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Physiology and biochemistry of leaf bleaching in prematurely aging maple (*Acer saccharinum* L.) trees. II. Functional and molecular adjustment of PSII.

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Abstract – In the present study we aimed to investigate physiological and molecular mechanisms of photosynthetic performance decline in prematurely aged bleached leaves of silver maple (*Acer saccharinum* L.) trees. We used *in vivo* chlorophyll *a* fluorescence measurement to analyze changes in PSII photochemistry, relative abundance of photosynthetic proteins (D1, LHCII, Cyt_f and Rubisco LSU), relations between chlorophylls and their precursor protochlorophyllide as well as elemental composition of the leaves. Decreases in Al, Cr, Cu, Fe, K, Zn and an increase in S concentrations were found in bleached leaves in comparison to healthy green ones. The bleached leaves were visually expressing symptoms characteristic of Fe deficiency. Further, they had considerably decreased chlorophyll contents and protochlorophyllide contents, overall photosynthetic activity and relative abundance of major photosynthetic proteins. All the results indicate that modifications in the molecular organization of photosynthetic electron-transport chain components in bleached leaves led to functional adaptation of the PSII achieved by modifications of some reaction centres (RCs), turning them from active to dissipative. This provided efficient adaptation of bleached leaves to high-light induced oxidative damage during summer.

Key words: *Acer saccharinum*, bleached leaves, chlorophyll, photosynthesis, premature aging

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Abbreviations: BCIP – 5-bromo-4-chloro-3-indolyl phosphate, **BSA** – bovine serum albumin, **Chl *a+b*** – total chlorophylls, **Cyt$_f$** – cytochrome f, **DTT** – dithiothreitol, **$\Delta F/F'_m$** – effective quantum yield of the photosystem II, **F_0** – minimal fluorescence yield, **F_m** – maximal fluorescence yield, **F_v/F_m** – maximum quantum yield of the photosystem II, **FM** – mass of fresh tissue, **ICP-OES** – inductively-coupled plasma optical emission spectroscopy, **LHCII** – light harvesting complex of photosystem II, **NBT** – nitroblue tetrazolium, **OJIP** test – chlorophyll *a* fluorescence transient measurement, **PChl** – protochlorophyllide, **PPFD** – photosynthetically active photon flux density, **PSII** – photosystem II, **RCs** – reaction centres, **Rubisco LSU** – large subunit of Rubisco, **SDS** – sodium dodecyl sulfate

Introduction

Silver maple (*Acer saccharinum* L.) is a widely distributed tree species. It constitutes sustainable resource for the production of maple syrup, wood and lately has been seen as good quality source for the production of biofuel (SHAPOURI and DUFFIELD 1993). Because of its marked tolerance to different kind of stresses, silver maple trees also have horticultural value and are often used for ornamentation of urban parks and landscapes (DAY et al., 2000, HARDIN et al., 2001). However, a considerable number of chlorotic silver maple trees were noticed in the city park of Osijek (Croatia) growing besides healthy silver maple trees with green leaves. Leaves in these trees of decreased vitality became bleached as early as late spring (May) and kept such status during the whole vegetation season. Such symptoms observed in trees of decreased vitality correlate with symptoms of premature aging, sometimes called decline, which is characterized by chlorosis of foliage (bleaching), crown thinning and tree dieback (HOUSTON 1999). Decline could be caused by various abiotic, biotic and unknown factors which are the source of stress to a tree. Silver maple is sensitive to nutrient deficiency, e.g. iron deficiency will cause a distinctive pattern on the leaves – cells close to the veins are green and the rest of the leaf area is bleached (SINCLAIR et al. 1987). Such a pattern can be seen in the maple leaves we investigated here (UŽAREVIĆ et al. 2011).

Our previous investigation on the regulation of hydrogen peroxide level in silver maple bleached leaves revealed reduced antioxidative enzyme specific activity levels as well as decreased ascorbic acid content (UŽAREVIĆ et al. 2011). Also, bleached leaves had lower levels of chlorophyll *a*, chlorophyll *b*, total carotenoids and F_v/F_m value (maximum quantum yield of photosystem II) than green leaves. Premature aging involves various mechanisms of adaptation in plants including changes in cell structure, metabolism, degradation of macromolecules and changes in gene expression (JING et. al. 2003). PSII, which functions as water-plastoquinone oxidoreductase, is one of major regulatory components in photosynthetic apparatus (BARBER et al. 1997). Accordingly, we aimed to investigate changes in PSII photochemistry, relative abundance of major photosynthetic proteins (D1, LHCII, Cyt$_f$ and Rubisco LSU), relations between chlorophyll and its precursor protochlorophyllide as well as leaf elemental composition in order to understand functional and molecular adjustments of PSII in bleached leaves.

