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Plant shading increases lipid peroxidation and intensifies senescence-induced changes in photosynthesis and activities of ascorbate peroxidase and glutathione reductase in wheat

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Abstract

Plants of spring wheat (*Triticum aestivum* L. cv. Saxana) were grown during the autumn. Over the growth phase of three leaves (37 d after sowing), some of the plants were shaded and the plants were grown at 100 (control without shading), 70, and 40 % photosynthetically active radiation. Over 12 d, chlorophyll (Chl) and total protein (TP) contents, rate of CO_2 assimilation (P_N), maximal efficiency of photosystem 2 photochemistry (F_V/F_P), level of lipid peroxidation, and activities of antioxidative enzymes ascorbate peroxidase (APX) and glutathione reductase (GR) were followed in the 1st, 2nd, and 3rd leaves (counted according to their emergence). In un-shaded plants, the Chl and TP contents, P_N, and F_V/F_P decreased during plant ageing. Further, lipid peroxidation increased, while the APX and GR activities related to the fresh mass (FM) decreased. The APX activity related to the TP content increased in the 3rd leaves. The plant shading accelerated senescence including the increase in lipid peroxidation especially in the 1st leaves and intensified the changes in APX and GR activities. We suggest that in the 2nd and 3rd leaves a degradation of APX was slowed down, which could reflect a tendency to maintain the antioxidant protection in chloroplasts of these leaves.

Additional key words: ageing; chlorophyll fluorescence; CO_2 assimilation; plant topography; *Triticum aestivum*.

Introduction

Plant senescence is a systemically organized heterogeneous process within the whole plant organism. Both plant signalling and gene expression are among the regulating factors in whole plant senescence, however, it is still discussed to what degree plant organ senescence is a matter of pure genetic, hormone, or combined control (Thomas *et al.* 2003).

There is an insertion gradient of leaf characteristics within a plant determined by different phases of ontogeny of subsequently formed leaves and by various micro-environmental conditions, especially irradiance (Šesták 1985). A typical phenomenon of whole plant senescence is a remobilization of nutrients from the lower (older) leaves to the upper (younger) ones. For this reason, the topography of a plant should be followed when studying whole plant senescence. The topographic measurement of leaf characteristics (*i.e.* measurement on several leaves of different insertion) makes possible to determine structural and functional heterogeneity within the plant that can differ under different conditions. The concept of topography gradients of measured parameters [*e.g.* the chlorophyll (Chl) fluorescence ones] might be of relevance (Matoušková *et al.* 1999). There is also an age gradient of structural and functional parameters within one leaf (*e.g.* Buschmann 1981, Nauš *et al.* 1985, Šesták and Šiffel 1997).

Leaf senescence is characterized by a decrease in the rate of photosynthesis. This decrease is associated with a progressive loss of Chls and with a decline in activities of the photosystems and reactions of the Calvin cycle. The senescence-induced inhibition of the Calvin cycle reactions (caused mainly by a decline in amounts and activities of stromal enzymes, especially ribulose-1,5-bisphosphate carboxylase/oxygenase) usually precedes

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Abbreviations: APX, ascorbate peroxidase; Chl, chlorophyll; DM, dry mass; FM, fresh mass; GR, glutathione reductase; LA, leaf area; PAR, photosynthetically active radiation; PS2, photosystem 2; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TP, total proteins.
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a decrease in function of the pigment-protein complexes in thylakoid membranes (Camp *et al.* 1982, Grover 1993). In particular, photochemistry of photosystem 2 (PS2) is diminished very little during senescence of leaves (Špundová *et al.* 2003).

A substantial decrease in net photosynthetic rate (P_N) accompanied by only a slight decrease in PS2 photochemistry can lead to photoinhibition and an increased generation of reactive oxygen species (ROS) in senescent leaves. Upon weakening of the anti-oxidative defence mechanisms oxidative degradation of pigments, proteins, and lipids is initiated (Munné-Bosch and Allegre 2002, Jing *et al.* 2003). From this point of view, a decrease in irradiance should lead to a lower photo-generation of ROS and a lower oxidative damage. The leaves of low insertion usually grow under low irradiance so a low oxidative damage could be expected. On the other hand, a lower P_N due to limitation of excitation energy may cause a shortage of cell energy and a failure of anti-oxidative defence mechanisms so that oxidative damage could increase in these leaves.

