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Physiology and biochemistry of leaf bleaching in prematurely aging maple (*Acer saccharinum* L.) trees: I. Hydrogen peroxide level, antioxidative responses and photosynthetic pigments

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Abstract – Essential signaling processes such as changes in calcium mobilization, protein phosphorylation and gene expression are known to be modulated by hydrogen peroxide (H_2O_2). A lot of silver maple trees in the city of Osijek (Croatia) were noticed to have bleached leaves by early summer as well as during the whole vegetation season. In this study we aimed to investigate the processes that regulate H_2O_2 levels in healthy (green) and prematurely aged (bleached) leaves. For that purpose, photosynthetic performance and antioxidative response of green and bleached silver maple leaves were studied. Bleached leaves had higher hydrogen peroxide level, a three-fold level of total soluble proteins as well as a lower level of ascorbic acid. Concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophylls and total carotenoids as well as maximum quantum yield of photosystem II were lower in bleached leaves. This indicated their impaired photosynthetic performance. Further more, bleached leaves were characterized by lower specific activities of the main antioxidative enzymes, which influenced their reactive oxygen species scavenging capability. The higher level of H_2O_2 content in bleached leaves as the consequence of reduced antioxidative enzyme specific activities as well as ascorbic acid level could be the reason for the down-regulation of photosynthetic performance and premature aging of those leaves.

Keywords: antioxidative enzymes, reactive oxygen species, photosynthesis, premature aging, *Acer saccharinum*

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Abbreviations: AA – ascorbic acid, **APX** – ascorbate peroxidase, **BSA** – bovine serum albumin, **Car** – total carotenoids, **CAT** – catalase, **Chl *a*** – chlorophyll *a*, **Chl *b*** – chlorophyll *b*, **Chl *a+b*** – total chlorophylls, **DM** – dry mass, **EDTA** – ethylenediamine tetraacetic acid, **FM** – fresh mass, F_v/F_m – maximum quantum yield of photosystem II, **GPOD** – guaiacol peroxidase, **NBT** – nitroblue tetrazolium, **PSII** – photosystem II, **PVP** – polyvinylpyrrolidone, **ROS** – reactive oxygen species, **SOD** – superoxide dismutase, **TBA** – thiobarbituric acid, **TBARS –** thiobarbituric acid reactive substances

Introduction

Hydrogen peroxide (H_2O_2) is reactive oxygen species (ROS), continually generated from various sources during normal metabolism and also as a result of oxidative stress (NEILL et al. 2002a, NEILL et al. 2002b, YAN et al. 2010). H_2O_2 is mostly generated via superoxide during electron transport processes in chloroplasts and mitochondria (DAT et al. 2000, ARORA et al. 2002). Also, H_2O_2 generation is induced in plants following exposure to a wide variety of abiotic and biotic stimuli. These include extremes of temperatures, drought, salinity, UV-B irradiation, excess excitation energy, ozone exposure, wounding, pathogens and herbivores (LAMB and DIXON 1997, COSTA et al. 2002, KARPINSKI et al. 2003, ŚLESAK et al. 2007). Oxidative stress arises from an imbalance in the metabolism of ROS, with more ROS being produced than are catabolized (NEILL et al. 2002b). The high rates of H_2O_2 production are normally balanced by very efficient antioxidant mechanisms, both enzymatic and non-enzymatic antioxidants, by which it is removed from the cell (NOCTOR and FOYER 1998, CHEN and GALLIE 2006, ŚLESAK et al. 2007). The balance between superoxide dismutase (SOD) and ascorbate peroxidase (APX) or catalase (CAT) activities in cells is crucial for determining the steady-state level of hydrogen peroxide and superoxide radicals. These defence processes are not restricted to the intracellular compartments, but are also found in the apoplast to a limited extent (ARORA et al. 2002). Abiotic and biotic stresses can disturb this balance, by increased H_2O_2 initiating signaling responses that include enzyme activation, gene expression, programmed cell death and cellular damage (NEILL et al. 2002a, HUNG et al. 2005, van DORN 2008). Well established deleterious effects of ROS include damage to nucleic acids, protein oxidation, enzyme inhibition, and membrane lipid peroxidation (MITTLER 2002).

