in PI_{ABS} (Fig. 4A). Also, values of these three parameters, as well as PI_{ABS} itself, were recovered at 7 p.m. in comparison to 1 p.m. This suggested that both leaf types had functional mechanisms for acclimation to high light intensity and that increased temperature and high light downregulated PSII function without any irreversible damage.

Further, we investigated to what extent described photochemical adaptations of PSII in YL and ML were regulated due to the differential diurnal accumulation of the main PSII proteins, LHCII and D1. Both LHCII and D1 accumulations are influenced by changes in light and temperature conditions (Riesselmann and Piechulla, 1992). Our results showed rather stable LHCII relative abundance in ML as well as its increase in YL during the day course (Fig. 5). It was shown in greening barley seedlings that availability of chlorophyll pigments limits accumulation of LHCII protein (Mathis and Burkey, 1989). Busheva et al. (1991) reported that during the development of fully functional photosynthetic apparatus LHCII level is related to chlorophyll content rather than to its mRNA level. Formation of chlorophylls is developmentally dependent and mostly induced by light. Variations of chlorophyll content show circadian and diurnal control (Eckhardt et al., 2004). Fig ML were shown to have 3–4 times more chlorophylls than YL (Table 2) what corroborated with relatively constant accumulation of LHCII in ML during the day course while, YL revealed a decrease of LHCII accumulation during the night (Fig. 5). Chl *a* content had higher variations during the day than Chl *b* in ML. Accordingly, the Chl *a* to Chl *b* ratio varied (data not shown). Those variations are caused by larger instability of Chl *a* due to fast interconversion of Chl *b* to Chl *a* what enables plants to respond rapidly to changes in environmental conditions (Eckhardt et al., 2004) contributing to better acclimation of leaves to increased irradiation and temperature. In YL variations in chlorophylls ratio were less prominent due to their far lower amount. In our previous work (Mlinarić et al., 2016) it was shown that ML had lower Chl *a*/*b* ratio compared to YL. Such low ratios in ML came from the fact that ML had about five times more Chl *b* than YL. Such extreme Chl *b* amount might cause replacement of Chl *a* with Chl *b* by unusual binding to Chl *a*-binding sites in core PSII com-

plexes (Tanaka and Tanaka, 2011). Although the Chl *a*/*b* ratio is closely associated with changes of LHCII (Rüdiger, 2002), Tanaka and Tanaka (2011) reviewed that accumulation of LHC proteins in thylakoid membranes is specifically dependent on the levels of chlorophyll *b*. So, higher LHCII protein accumulation in ML (Fig. 5A) could be connected with elevated Chl *b* content. Different LHCII relative abundance during the day course in fig YL (Fig. 5A) is the most likely related to increasing peak in both, Chl *a* and Chl *b* content at midday what was reflected in the light harvesting processes (Fig. 3A).