Several enzyme cycles participate in the anti-oxidative defence of plants. The antioxidant enzymes ascorbate peroxidase (APX) and glutathione reductase (GR) are the main enzymes of the ascorbate-glutathione cycle that is a part of the water-water cycle in chloroplasts (Asada 1999, 2000) but also appears in other organelles (Jiménez *et al.* 1997). The water-water cycle scavenges ROS (superoxide and hydrogen peroxide) photo-generated mostly by thylakoid membranes. Senescence of detached leaves (Feng *et al.* 2003), seeds (Goel and Sheoran 2003), or stressed plants (Jiang and Huang 2001) is usually accompanied by a decrease in APX and GR activities in parallel with an increase of lipid peroxidation. In naturally senescing cucumber cotyledons, the GR activity decreased whereas APX activity increased (Kanazawa *et al.* 2000). Irradiance can influence significantly the senescence-induced changes in anti-oxidative enzyme activities (Kar *et al.* 1993, Kanazawa *et al.* 2000, Yamazaki and Kamimura 2002). This phenomenon is not usually taken into account in senescence studies.

In this project we investigated the effect of reduced irradiance on senescence of wheat leaves in different insertion. We focused on changes in lipid peroxidation and in the APX and GR activities in order to determine how the decrease in irradiance affects these changes in the senescing leaves.

Materials and methods

Plants and their shading: Plants of spring wheat (*Triticum aestivum* L. cv. Saxana) were grown in pots with an artificial medium composed of perlite and Knop's solution at autumn season (October, November) in a greenouse in Olomouc, Czech Republic. Irradiance changed according to weather conditions; the maximal irradiance at midday was around 500 μmol(photon) m^{-2} s^{-1} of photosynthetically active radiation (PAR). The day temperature varied between 16 and 24 °C and the relative air humidity was 33–52 %. 37 d after sowing the pots with plant seedlings were divided into three groups. Two of them were shaded with a non-woven fabric (permeable to water) so that three irradiances were set: 100 % (un-shaded plants), 70 %, and 40 % of PAR. The first, second, and third leaves of the plants (counted from the shoot base) were used for measurements that were carried out on the day of shading (0 d) and on the 6^{th} and 12^{th} d of shading. In order to eliminate (at least partly) an effect of age gradient within the leaf, only middle part of leaf blades was used for measurements. The shading slowed down the growth rate of plants. The growth phases according to Feekes (1941) are shown in Table 1.

Chl and total protein (TP) contents: Chl content was determined spectrophotometrically (*Unicam UV550, Thermospectronic*, Cambridge, UK) in 80 % acetone extract prepared from the middle part of leaf blades (centrifugation: 5 min, 3 600×*g*) according to Lichtenthaler (1987). The content of TP was estimated by a modified method of Lowry *et al.* (1951) using ovalbumin as a protein standard.

P_N was measured using an open gasometric system (*LCA–4, ADC*, Hoddesdon, UK). Middle parts of blades (625 mm^2) of attached wheat leaves were placed into a leaf chamber (CO_2 concentration 350 μmol mol^{-1}, temperature 24 °C) and dark-adapted for 7 min, then the "white" actinic irradiation [290 μmol(photon) m^{-2} s^{-1}] was switched on. P_N was measured within the 19^{th} and 22^{nd} min of the actinic irradiation every 30 s and the mean value was estimated.