Silver maple (*Acer saccharinum* L.) is a relatively fast-growing tree that is also highly adaptable, although it has higher sunlight requirements than other maples. This species is highly tolerant to a wide range of soils, drought, seasonal flooding and urban conditions; therefore it is frequently planted next to streets. Also, it is widely used as ornamental tree in parks especially because of its ease of propagation and transplanting (DAY et al. 2000, HARDIN et al. 2001). However, a lot of silver maple trees in the city of Osijek (Croatia) were noticed to have bleached leaves by early summer as well as during the whole vegetation season. In this study we aimed to investigate the processes that regulate H_2O_2 levels in healthy (green) and prematurely aged (bleached) leaves. For that purpose, hydrogen peroxide level, antioxidative responses and photosynthetic pigment content in green and bleached silver maple leaves were studied.

Material and methods

Plant material and sampling

The materials for study were healthy (green) and prematurely aged (bleached) silver maple (*Acer saccharinum* L.) leaves (Fig. 1). Leaves were sampled from trees grown in the city of Osijek (Croatia). The sampling was done in July 2006. Branches from ten trees of each type were sampled from the lower part of the crown, put in a plastic bag and delivered to the laboratory within one hour. 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.

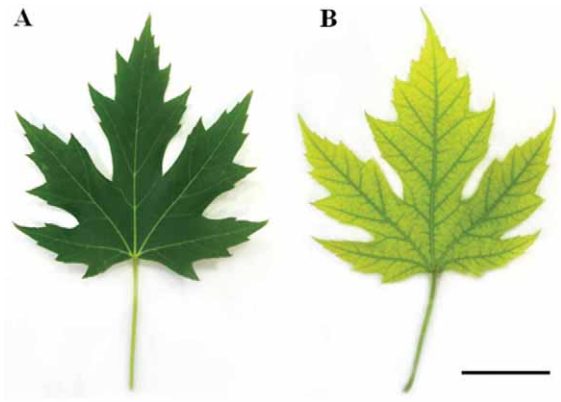

Fig. 1. Silver maple (*Acer saccharinum* L.) leaves: (A) – healthy (green), (B) – prematurely aged (bleached). Bar = 2 cm.

Determination of photosynthetic parameters

Leaf pigments were extracted from about 0.1 g of liquid-nitrogen-powdered leaves with absolute ice-cold acetone in the presence of magnesium hydroxide carbonate. The contents of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophylls (Chl *a*+*b*) and total carotenoids (Car) per dry mass of tissue (DM) were determined spectrophotometrically (Specord 40 Analytic Jena) at 470, 644.8 and 661.6 nm, respectively (LICHTENTHALER 1987). Fluorescence measurements were done on dark-adapted (for 30 minutes) plant material. Maximum quantum yield of photosystem II (PSII) (F_v/F_m) was measured by the saturating pulse method (Mini-PAM, Waltz) according to SCHREIBER et al. (1994).

Enzyme assays

Activities of ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (GPOD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) were assayed in leaf tissue extracts. The extraction of proteins for APX activity measurements was done in ice-cold 100 mM potassium phosphate buffer (pH 7.0) with 5 mM sodium ascorbate and 1 mM ethylenediamine tetraacetic acid (EDTA), with the addition of polyvinylpyrrolidone (PVP). The extractions for GPOD, CAT and SOD activity determi-

nation were done in ice-cold 100 mM potassium phosphate buffer (pH 7.5) with the addition of PVP.

The APX activity was measured spectrophotometrically by monitoring the decrease in absorbance at 290 nm, after NAKANO and ASADA (1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) with 0.1 mM EDTA, 50 mM ascorbic acid (AA) and enzyme extract. The reaction was started with the addition 12 mM H_2O_2.

The GPOD activity was measured at 470 nm as described by SIEGEL and GALSTON (1967). The reaction mixture consisted of 5 mM guaiacol and 5 mM H_2O_2 in 200 mM phosphate buffer (pH 5.8) and enzyme extract.