Although ML revealed considerably higher D1 abundance (Fig. 5B) during the night compared to YL, both leaf types were able to accumulate sufficient amount of protein at 7 a.m. for efficient functioning during the day course. After 7 a.m., D1 protein content in both leaf types continuously decreased. Degradation of D1 at increased irradiation in both leaf types was followed by the rapid decrease of F_m values at 1 a.m. (Table 2). It was suggested that inactivation of PSII RC could be connected with reduction of F_m values (Perales-Vela et al., 2016). Since the Q_B-binding site at D1 protein is essential for electron transport through PSII, the changes in D1 accumulation could be associated with photoreduction of PQ pool in Q_B-reducing centers (Tomek et al., 2003). So, a decrease of D1 content is connected with inhibition of electron transport between Q_A and Q_B (Cheng et al., 2016). This implies that there are more parameters that can be attributed to the status of D1 protein. However, fluorescence measurements of the most parameters take into account only active reaction centers. Therefore, fluorescence measurements of photosynthetic parameters, such as F_v/F_m and PI_{ABS}, show the capacity of photosynthetic apparatus to perform photosynthesis, rather than an actual photosynthetic rate. ML revealed much faster D1 protein degradation at high light in comparison to YL (Fig. 5B) what could be attributed to more pronounced excitation energy pressure to the RCs due to theirs better connectivity with LHCII. It is known that high irradiation impairs the functioning of the D1 protein by inducing its structural damage that leads to the functional disability and consequently to its degradation (Aro et al., 1993; Khatoon et al., 2009). Efficient D1 turnover rate is an important regulatory process that maintains functional PSII during high light conditions (Krause et al., 1995). It was reported that damaged D1 proteins need to be replaced before another light period to maintain an optimal amount of functional proteins in PSII (Riesselmann and Piechulla, 1992; Booij-James et al., 2002) what corresponded to the highest accumulation of D1 at 7 a.m. in both investigated leaf types. Slower recovery of D1 in YL during the night compared to ML might be the result of greater overall inhibition of YL during the day. Photosynthetic protein complexes included in light reactions are regenerating with different dynamics (Baena-González and Aro, 2002). Therefore, it is possible that the rate of D1 protein degradation was much faster during the day than *de novo* synthesis. So, longer time was needed for reaching the level of abundance at 7 a.m. of D1 what is explaining its lower abundance at 1 a.m., while capacity for efficient photosynthesis described by F_v/F_m and PI_{ABS} was reached at 1 a.m.

Unlike ML that revealed stable Rubisco LSU accumulation during the day course, YL showed its continuous decrease during the light period (Fig. 5C). Observed difference in Rubisco LSU accumulation is the most probable reason for the differential capability of ML and YL for photosynthetic electron transport during the high light period (Fig. 3C) as well as for their different overall photosynthetic performance (Fig. 4A). Young developing maple leaves were previously shown to have lower PSII efficiency accompanied with lower Rubisco LSU levels in comparison to fully developed mature leaves (Lepeduš et al., 2011). Reduced electron transport that was observed in YL (Fig. 3C) that was directly influenced by reduced electron transfer from Q_A^- to Q_B (Table 2) could decrease trans-thylakoid proton gradient (Ruuska et al., 2000), what is involved in decrease of Rubisco accumulation on the one hand and in heat dissipation increase (Fig. 3D), on the other hand.

5. Conclusion

Different photochemical and molecular adjustment of PSII was observed in young and mature leaves of common fig during the day course. Although both leaf types revealed efficient primary photochemistry of PSII in the morning, midday depressions of most parameters indicated certain level of photoinhibition. The major difference between young and mature leaves was observed in the differential diurnal accumulation of the main photosynthetic proteins, D1, LHCII and Rubisco LSU, what was connected with the regulation of photosynthetic activity. Relatively stable LHCII accumulation in ML together with its better connectivity to PSII RC (negative L-band) enabled them efficient utilization of the excitation energy. This, in turn, triggered faster D1 protein degradation at high light in comparison to YL. Also, greater capacity for D1 accumulation in ML during the night led to their quicker recovery from photoinhibition. On the other hand, YL showed reduced connectivity of LHCII to PSII RC (positive L-band) in comparison to mature leaves, what resulted in their adjustment to dissipative mode (increase in DI_0/RC), causing lower pressure on D1 protein and subsequently its slower degradation in comparison to ML. A further distinction between YL and ML was in the regulation of their electron transport at high light. Unlike ML, which revealed stable Rubisco LSU accumulation and increase in electron transport (ET_0/RC) at midday, YL had decreased electron transport due to reduced electron transfer from Q_A^- to Q_B what could induce further degradation of Rubisco LSU during the day course. All together, led to the stronger decrease of overall photosynthetic efficiency in YL (PI_{ABS}) during the midday. It can be concluded that mechanisms for acclimation of PSII to high light were present in ML and developing YL. However, since efficient recovery after photoinhibition in both leaf types was accomplished, those mechanisms can be regarded as sufficiently effective in diurnal PSII adjustment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2016.12.002.

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