Maximal efficiency of PS2 photochemistry [$F_V/F_P = (F_P - F_0)/F_P$] was measured at room temperature with a fluorometer *PEA* (*Hansatech*, King's Lynn, UK) from the adaxial side of pre-darkened (15 min) leaves. F_0 was the minimal Chl fluorescence intensity and F_P was the fluorescence intensity at a P-level of the O-J-I-P transient (Strasser and Govindjee 1992). The excitation irradiance was 4 800 μmol(photon) m^{-2} s^{-1}, time of detection was 2 s. General statistical description (medians and quartiles) was used for the F_V/F_P ratio (Lazár and Nauš 1998).

Fresh mass (FM) and dry mass (DM) were determined from a set of 15 leaf blade segments cut 1 cm from apex and 1 cm from the leaf base. FM was determined immediately after detachment of the segments, DM was determined after oven-drying of the segments for 1 h at 105 °C. Both quantities were related to the leaf area (LA).

Lipid peroxidation in wheat leaves was determined from the content of thiobarbituric acid (TBA) reaction products according to a modified procedure of Dan *et al.* (1996): 0.5 g of segments from the middle part of leaf blades was homogenized in 5 cm^3 of 0.1 % trichloroacetic acid (TCA). A filtrated homogenate was centrifuged at 10 000×*g* for 10 min at 4 °C. A 1 cm^3 of supernatant was mixed with 4 cm^3 of 20 % TCA containing 0.5 % TBA. The mixture was heated in boiling water for 30 min and then quickly cooled in an ice-bath. After centrifugation at 10 000×*g* for 10 min at 4 °C, the absorbances of the supernatant at 532 nm (A_{532}) and 600 nm (A_{600}) were measured (*Unicam UV550, ThermoSpectronic*, Cambridge, UK) using 0.5 % TBA in 20 % TCA as a blank. A 1 cm cuvette was used. A difference of absorbances ($\Delta A = A_{532} - A_{600}$) reflecting the content of TBA reaction products was used as an indicator of lipid peroxidation (Dan *et al.* 1996).

Leaf homogenate was prepared from the leaf blade segments cut 1 cm from apex and 1 cm from the leaf base. Two grams of segments were placed in aluminium foil, immersed in liquid nitrogen (see also Jiang and Huang 2001) for 2 min, and then ground. The leaf powder was overlaid by 10 cm^3 of the extraction buffer (5 °C). The composition of extraction buffer was 0.1 M Tris (pH 7.8), 1 mM dithiothreitol, 1 mM $EDTANa_2$, 1 % (m/v) *Triton X-100*, 4 % (m/v) polyvinylpolypyrrolidone, and 5 mM ascorbic acid. The mixture was homogenized in an interrupted regime (*Ultra-Turrax T 25B, IKA–Labortechnik*, Germany) for 1 min (13 500 rotations per min). Then the homogenate was exposed for 1 min to ultrasound (*Transsonic T 460 H, Elma*, Germany). After 30 min incubation on ice the homogenate was centrifuged (21 000×*g* for 10 min at 4 °C). The supernatant was filtered through 4 layers of gauze and placed to Eppendorf cuvettes. All the steps were done at about 4 °C. The homogenate was then stored at –80 °C for subsequent enzymatic assays and TP content determination.

APX and GR activities: The APX activity was assayed according to Nakano and Asada (1981) by measuring the decrease in absorbance at 290 nm that reflects the ascorbate oxidation. The reaction mixture for measuring of the APX activity contained 95 mM HEPES-EDTA (pH 7.0), 0.5 mM Na-ascorbate, 0.88 mM H_2O_2, and 50 mm^3 of the leaf homogenate in 3 cm^3 of the mixture. The GR activity was assayed using a modified method of Foyer and Halliwell (1976) by measuring the decrease in absorbance at 340 nm that reflects the consumption of NADPH. The reaction mixture for measuring of the GR activity contained 91 mM Tris-EDTA (pH 7.8), 0.1 mM NADPH, 1.0 mM glutathione (oxidized form, GSSG), and 100 mm^3 of the leaf homogenate in 3 cm^3 of the mixture. The absorbance changes were measured by a spectrophotometer (*Unicam UV550, Thermospectronic*, Cambridge, UK) during the 2 min of the reaction. A 1 cm cuvette was used; the exact temperature 25 °C and sample stirring were insured by a special cuvette holder. The spectral slit-width was 1 nm.