The CAT activity was determined in the presence of 50 mM potassium phosphate buffer (pH 7.5), 10 mM H_2O_2 and enzyme extract according to AEBI (1984). The decrease in absorbance at 240 nm was monitored.

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by GIANNOPOLITIS and RIES (1977). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.5), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and enzyme extract. The reaction mixture that was not exposed to light did not develop color, and served as control. The absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560 nm.

Hydrogen peroxide and ascorbic acid determination

Concentrations of hydrogen peroxide (H_2O_2) and ascorbic acid (AA) were determined by method described in MUKHERJEE and CHOUDHURI (1983). For the determination of H_2O_2 level, the absorbance was measured at 415 nm and concentration was calculated using an extinction coefficient of 1.878 nM^{-1} cm^{-1}. For the measurement of AA level the absorbance was measured at 530 nm and concentration was calculated using an extinction coefficient of 226.2 mM^{-1} cm^{-1}.

Thiobarbituric acid reactive substances determination

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) determined by the thiobarbituric acid (TBA) reaction as described by VERMA and DUBEY (2003). The absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Soluble proteins, organic nitrogen and tissue dry weight determination

The soluble proteins were assayed as described by BRADFORD (1976) using bovine serum albumin (BSA) as a standard. Organic nitrogen was determined by standard Kjeldahl method. The amount of the tissue dry mass (DM) was determined by drying on 105 °C for 24 hours.

Statistical analysis

Obtained data were analyzed using Student's *t*-test modified for small samples ($n = 10$, $P < 0.05$) (PETZ 1997).

Results

Hydrogen peroxide level in green leaves was 39.94 ± 2.96 nmol g^{-1} DM, while in bleached leaves it was 45.99 ± 6.42 nmol g^{-1} DM (Fig. 2). That is 15.16% higher in respect to green leaves. Oppositely, the level of ascorbic acid in bleached leaves was 1.11 ± 0.15 mmol g^{-1} DM and in green ones 1.69 ± 0.15 mmol g^{-1} DM (Fig. 2). So, the amount of AA in bleached leaves was 65.69% of the value in green leaves. TBARS level in green leaves was 19.01 ± 4.80 nmol g^{-1} DM and in bleached ones it was 20.42 ± 6.93 nmol g^{-1} DM (Fig. 2) showing no difference between green and bleached leaves.

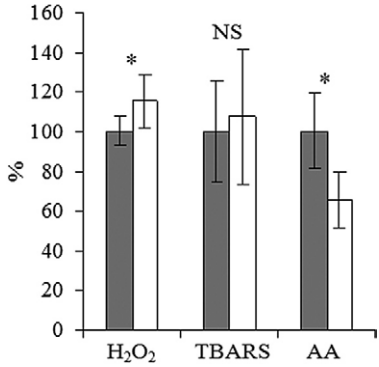

Fig. 2. The levels of hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS) and ascorbic acid (AA) in silver maple leaves (green leaves – grey columns, bleached leaves – white columns). Collumn lengths indicate mean values expressed as 100% in green leaves giving relative levels in white leaves in comparison to green ones. Vertical bars indicate ± S.D., n = 10, P(t) < 0.05, * – significant difference, NS – not significant difference.

The mean values of antioxidative enzyme specific activities, ascorbate peroxidase, guaiacol peroxidase, catalase and superoxide dismutase in green and bleached maple leaves are shown on figure 3. The specific activities of APX were 1.82 ± 0.45 ΔA min^{-1} mg^{-1} proteins in green leaves and 0.78 ± 0.21 ΔA min^{-1} mg^{-1} proteins in bleached leaves. GPOD specific activity was 1.84 ± 0.50 ΔA min^{-1} mg^{-1} proteins in green leaves towards 0.63 ± 0.16 ΔA min^{-1} mg^{-1} proteins in bleached ones. In green leaves the specific activity of CAT was 1.18 ± 0.13 ΔA min^{-1} mg^{-1} proteins while in bleached leaves it was 0.52 ± 0.11 ΔA min^{-1} mg^{-1} proteins. Relative activity of SOD was 15.62 ± 2.32 U mg^{-1} proteins in green and 8.79 ± 2.19 U mg^{-1} proteins in bleached leaves. The enzyme specific activities of APX, GPOD, CAT and SOD in green leaves were almost twice those in bleached leaves.