Materials and methods

Plant materials

Branches from the lower parts of the crowns of ten different trees (five green and five bleached) of silver maple (*Acer saccharinum*) were sampled, put in a plastic bag and deliv-

ered to the laboratory within one hour. Sampling was done in May 2008, in the city park in Osijek (Croatia) every day at 10.30 am. For all extractions, leaves were cut into small pieces without main veins and ground with liquid nitrogen in a mortar in order to obtain fine tissue powder.

Determination of photosynthetic pigment content

For determination of photosynthetic pigment content combined samples of 5 replicates for every leaf type were used. Fine tissue powder was extracted in absolute ice-cold acetone for 15 minutes at +4 °C and centrifuged for 10 minutes at 3000 rpm. This procedure was repeated until the leaf material was completely uncoloured. The concentrations of total chlorophylls (Chl *a*+*b*) and protochlorophyllide (PChl) per mass of fresh tissue (FM) were determined spectrophotometrically (Specord 40, Analytic Jena, Germany) according to LICHTENTHALER (1987).

Protein extraction, SDS-PAGE and immunodetection of D1, LHCII and Rubisco LSU

Fresh leaf tissue was ground in liquid nitrogen and 0.5 g of powder was used for extraction. Soluble and membrane proteins were extracted by adding solution containing 0.013M Tris pH=8, 4.6% SDS, 1.54% DTT and 15% glycerol pre-warmed at 80 °C. After 10 minutes of extraction at 80 °C, samples were centrifuged at +4 °C for 10 minutes at 18000*g*. The concentration of total protein content was determined spectrophotometrically according to BRADFORD (1976) using bovine serum albumin (BSA) as a standard. Aliquots containing 30 μg proteins per lane were mixed with Laemmli sample buffer (0.065 M Tris-HCl buffer containing 6% SDS, 6% β-mercaptoethanol, 30% glycerol and 0.01 % bromphenol blue), boiled for 5 minutes and loaded on the gel. The samples were separated by SDS-PAGE (Mini-gel Twin, Biometra, Germany) using 12% polyacrylamide gels and blotted onto a nitrocellulose membrane (Fastblot B43, Biometra, Germany). The membranes were blocked with 5% non-fat powdered milk solution in PBS buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl) pH = 7.4 containing 1% Tween 20 over night at 4 °C and incubated with rabbit monoclonal antibodies raised against the pea Rubisco LSU, D1, LHCII and cytochrome *f* for 2 hours at room temperature. After washing, membranes were probed with an anti-rabbit alkaline – phosphatase conjugated secondary IgG antibody diluted 1:30 000 in PBST. Protein bands were visualized using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium and images were analyzed using Kodak 1D 3.6 software (LEPEDUŠ et al. 2005, 2008).

Measurement of chlorophyll *a* fluorescence

In vivo chlorophyll *a* fluorescence measurements were performed using a pulse-amplitude-modulated photosynthesis yield analyser (Mini-PAM, Waltz). The plant material (10 leaves (n=10) taken from 5 different trees) was dark-adapted for approximately 30 minutes before measurement. Minimal (F_0) and maximal (F_m) fluorescence yields were measured in the dark-adapted leaves. The same parameters (F) and (F'_m) were measured upon light applications (photosynthetic photon flux density (PPFD) of 100, 500 and 1100 μmol m^{-2} s^{-1}). The radiation was maintained until both, F and $F'm$ were stable. The maximum quantum yield of the photosystem II (F_v/F_m) as well as the effective quantum yield of the photosystem II ($\Delta F/F'm$) were calculated according to SCHREIBER et al. (1994).

The chlorophyll *a* fluorescence transient measurement (OJIP test) was performed with the use of a Plant Efficiency Analyser (PEA, Hansatech). Plant material (25 leaves (n=25) taken from 5 different trees) was dark-adapted for about 30 minutes before measurements. Chlorophyll fluorescence transients were induced by applying the pulse of saturating red light (peak at 650 nm, 3000 μmol m^{-2} s^{-1}). Changes in fluorescence were measured for 1s, starting from 50 μs after onset of illumination. During the first 2 ms changes were recorded every 10 μs and every 1 ms afterward. The obtained data were used in OJIP test (Strasser et al. 2004) in order to calculate several parameters of PSII photochemistry (Tab. 1).