Results

In order to describe the situation of the whole plant during senescence, a method of plant topography (see Šesták 1985, Šesták and Šiffel 1997, Matoušková *et al.* 1999) was used. The first three fully developed leaves of wheat plants were followed. The youngest developing leaves (4th and 5th) were not measured (see Table 1).

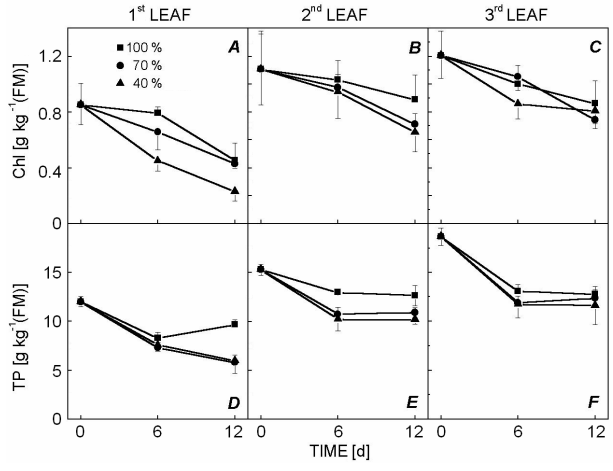

Fig. 1. Chlorophyll (Chl) (*A–C*) and total protein (TP) (*D–F*) contents related to the fresh mass (FM) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, $n = 6$.

Table 1. The growth phase, *i.e.* number of developed leaves (Feekes 1941) of wheat plants (*Triticum aestivum* L. cv. Saxana) grown under different irradiances. In brackets, the number of developed leaves is shown.

Time of experiment [d]	Growth phase 100 % PAR	70 % PAR	40 % PAR
0	1.3 (3)	1.3 (3)	1.3 (3)
6	1.4 (4)	1.4 (4)	1.3 (3)
12	1.5 (5)	1.4 (4)	1.4 (4)

A decrease in Chl and TP contents was observed in all the measured leaves during the experiment (Fig. 1). The lowest values were found in the 1st leaf. The plant shading stimulated the decrease in Chl and TP contents, especially in the 1st leaves (Fig. 1*A,D*). Similar trends were found in P_N (Fig. 2*A-C*) and maximal efficiency of PS2 photochemistry (F_V/F_P) (Fig. 2*D–F*). In all the measured leaves, a relative decrease in P_N with plant ageing was

higher than a relative decrease in F_V/F_P, which indicated a more pronounced senescence-induced inhibition of stromal reactions in comparison with PS2 photochemistry.

The FM (related to the LA) was mostly slightly higher in the 1st leaves in comparison with the 2nd and 3rd ones (Fig. 3*A–C*). This observation indicates that the plants were sufficiently supplied with water and did not relocate it from the oldest leaves to the younger ones. The DM (related to the LA) was usually slightly higher in the 3rd leaves compared with the older leaves (Fig. 3*D–F*). No pronounced changes (up to about 15 %) were found in the FM and DM during plant ageing or after plant shading.