The mean values of chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoid concentrations in bleached leaves were significantly lower than in green leaves (Tab. 1). Concentration of chlorophyll *a* in bleached leaves was 33.69% of that in green leaves. Chlorophyll *b* concentration in bleached leaves was even lower, only 23.32% of the concentration in green leaves. Consequently, the concentration of total chlorophylls in bleached leaves was 29.13% of that in green leaves. Concentration of total carotenoids in bleached leaves was 59.45% of that for green leaves. Chlorophyll *a* to chlorophyll *b* ratio was 47.95% higher in bleached leavesthan green leaves). The total chlorophylls to total

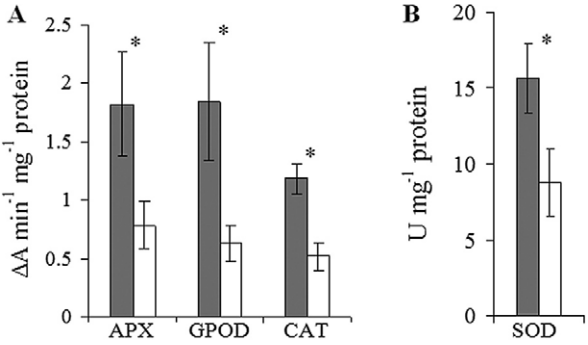

Fig. 3. The antioxidative enzyme specific activity in silver maple leaves (green leaves – grey columns, bleached leaves – white columns): (A) – ascorbate peroxidase (APX), guaiacol peroxidase (GPOD) and catalase (CAT), (B) – superoxide dismutase (SOD). Vertical bars indicate ± S.D., n = 10, P(t) < 0.05, * – significant difference.

Tab. 1. Mean values (± standard deviation) of photosynthetic parameters in green (G) and bleached (B) leaves of silver maple (*Acer saccharinum*). Standard deviation (± S.D) is given in parentheses, n = 10. Chlorophyll *a* – Chl *a*, chlorophyll *b* – Chl *b*, total chlorophylls – Chl *a*+*b*, total carotenoids – Car, maximum quantum yield of PSII – F_v/F_m, P(t) – percent of similarity, DM – dry mass.

Parameter	G	B	P (t)
Chl *a* (mg g^{-1} DM)	5.18 (± 0.62)	1.75 (± 0.31)	< 0.05
Chl *b* (mg g^{-1} DM)	4.07 (± 0.78)	0.95 (± 0.38)	< 0.05
Chl *a*+*b* (mg g^{-1} DM)	9.25 (± 0.93)	2.69 (± 0.63)	< 0.05
Car (mg g^{-1} DM)	1.17 (± 0.26)	0.69 (± 0.09)	< 0.05
Chl *a*/Chl *b*	1.32 (± 0.34)	1.96 (± 0.41)	< 0.05
Chl *a*+*b*/Car	8.26 (± 1.85)	3.92 (± 0.97)	< 0.05
F_v/F_m	0.81 (± 0.01)	0.64 (± 0.08)	< 0.05

carotenoids (Chl *a*+*b*/Car) ratio was 52.50% lower in bleached than in green leaves. The mean value of maximum quantum yield of photosystem II (F_v/F_m) in bleached leaves was also significantly lower, 78.59% of the value in green leaves.

In green leaves the amount of organic nitrogen was 2.72 ± 0.14 m/m% while in bleached leaves it was 3.26 ± 0.38 m/m%, or 19.85% more (Fig. 4). The amount of soluble proteins was almost 3 times higher in bleached leaves (12.21 ± 1.61 mg g^{-1} FM) than in green leaves (3.97 ± 0.90 mg g^{-1} FM). The amount of dry mass was lower in bleached leaves (0.29 ± 0.02 g g^{-1} FM) than in green leaves (0.34 ± 0.02 g g^{-1} FM).