Tab. 1. OJIP test parameters and expressions.

OJIP TEST DATA AND PARAMETERS	
F_0	– fluorescence intensity at 50 μs (O step)
F_{300}	– fluorescence intensity at 300 μs
F_J	– fluorescence intensity at 2 ms (J step)
F_I	– fluorescence intensity at 30 ms (I step)
F_m	– maximal fluorescence intensity (P step)
F_v	– maximal variable fluorescence; $F_v = F_m - F_0$
V_J	– variable fluorescence at J step; $V_J = (F_J - F_0)/(F_m - F_0)$
V_I	– variable fluorescence at I step; $V_I = (F_I - F_0)/(F_m - F_0)$
S_m	– normalized total complementary area above OJIP transient; S_m = AREA/$(F_m - F_0)$
N	– turnover number; $N = S_m \times [(dV/dt)_0] / V_J$
RC/CS_0	– density of reaction centers; $RC/CS_0 = F_v/F_m \times (V_J/M_0) \times ABS/CS_0$
ABS/RC	– absorption per active reaction center; $ABS/RC = M_0 \times (1/V_J) \times [1/(F_v/F_m)]$
TR_0/RC	– trapping per active reaction center; $TR_0/RC = M_0 \times (1/V_J)$
ET_0/RC	– electron transport per active reaction center: $ET_0/RC = M_0 \times (1/V_J) \times (1\text{-}V_J)$
DI_0/RC	– dissipation per active reaction center; DI_0/RC = (ABS/RC) – (TR_0/RC)
PI_{ABS}	– performance index; $PI = (RC/ABS) \times (TR_0/DI_0) \times [ET_0/(TR_0/ET_0)]$
RC/ABS	– density of reaction centers on chlorophyll basis; $RC/ABS = (RC/TR_0) \times (TR_0/ABS) = [(F_J - F_0)/4(F_{300} - F_0)] \times (F_v/F_m)$
TR_0/DI_0	– flux ratio trapping per dissipation; $TR_0/DI_0 = F_v/F_0$
$ET_0/(TR_0/ET_0)$	– electron transport beyond Q_A^-; $ET_0/(TR_0/ET_0) = (F_m - F_J)/(F_J - F_0)$

Oxygen evolution

Oxygen evolution was measured using the gas-phase Clark-type oxygen electrode (Hansatech). Five bleached and five green leaves were analyzed, each taken from a different tree. The leaf discs (2.5 cm^2) were placed in the reaction chamber and the oxygen evolution was measured at three different light levels 100, 500 and 1100 μmol m^{-2} s^{-1}. The temperature inside the reaction chamber was 25 °C.

Inductively-coupled plasma optical emission spectroscopy (ICP-OES) analysis for mineral concentrations in leaves

For determination of mineral concentration content combined samples of 3 replicates for every leaf type were used. Leaves were dried at 105 °C for 72 hours and ground into a powder. Mineral concentrations in leaves were determined by inductively-coupled plasma optical emission spectroscopy (ICP-OES) after microwave digestion. Leaves were digested in 65% nitric acid (HNO_3) + 30% hydrogen-peroxide (H_2O_2) with a Milestone MLS 1200 microwave (ZARCINAS et al. 1987). The analyses were performed with a Jobin-Yvon Ultrace 238 ICP-OES spectrometer.

Statistical analysis

Data on Chl *a*+*b*, PChl, elemental analysis and OJIP parameters were analyzed by t-test. Data on the maximum quantum yield of the photosystem II (F_v/F_m), effective quantum yield of the photosystem II ($\Delta F/F'_m$) and oxygen evolution were analyzed by one-way analysis of variance (ANOVA) and Fischer LSD (Least significant difference) for post hoc analysis. Differences were considered significant at $p \leq 0.05$. All statistical analyses were done with Statistica 7.1. Software (StatSoft, Inc. 2005).

Results

Total chlorophyll (Chl *a*+*b*) concentration, protochlorophyllide (PChl) concentration and total chlorophyll to protochlorophyllide ratio in green leaves were significantly higher (72 %) than in bleached leaves (Tab. 2).