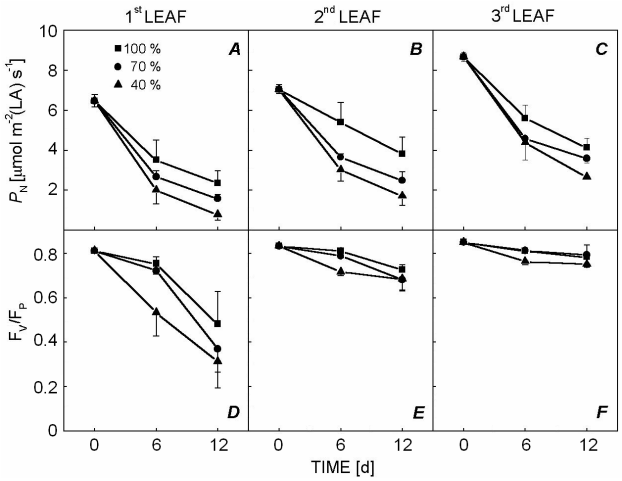

Fig. 2. The net photosynthetic rate (P_N) related to the leaf area (LA) (*A–C*) and the maximal efficiency of photosystem 2 photochemistry (F_V/F_P; *D–F*) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. For P_N, means and SD (n = 3), and for F_V/F_P, medians and quartiles (n = 7) are shown, respectively.

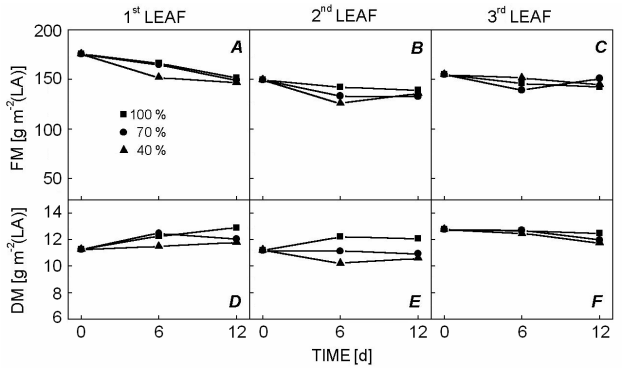

Fig. 3. The fresh mass (FM; *A–C*) and dry mass (DM; *D–F*) related to the leaf area (LA) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances.

The lipid peroxidation increased during ageing in all the measured leaves (Fig. 4). In the un-shaded plants, the highest relative increase (of about 160 %) was found in the 3rd leaves (Fig. 4*C*) whereas a relative increase found in the 1st leaves was smaller (of about 40 %) because of a relatively high lipid peroxidation at the beginning of experiment (Fig. 4*A*). Plant shading stimulated the increase in lipid peroxidation slightly in the 3rd leaves and considerably in the 1st leaves (Fig. 4*A*,*C*).

The APX activity related to FM was lower in the 1st leaves compared with the 2nd and 3rd ones during whole experiment (Fig. 5*A–C*). During plant ageing, the APX activity decreased slightly in all the measured leaves. In the 1st leaves, this decrease was stimulated by plant shading (Fig. 5*A*). The APX activity related to the TP content did not almost change in the 1st and 2nd leaves and

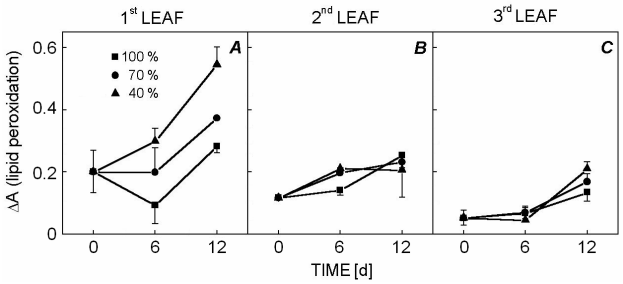

Fig. 4. Lipid peroxidation (expressed as the difference of absorbances $\Delta A = A_{532} - A_{600}$ reflecting the content of products of thiobarbituric acid reaction) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, n = 3.

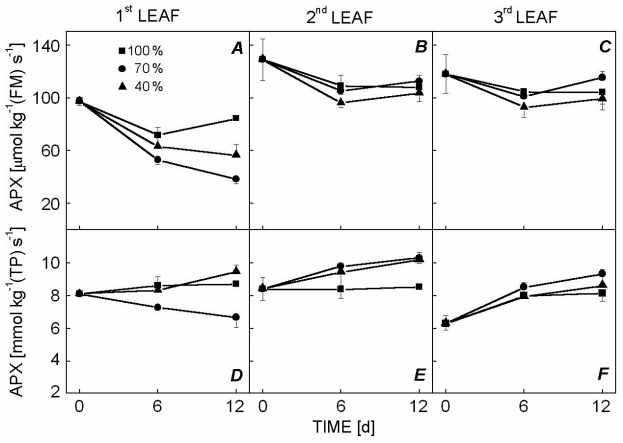

Fig. 5. The activity of ascorbate peroxidase (APX) related to the fresh mass (FM; *A–C*) and total protein content (TP; *D–F*) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, n = 3.