Discussion

Hydrogen peroxide level in bleached leaves was 15.16% higher in respect to green leaves (Fig. 2). Higher level of H_2O_2 content in bleached leaves could be due to decline in its antioxidant response. Ascorbic acid level in bleached leaves was lower (65.69%) than

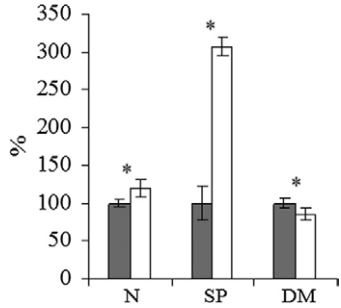

Fig. 4. Organic nitrogen (N), soluble proteins (SP) and dry mass (DM) in silver maple leaves (green leaves – grey columns, bleached leaves – white columns). Column lengths present mean values expressed as 100% in green leaves giving relative levels in white leaves in comparison to green ones. Vertical bars indicate ± S.D., n = 10, P(t) < 0.05, * – significant difference.

the value in green leaves (Fig. 2). AA has multiple functions in photosynthesis and photoprotection, and it plays an important role in the antioxidant defense system in plants (NOCTOR and FOYER 1998, DAVEY et al. 2000). As the AA is involved in the detoxification of ROS including H_2O_2 (CHEN and GALLIE 2006) reduced AA level in bleached leaves could contribute to an increased level of H_2O_2 content in those leaves. Also, the correlation between increase in ROS and suppressing dehydroascorbate reductase expression resulting in less efficient AA recycling was shown (CHEN and GALLIE 2006).

The APX, GPOD, CAT and SOD enzyme specific activities were lower in bleached than in green leaves: 43.05, 34.08, 43.66 and 56.27% of the values in green, respectively. Bleached leaves characterized by lower specific activities of the antioxidative enzymes showed declined antioxidative response and ROS scavenging capability. Free radicals and antioxidants play a significant role during the natural senescence process (ARORA et al. 2002). PROCHAZKOVA et al. (2001) have reported in the case of maize that early senescence was due to enhanced H_2O_2 production and lipid peroxidation, and lower SOD, APX and CAT activity towards aging. Similar results were reported for ginkgo, and birch leaves were H_2O_2 production increased and CAT activity decreased in the early phase of leaf senescence (KUKAVICA and VELJOVIC-JOVANOVIC 2004). Decline in antioxidant enzymes activity has been reported as the possible cause for leaf senescence in plants (YE et al. 2000).

The mean values of concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophylls and total carotenoids in bleached leaves were lower: 33.69, 23.32, 29.13 and 59.45% of the values in green leaves, respectively (Tab. 1). In chloroplasts, the chlorophyll pigments associated with the electron transport system are the primary source for production and scavenging of ROS in leaf cells (ARORA et al. 2002). The lower pigment contents in maize leaves were described previously as the consequence of increased hydrogen peroxide content (PROCHAZKOVA et al. 2001). Chlorophyll *a* to chlorophyll *b* ratio was 47.95% higher in bleached than in green leaves (Tab. 1). Such increased Chl *a*/Chl *b* ratio showed that chlorophyll *b* was degraded faster in bleached leaves. Total chlorophylls to total carotenoids (Chl *a*+*b*/Car) ratio was lower in bleached (for 52.50%) than in green leaves (Tab. 1), due to faster chlorophyll degradation. Lower chlorophyll content in bleached leaves reflected on its maximum quantum yield of PSII (Fv/Fm). The mean value of Fv/Fm in bleached leaves was significantly lower, 78.59% of the value in green leaves (Tab. 1). The Fv/Fm in

bleached leaves was below 0.75, which is considered the boundary value for fully functional PSII (BOLHÁR-NORDENKAMPF et al. 1989). A lower Fv/Fm value in bleached leaves indicates a stress situation as well as declined photosynthesis. The lowering of photosynthetic parameters was previously reported in leaves of maize, ginkgo and birch that also contained higher level of H_2O_2 (FOYER et al. 2002, KUKAVICA and VELJOVIC-JOVANOVIC 2004, OUGHAM et al. 2008).