Tab. 2. Mean values (± standard deviation) of total chlorophyll (Chl *a*+*b*) concentration, protochlorophyllide (PChl) concentration and total chlorophylls to protochlorophyllide ratio in green (G) and bleached (B) leaves of silver maple (*Acer saccharinum*). P(t) – percent of similarity.

Parameters	G	B	t	P(t)
Chl *a*+*b* (mg g^{-1} FM)	2.57±0.09	0.72±0.05	60.514	<0.1%
PChl (mg g^{-1} FM)	0.33±0.06	0.19±0.03	6.502	<0.1%
Chl *a*+*b* / PChl	8.07±1.71	3.84±0.31	7.696	<0.1%

Bleached leaves had lower Al, Cr, Cu, Fe, K, Zn and higher S concentrations than green leaves (Tab. 3).

In comparison to green, bleached leaves had 34.6, 35.7, 38 and 27.7 % reduced abundances of D1, LHCII, cytochrome *f* and Rubisco LSU, respectively (Fig. 1).

Down-regulation of PSII photochemical efficiency was found (Fig. 2A). Maximum quantum yield of PSII (F_v/F_m) was 0.81 in green leaves and 0.72 in bleached leaves. Effective quantum yields of PSII ($\Delta F/F'_m$) were also decreased in bleached leaves. The values of $\Delta F/F'_m$ at PPFD of 100, 500, 1000 μmol m^{-2} s^{-1} were 0.72, 0.52 and 0.37, respectively, in green leaves. In bleached leaves the values of $\Delta F/F'_m$ at the same light intensities were

Tab. 3. Mean values (± standard deviation) of element concentrations (mg kg-1 dry mass) in green (G) and bleached (B) leaves of silver maple (*Acer saccharinum*). P(t) – percent of similarity, NS – not significant.

Elements	G	B	t	P(t)
Al	70.97±0.68	45.67±2.94	11.390	<5%
B	117.19±8.32	10.78±3.29	3.068	NS
Ba	2.70±0.51	2.70±0.04	2.393	NS
Ca	10617.46±676.84	10617.46±96.18	6.594	NS
Cr	1.88±0.30	0.50±0.03	8.549	<5%
Cu	12.21±0.32	5.61±0.05	37.933	<5%
Fe	116.77±4.71	57.99±2.17	19.835	<5%
K	11187.92±895.99	14770.64±88.62	7.514	<5%
Mg	3783.79±95.05	3889.90±60.19	1.578	NS
Mn	33.55±1.58	27.59±0.16	7.082	NS
Mo	0.19±0.07	0.18±0.01	0.323	NS
Na	11.81±4.10	7.97±1.47	1.585	NS
0Ni	0.85±0.32	0.32±0.09	2.859	NS
P	3574.91±214.73	3255.21±52.24	2.671	NS
Sr	8.42±1.19	8.96±0.09	0.856	NS
Zn	36.44±0.22	21.01±0.35	54.242	<5%
S	2131.34±55.53	2419.62±26.37	8.178	<5%

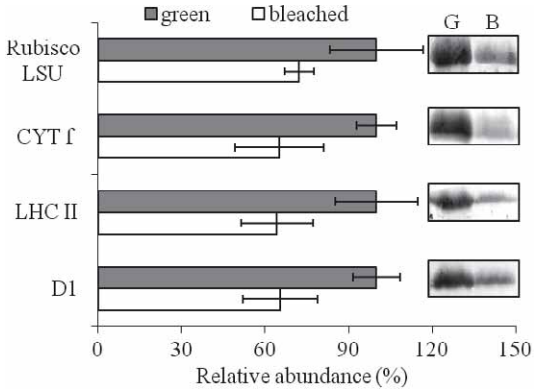

Fig. 1. Relative abundance of D1, Rubisco LSU, cytochrome *f* and LHCII proteins on imunoblots in green (G) and bleached (B) leaves of silver maple (*Acer saccharinum* L.). The abundance of each protein in G leaves was taken as 100 %. Horizontal bars indicate relative standard deviation.