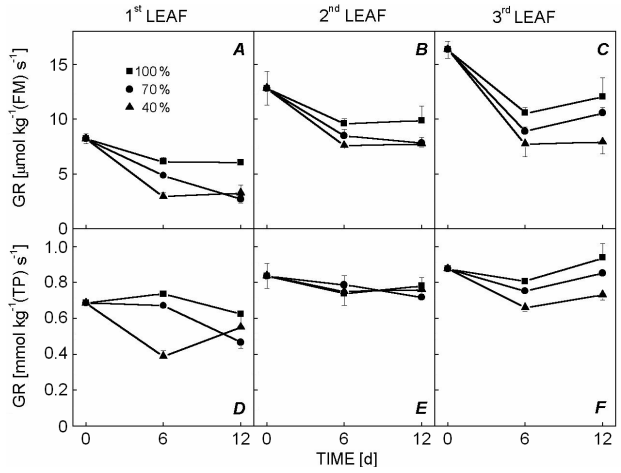

Fig. 6. The activity of glutathione reductase (GR) related to the fresh mass (FM; *A–C*) and total protein content (TP; *D–F*) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, $n = 3$.

Discussion

The changes in parameters measured in the un-shaded wheat plants revealed that these plants were undergoing the senescence process during the experiment despite their relatively low age (37–49 d). Probably the plants suffered from the low irradiance and short day due to their growing in the autumn. During the 12 d of the experiment, the Chl and TP contents (Fig. 1), and P_N and maximal efficiency of PS2 photochemistry (Fig. 2) decreased while the rate of lipid peroxidation increased (Fig. 4) in all the measured leaves. The values of the mentioned parameters indicated that senescence of the 1st leaves had already started before the beginning of experiment and became more pronounced during it. The most pronounced senescence of the oldest leaves corresponds to the expected remobilization of nutrients from these leaves to the younger parts of the plant.

Senescence is accompanied by an increasing generation of ROS and consequent oxidative damage (Munné-Bosch and Allegre 2002, Jing *et al.* 2003). One of the reasons for this phenomenon is an increasing imbalance between generation and consumption of electrons in the photosynthetic electron transport chain caused by a preferential inhibition of stromal reactions compared with the PS2 photochemistry (Camp *et al.* 1982, Grover 1993, Špundová *et al.* 2003). The inhibition of stromal reactions leads to an increasing probability of the electron flow to molecular oxygen, consequently ROS accumulate and photo-damage to the chloroplast components increases. Most probably that was the situation in the un-shaded wheat plants—the relative decrease in P_N during plant ageing was much higher than the relative decrease in the PS2 photochemistry (F_V/F_P) (Fig. 2) and lipid peroxidation increased (Fig. 4).

Oxidative damage to the cellular components can be mitigated by operation of anti-oxidative enzymes. We investigated senescence-induced changes in the activities of APX and GR, the main enzymes of the ascorbate-glutathione cycle that is a part of the water-water cycle in chloroplasts (Asada 1999, 2000). In order to interpret changes in the APX and GR activities correctly, attention was paid to the choice of reference quantity because it can influence the nature of trends. The enzyme activities were related to FM, DM, and TP contents. The FM and DM changed minimally during senescence (Fig. 3) therefore they both could be taken as the proper reference quantities. The changes of APX and GR activities related to both the FM and DM were nearly the same so only the FM was chosen as the representative quantity (Figs. 5*A–C*, 6*A–C*). The APX and GR activities related to the TP content (Figs. 5*D,E*, 6*D,E*) should indicate their relative degradation in comparison with the rate of general degradation of proteins.