Accumulation of thiobarbituric acid-reactive substances is often used as an indicator of lipid peroxidation. In the present study, TBARS level did not show a difference between green and bleached leaves (Fig. 2). Because of the unchanged TBARS level, increased H_2O_2 level seems to be not very harmful for bleached leaves. Generally, a coordinated regulation of the free radical scavenging system comprising CAT, SOD, APX, ascorbate and glutathione is essential (ZIMMERMANN and ZENTGRAF 2005). The promoted ability in scavenging does not always prevent the increase in H_2O_2 content (LU et al. 2009). Also, among the different ROS, only H_2O_2 is relatively stable and able to penetrate the plasma membrane as an uncharged molecule. In addition to being a toxicant H_2O_2 has been regarded as a signaling molecule acting in initiating transduction pathway towards plant cell death (CHAKRABARTY et al. 2009). The increased radical levels displayed during senescence are not only caused by the elevated production of radicals but also by a loss in antioxidant capacity. The degradation of chlorophylls and the membranes causes an increase in the production of free radicals. In addition, the amount of reduced oxygen, e. g. H_2O_2, increases greatly during senescence. Lipid peroxidation, measured as TBARS level leads to the generation of free radicals which in turn initiates an increase in ethylene formation leading to the promotion of senescence (ZIMMERMANN and ZENTGRAF 2005, YAN et al. 2010). Although our investigations showed an increase of H_2O_2 and no significant increase of TBARS level, we could speculate that the increase of 15% in H_2O_2 and increase of 7% in TBARS together with the lowering of CAT, GPOD, APX and SOD activity leads to a certain loss in antioxidant capacity and the slow promotion of senescence. Accelerated leaf senescence is one of the harmful effects of elevated troposferic ozone concentrations on plants (GIELEN 2007, YAN et al. 2010). Moreover, according to the data on the photosynthesis performance and biochemistry this could indicate that bleaching was not a simple symptom of premature aging but also an adaptation to high-light induced oxidative stress during summer (LEPEDUŠ et al. 2011).

The relative levels of organic nitrogen (for 19.85%) and soluble proteins (for 208.05%), were higher in bleached leaves while dry mass was lower than in green ones (85.49% of the value in green leaves) (Fig. 4). PERRY and HICKMAN (2001) have reported that mean leaf nitrogen concentrations for silver maple healthy leaves was 2.52%. data revealed that the nitrogen level in bleached leaves (3.26%) was higher than that mean value (PERRY and HICKMAN 2001) and higher than the value in green leaves (2.72%). Natural senescence is characterized by protein degradation and mobilization of released nitrogen to the developing parts of the tree or to the storage tissues (VALJAKKA et al. 1999, VANACKER et al. 2006). In our study, bleached leaves do have more nitrogen and especially more soluble proteins, although antioxidative enzymes specific activities were reduced showing that some other proteins were having a more intensive biosynthesis. This is rather the indication of some type of stress situation leading to premature aging of bleached leaves, than of the natural senescence process.

It can be concluded that decreased chloroplast pigments concentrations and reduced maximum quantum yield PSII (F_v/F_m) in bleached leaves indicated their impaired photosynthetic performance. Bleached leaves were also characterized by lower specific activities of the antioxidative enzymes showing declined antioxidative response and ROS scavenging capability. Increased level of hydrogen peroxide content in bleached leaves as the consequence of deficient antioxidative response could be the reason for the down-regulation of photosynthetic performance as well as premature aging of those leaves. Judging from unchanged the TBARS level, the increased H_2O_2 level was not deleterious to bleached leaves. It can be concluded that the down-regulation of photosynthetic performance was sufficient to enable the bleached leaves to keep their functionality in the beginning of summer. Extended investigations will comprise detailed analysis of PSII photochemistry and abundance of key photosynthetic proteins in order to investigate mechanisms of photosynthetic down regulation in bleached leaves.

Acknowledgments

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