0.59, 0.35 and 0.24, respectively. Relative electron transport rates (rel. ETR), shown in figure 2B, at higher light intensities were lower in bleached than in green leaves. At 100, 500, 1000 μmol m^{-2} s^{-1} measured relative electron transport rates were 35.86, 130.80 and 202.62

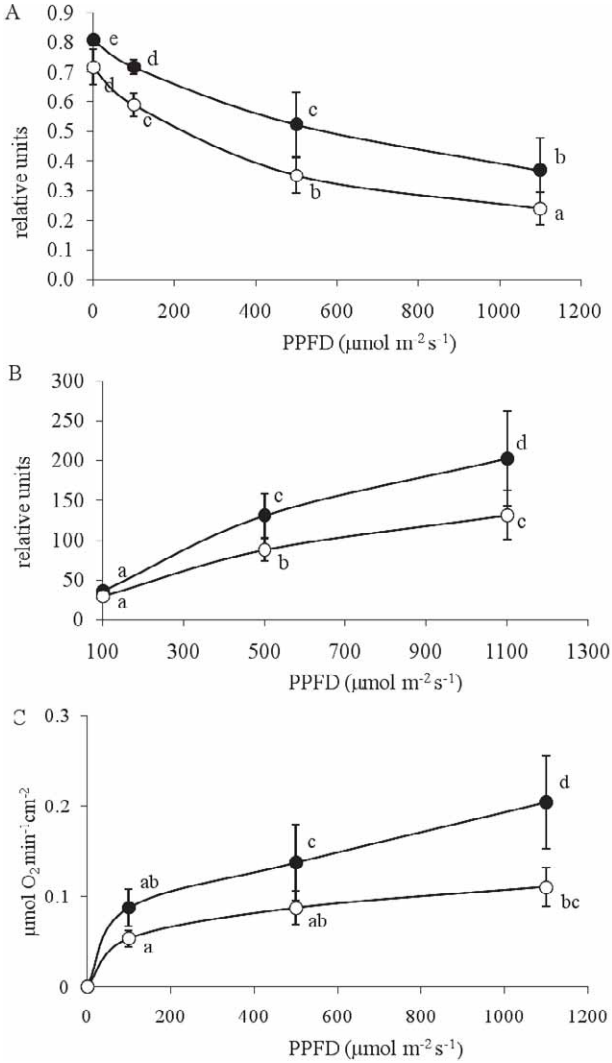

Fig. 2. The arithmetic mean values and standard deviations of photosystem II efficiency (given as maximum quantum yield (F_v/F_m) at 0 PPFD and effective quantum yields ($\Delta F/F'_m$) (A), relative electron-transport rate (rel. ETR) (B) and PSII capacity for oxygen evolution (C) in green (grey circles) and bleached (white circles) leaves of silver maple (*Acer saccharinum* L.). Significant differences between G, YG and Y leaves were designated by different letters (a, b, c) placed near the circles.
PPFD (in µmol m^{-2} s^{-1}) – photosynthetically active photon flux density; vertical bars indicate standard deviation.

in green leaves, respectively while measured relative electron transport rates in bleached leaves were 29.53, 87.95 and 131.96, respectively. In figure 2C capacity for oxygen production is shown. At PPFD of 500 and 1100 µmol m^{-2} s^{-1} it was lower in bleached than in green leaves, while no difference was observed at low light level (100 µmol m^{-2} s^{-1})

The chlorophyll *a* fluorescence transient, revealed a usual OJIP curve shape in both leaf types (Fig. 3A). O-P normalized curves revealed the increase of fluorescence in J and I steps in bleached leaves (Fig. 3B).

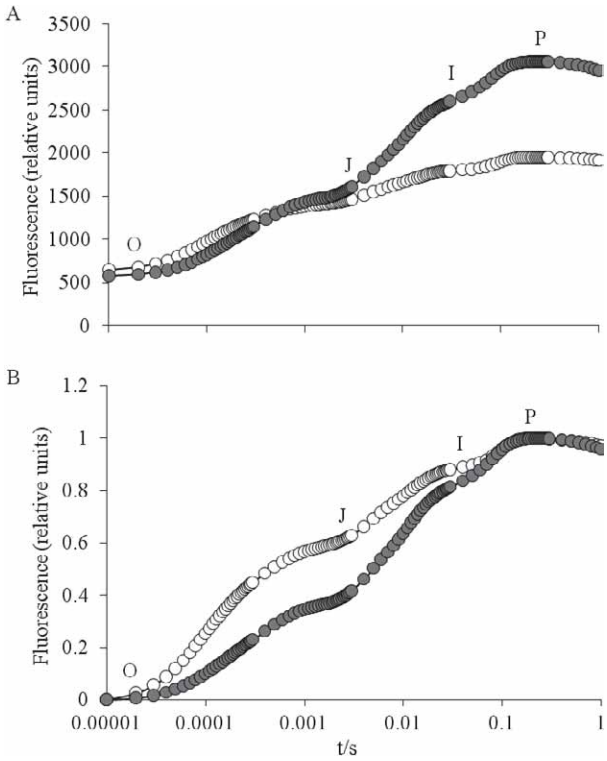

Fig. 3. OJIP chlorophyll *a* fluorescence transients without normalization (A) and O-P normalized (B) in green (grey circles) and bleached (white circles) leaves of silver maple (*Acer saccharinum* L).