In the un-shaded plants, the activity of anti-oxidative system seemed not to be stimulated during senescence as the APX and GR activities related to the FM decreased with the increase in leaf age and lipid peroxidation. However, the APX activity related to the TP content barely changed with ageing in the 1st and 2nd leaves and even increased in the 3rd leaves (Fig. 5*D–F*). Hence the degradation of the APX might be slower than degradation of total proteins, especially in the 3rd leaves. The slower degradation of the APX could reflect a tendency to maintain the antioxidant protection of chloroplast components as about 90 % of the total leaf APX activity is in the increased in the 3rd leaves (by about 30 %) during ageing of the un-shaded plants. Shading of the plants stimulated this increase in both the 2nd and 3rd leaves (Fig. 5*E,F*). The smallest stimulation of increase or even decrease (in 40 % PAR) was found in the 1st leaves (Fig. 5*A*).

The GR activity related to the FM was again lowest in the 1st leaves (Fig. 6*A*) in comparison with the younger leaves and decreased with plant ageing in all the measured leaves (Fig. 6*A–C*). Shading of the plants stimulated this decrease. A relative decrease in activity related to the FM was more pronounced in the case of GR in comparison with APX, especially in the shaded plants (compare Figs. 5 and 6). The GR activity related to the TP content decreased only slightly in the 1st and 2nd leaves of un-shaded plants (Fig. 6*D,E*), in the 3rd ones even slightly increased (by about 6 %) (Fig. 6*F*). Plant shading stimulated the decrease in the 1st leaves and decreased the activity in the 3rd leaves (Fig. 6*D,F*).

chloroplasts (Gillham and Dodge 1986). The GR activity related to TP did not increase significantly with ageing, which indicates that the rate of GR degradation was probably similar to the rate of TP degradation but higher than the rate of APX degradation.

Shading of the plants intensified the senescence-induced decrease in Chl and TP contents, P_N, and maximal efficiency of PS2 photochemistry. This effect was most pronounced in the 1st leaves. A decrease in supply of excitations due to plant shading should slow down the photosynthetic electron generation and consequently suppress the photo-generation of ROS and oxidative damage. Contrary to this expectation, the rate of lipid peroxidation mostly increased in the shaded plants, especially in the 1st leaves (Fig. 4*A*). The high increase of lipid peroxidation in the 1st leaves might be caused by the more pronounced degradation of the APX and GR: their activities related to the FM (and to the TP content too in the case of GR) decreased faster (in most cases) than in the 1st leaves of unshaded plants. Similarly, the APX activity related to the FM decreased with decreasing irradiance in leaves of *Cucurbita pepo* and *Vinca major* (Logan *et al.* 1998) or pea (Gillham and Dodge 1987). Yamazaki and Kamimura (2002) also explained a decrease in the APX activity (related to the LA) within rice plants from the top to bottom leaves as a consequence of decreasing irradiance but the lower activity in the bottom leaves seemed to be caused simply by senescence as in our un-shaded plants.

On the other hand, the decrease in APX activity related to FM was not markedly stimulated in the 2nd and 3rd leaves of the shaded plants in comparison with the unshaded ones (Fig. 5*B*,*C*). Moreover, the increase of APX activity related to the TP content was intensified in these leaves (Fig. 5*E*,*F*). These results indicate a tendency in the younger leaves of shaded plants to slow down a senescence-induced decrease in the APX activity.

Summing up, the shaded plants accelerated senescence of the 1st leaves probably in order to maintain the functionality of the younger leaves. In the latter leaves we suppose a more pronounced tendency to maintain the antioxidant protection in chloroplasts. However, the "low-light" stress was probably too strong because the situation of the 2nd and 3rd leaves got worse too, as compared with the un-shaded plants. The main reason of the accelerated senescence of the oldest leaves was probably the pronouncedly increased oxidative damage due to weakening of anti-oxidative protection.

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