Chlorophyll *a* fluorescence parameters revealed that the F_0 value corresponded in green and bleached leaves, but maximal fluorescence intensity, F_m values were lower in bleached leaves (Tab. 4). Also, the turnover number (N), S_m value and density of active reaction centres (RC/CS_0) significantly decreased in bleached leaves.

Specific fluxes or specific activities per active reaction centre were calculated (Tab. 5) to show functioning of PSII reaction centres. Parameters describing absorption (ABS/RC) and trapping (TR_0/RC) of light energy per active reaction centre were higher in bleached leaves than in green leaves, as was dissipation of excess energy (DI_0/RC). The capability of bleached leaves for electron transport beyond Q_A – (ET_0/RC) was significantly lower.

Values of performance index (PI_{ABS}) and its components in green and bleached leaves revealed that green leaves had higher PI_{ABS}, as well as density of reaction centres on chlorophyll basis (RC/ABS), ratio of trapping and dissipation fluxes (TR_0/DI_0) and efficiency of the conversion of excitation energy to electron transport ($ET_0/(TR_0\text{-}ET_0)$ (Tab. 6).

Tab. 4. The mean values (± standard deviation) of chlorophyll *a* fluorescence parameters in green (G) and bleached (B) leaves of silver maple (*Acer saccharinum*). All parameters are expressed in relative units. P(t) – percent of similarity, NS – not significant.

Parameters	G	B	t	P(t)
F_0	565.36±92.09	650.92±206.35	12.940	NS
F_m	3133.56±308.72	1908.12±359.03	1.893	<0.1%
Vj	0.383±0.02	0.588±0.05	17.896	<0.1%
Vi	0.820±0.03	0.883±0.03	7.322	<0.1%
Sm	17.82±2.99	12.45±2.59	6.785	<0.1%
N	44,09±5.61	37,06±7.78	3.662	<1%
RC/CS_0	184.95±18.45	141.57±36.43	5.312	<0.1%

Tab. 5. The mean values (± standard deviation) of specific fluxes or specific activities per active reaction centre in green (G) and bleached (B) leaves of silver maple (*Acer saccharinum*). All parameters are expressed in relative units. P(t) – percent of similarity.

Parameters	G	B	t	P(t)
ABS/RC	3.044±0.24	4.558±0.54	12.870	<0.1%
TR_0/RC	2.493±0.16	2.981±0.16	11.574	<0.1%
ET_0/RC	1.539±0.10	1.228±0.16	8.173	<0.1%
DI_0/RC	0.550±0.09	1.577±0.52	9.788	<0.1%

Tab. 6. The mean values (± standard deviation) of performance index (PIABS) and its components in green (G) and bleached (B) leaves of silver maple (*Acer saccharinum*). All parameters are expressed in relative units. P(t) – percent of similarity.

Parameters	G	B	t	P(t)
PI_{ABS}	2.475±0.44	0.324±0.11	23.812	<0.1%
RC/ABS	0.330±0.03	0.222±0.02	15.475	<0.1%
TR_0/DI_0	4.602±0.48	2.064±0.58	17.085	<0.1%
$ET_0/(TR_0\text{-}ET_0)$	1.619±0.12	0.716±0.16	22.687	<0.1%

Discussion

Numbers of reports have revealed that changes in chlorophylls content and photosynthetic performance can be considered as very sensitive indicators for different biotic and abiotic stresses as well as developmental processes in higher plants (MUNNE-BOSCH 2008, TAKAHASHI and MURATA 2008, ĆURKOVIĆ-PERICA et al. 2007, FULGOSI et al. 2005, LEPEDUŠ et al. 2005, PFANNSCHMIDT 2003, MITTLER 2002). Bleaching is often caused by disturbance in mineral nutrition. Our investigation of maple leaf premature aging (UŽAREVIĆ et al. 2011)

revealed increased levels of organic nitrogen (for 19.85%) and soluble proteins (for 208.05%). In this study we analyzed the abundance of some other elements in leaves (Tab. 3). Differences in elemental concentrations were found between green and bleached leaves. Bleached leaves had decreased Al, Cr, Cu, Fe, K, Zn and increased S concentrations in comparison to healthy green leaves. It can be speculated that increased S content might account for the increase in the synthesis of glutathione (MENDOZA-CÓZATL et al. 2005, RAUSCH and WACHTER 2005) and in this way enhance the protection against potential oxidative damage. Surprisingly, Mg and Mn content did not differ between green and bleached leaves, indicated that the bleaching symptom was not due to any deficiency of these elements. Chlorophyll degradation is an enzymatically regulated process (RODONI et al. 1998, 1997, MATILE et al. 1996,) accompanied by breakdown of pigment-protein complexes in photosynthetic membranes (TANG et al. 2005). The concentration of protochlorophyllide, a precursor in chlorophyll biosynthesis (SUZUKI et al. 1997), was decreased in bleached leaves to 57% of that in green leaves. The decreased total chlorophyll to protochlorophyllide ratio in bleached leaves indicated that lowering of total chlorophyll content was not brought about merely by degradation but also due to decreased biosynthetic capability of bleached leaves. Chlorophyll biosynthesis is to a large degree dependent on iron, which was shown to be deficient in bleached leaves (Tab. 3). Iron is known to be a co-factor of many enzymes of the chlorophyll biosynthetic pathway and also an important part of the electron-transport chain in chloroplasts. A deficiency is usually accompanied by bleaching symptoms and decrease in photosynthetic performance (ITURBE-ORMAEXTE et al. 1995). Decreased photosynthesis was due not only to decreased pigments content but also to changes in chloroplast proteome (BRIAT et al. 2007). Relative abundance of major photosynthetic proteins investigated here (Fig. 1) showed that in comparison to green leaves, bleached leaves had 34.6, 35.7, 38 and 27.7 % reduced abundances of D1, LHCII, cytochrome *f* and Rubisco LSU, respectively. ANDALUZ et al. (2006) reported reduced abundance of chloroplast electron-transport proteins and an increased amount of carbon assimilation proteins, in response to Fe deficiency. Since our results revealed that the abundance of Rubisco LSU was also decreased in bleached leaves (Fig. 1) it can be speculated that premature bleaching of the investigated maple leaves was influenced not by Fe deficiency alone, rather, in combination with some other ecological and/or physiological factors such as high light, drought and elevated temperatures. This also implies that the observed bleaching is most likely a pleiotropic effect influenced by many cellular energetic and metabolic processes.

In this study down-regulation of PSII photochemical efficiency was shown (Fig. 2A). Maximum quantum yield of PSII (F_v/F_m) in bleached leaves (0.72) was significantly decreased in comparison to green leaves (0.81). Since the value of 0.75 for F_v/F_m has been considered to be a boundary value for fully functional PSII (BOLHÀR-NORDENKAMPF et al. 1989) the values measured in bleached leaves indicated impaired PSII photochemistry. Effective quantum yields of PSII ($\Delta F/F'_m$) were also significantly decreased in bleached leaves (Fig. 2A). Since $\Delta F/F'_m$ corresponds to the proportion of the light absorbed by chlorophylls associated with PSII (MAXWELL and JOHNSON 2000), decrease in $\Delta F/F'_m$ (Fig. 2A) was in accordance with the observed chlorophylls content decrease (Tab. 2) in bleached leaves. Down-regulation of $\Delta F/F'_m$ in bleached leaves is reflected in PSII-driven electron transport (Fig. 2B). Decrease in relative electron transport rates (rel. ETR) at higher light intensities might be related to a reduced abundance of Rubisco enzyme, since a

clear relationship between CO_2 fixation and PSII driven electron transport was established (KRALL et al. 1992). In addition to marked down-regulation of photochemical efficiency, reduced capacity for oxygen production at 500 and 1100 μmol m^{-2} s^{-1} was also found in bleached leaves (Fig. 2C). Oxygen is produced as the by-product of photosynthesis during water oxidation at oxygen evolving complex (OEC) which is constitutive part of PSII (IWATA and BARBER 2004, RAYMOND and BLANKENSHIP 2008). It has been postulated that S state of OEC plays an important role in chlorophyll fluorescence rise at J-step of OJIP curve (STRASSER et al. 2004). The chlorophyll *a* fluorescence transient revealed usual OJIP curve shape in both leaf types (Fig. 3A). The F_0 did not differ between green and bleached leaves, but F_m values appeared to be much lower in bleached leaves (Tab. 4). Since F_m is obtained when all Q_A molecules are reduced (SCHREIBER et al. 1994), changes in F_m level usually reflect heat dissipation events in PSII (MAXWELL and JOHNSON 2000). O-P normalized curves revealed the increase of fluorescence in J and I steps (Fig. 3B). Rise in the O – J step in bleached leaves, seen as an increase in variable fluorescence at 2 ms (V_J), can be explained by the accumulation of reduced primary plastoquinone (Q_A^-) because of their inability to transfer electrons efficiently further than Q_A^-. Parameters that describe the accumulation of Q_A^- very well are the turnover number (N) representing the number of Q_A reduction events between F_0 and F_m and normalized total complementary area above OIJP transient (S_m), which corresponds to the energy needed to close all reaction centres (STRASSER et al. 2000). Both parameters were significantly lower in bleached (Tab. 4) than in green leaves, indicating the reduced transport from Q_A^- to Q_B. Also, there was a rise in the J – I step that was evident as increase in variable fluorescence at 30 ms (VI) (Tab. 3). While the O – J step represents the full reduction of Q_A (photochemical phase), the J – I – P steps represent thermal phases associated with reduction of secondary plastoquinone (non-photochemical phase) (STRASSER et al. 1995). The latest steps reflect the PSII heterogeneity due to the presence of fast and slow reducing plastoquinone (PQ) reaction centres (STIRBET et al 1998). In order to analyze the functioning of PSII reaction centres specific fluxes or specific activities per active reaction centre were calculated (Tab. 5). Parameters describing absorption (ABS/RC) and trapping (TR_0/RC) of light energy per active (Q_A reducing) reaction centre were higher in bleached leaves than in green leaves. The increase in average functional antenna size (ABS/RC) (Tab. 5) together with decreased density of active reaction centres (RC/CS_0) (Tab. 4) indicate the presence of non-Q_A reducing reaction centres in bleached leaves. Such non-Q_A reducing reaction centres are also called silent reaction centres and act as heat sinks (STRASSER et al. 2004). According to STRASSER et al. (2004) the fraction of remained active reaction centres in bleached leaves might be calculated using the expression: $RC_{control\ (green)}/RC_{stressed\ (bleached)} = (ABS/RC)_{stressed\ (bleached)} / (ABS/RC)_{control\ (green)}$ and it was 66.78 % of that in green leaves. Further, the capability of bleached leaves for electron transport beyond Q_A^- (ET_0/RC) was significantly impaired (Tab. 5) due to reduced transport from Q_A^- to QB. This resulted in an about three-fold increased dissipation of excess energy (DI_0/RC) (Tab. 5) in order to avoid oxidative damage of PSII. Described differences in the shape of chlorophyll *a* fluorescence transient curves (Fig. 3) between green and bleached leaves were reflected on values of performance index (PIABS) (Tab. 6). This parameter was established by STRASSER et al. (2000) and describes overall photosynthetic performance. It combines several parameters that describe three main functional characteristics of PSII reaction centre, namely RC/ABS (density of reaction centres on chlorophyll basis), TR_0/DI_0 (ratio of trapping and dissipation fluxes) and $ET_0/(TR_0\text{-}ET_0)$ (efficiency of the

conversion of excitation energy to electron transport). Green leaves had about two-fold higher PI_{ABS} than bleached, with all of its components decreased in bleached leaves (Tab. 6).

From the presented results, it can be concluded that maple leaves of lower vitality revealed bleaching caused by disturbed mineral nutrition with marked Fe deficiency but with Mg and Mn content comparable with green healthy leaves. Data on the photosynthesis performance and biochemistry indicated that bleaching was not a simple symptom of premature aging but rather an adaptation to high-light induced oxidative stress during summer. In that manner, down-regulation of total chlorophylls and major photosynthetic proteins content (D1, LHCII, cytochrome *f* and Rubisco LSU) occurred. These biochemical processes were accompanied with marked changes in PSII photochemistry. The most important feature of PSII was the down-regulation of its efficiency, which was achieved by modifications of a certain fraction of RCs, turning them from active to dissipative. Such dissipative RCs were characterised by the presence of non-Q_A and non-Q_B reducing RCs which resulted by in reduced overall photosynthetic performance, seen as decrease in oxygen evolution and PI_{ABS} value.

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