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# Changes in antioxidative protection in bean cotyledons during natural and continuous irradiation-accelerated senescence

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## Abstract

We employed continuous irradiation (CL) for induction of premature senescence caused by enhanced production of reactive oxygen species. As a model plant we used bean (*Phaseolus vulgaris* L. cv. Jantar) cotyledons because they have well defined and a quite short life span. Senescence of bean cotyledons induced by CL progressed more rapidly than natural senescence: the life span of CL cotyledons was 13 d compared to 16 d in controls (C). Chl content was significantly lower in 10- and 13-d-old CL plants than in C plants and the change with age was not statistically significant. Activities of all antioxidative enzymes declined either with senescence onset or during whole life span. Activity of antioxidative enzymes, except ascorbate peroxidase, was lower in CL plants compared to C plants. On the contrary, contents of non-enzymatic antioxidants β-carotene and ascorbate were higher in CL plants than in C plants. No significant difference, except in the youngest cotyledons, was observed in glutathione content.

*Additional key words*: antioxidants, *Phaseolus vulgaris* L., reactive oxygen species, senescence.

## Introduction

Senescence is a genetically regulated sequence of physiological and biochemical processes between maturity and death of a plant or a plant part. Senescence is not only a degenerative process, but it is also a recycling process in which nutrients are translocated from the senescing cells to young leaves, developing seeds or storage tissues (Gan and Amasino 1997). This is a reason why the rise of both degradative and protective activities is promoted in senescence.

Senescence is mainly characterized by a cessation of photosynthesis, disintegration of organelle structures, intensive losses of chlorophyll and proteins, and dramatic increases in lipid peroxidation and membrane leakiness (Buchanan-Wollaston 1997). During leaf senescence an increase in the concentration of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide ($H_2O_2$) was observed (Kar and Feierabend 1984, Thompson and Barber 1987, Leshem 1988, Strother 1988, Longa *et al*. 1994). To counteract the toxicity of reactive oxygen species a highly efficient antioxidative defense system, composed of both enzymatic and non-enzymatic constituents, is present in all plant cells (Foyer *et al*. 1994).

Superoxide dismutases (SOD; EC 1.15.1.1) are a group of metalloenzymes that catalyze the disproportionation of superoxide molecules (McCord and Fridovich 1969). SOD is contained mainly in chloroplasts, cytosol, mitochondria and peroxisomes (Rabinowitch and Fridovich 1983, Del Río *et al*. 1983). Ascorbate peroxidase (APOD; 1.11.1.11) is a hydrogen peroxide-scavenging enzyme, which is specific to plant cells (Asada 1992); it is localized in chloroplasts, mitochondria, cytosol and peroxisomes. Catalase (CAT; 1.11.1.6) catalyzes the disproportionation of $H_2O_2$ in peroxisomes to water and molecular oxygen as well as the reduction of $H_2O_2$ to water using a variety of two- and one- electron donors (Sichak and Dounce 1986).

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*Abbreviations*: APOD - ascorbate peroxidase; AsA - reduced ascorbate; C plants - control plants; CAT - catalase; Chl - chlorophyll; CL plants - continuously irradiated plants; DHA - oxidized ascorbate; DTT - dithiothreitol; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; PS 2 - photosystem 2; ROS - reactive oxygen species; SOD - superoxide dismutase.

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Glutathione reductase (GR; 1.6.4.2) catalyzes regeneration of non-enzymatic antioxidant glutathione (GSH) from its oxidized form (GSSG) (Schaedle and Bassham 1977). The decrease of SOD, APOD, CAT and GR activity during natural senescence has been described in various plants (Pauls and Thompson 1984, Jimenéz *et al*. 1998, Procházková *et al*. 2001, Feng *et al*. 2003).

Non-enzymatic antioxidant ascorbate is a major primary antioxidant, reacting directly with hydroxyl radicals, superoxide and singlet oxygen (Buettner and Jurkiewicz 1996, Noctor and Foyer 1998). Ascorbate plays an important role in preserving the activities of enzymes that contain prosthetic transition metal ions. It is also a powerful secondary antioxidant, reducing the oxidized form of α-tocopherol (Padh 1990, Noctor and Foyer 1998). Content of reduced ascorbate (AsA) decreased during senescence in oat leaf segments (Borraccino *et al*. 1994), cucumber cotyledons (Feng *et al*. 2003) and chrysanthemum petals (Bartoli *et al*. 1997). Glutathione, due to its sulphydryl group, reacts directly with ROS or with oxidized substrates reducing them (Tausz 2001). Ratio of oxidized (GSSG) to reduced (GSH) glutathione ratio increased with a concomitant decrease in reduced glutathione content during senescence of soybean nodule (Evans *et al*. 1999) and pea leaves (Jimenéz *et al*. 1998) and during ageing of tomato seeds (De Vos *et al*. 1994). Carotenoids are effective quenchers of singlet oxygen (Foyer and Harbinson 1999). β-carotene is an efficient quencher of the excited triplet state of chlorophyll molecules. Carotenoid content decreased during senescence of apricot leaves (Scebba *et al*. 2001), in sage leaves (Munné-Bosch *et al*. 2001), and in *Cistus clusii* leaves (Munné-Bosch and Alegre 2002).

Environmental stresses such as low or high temperature, drought, poor light or pathogen attack will all result in premature initiation of senescence (Buchanan-Wollaston 1997). Differences of gene expression in natural and artificially induced senescence were found in barley leaves (Becker and Apel 1993), whereas similarities were observed in asparagus (King *et al*. 1995, Kanazawa *et al*. 2000). It is also unclear whether changes in antioxidative enzymes in artificially induced senescent plant cells are similar to those found in naturally senescent cells (Kanazawa *et al*. 2000). Because the photosynthetic electron transport is the major source of ROS (Asada 1994), we used continuous irradiation for evocation of enhanced production of ROS: for example, a rapid ageing in continuous irradiation was described in *Arabidopsis thaliana* (Hensel *et al*. 1993). As a model plant we used *Phaseolus vulgaris* L. cv. Jantar cotyledons because they have well defined and a quite short life span.

## Materials and methods

**Plants:** French bean (*Phaseolus vulgaris* L. cv. Jantar) plants were grown in sand in plastic pots in a growth chamber (*Klimabox* 1300, Kladno, Czech Republic). The control plants (C plants) were grown under 16-h photoperiod, temperature 24/18 °C, air humidity 60/80 % and irradiation of 220 μmol(photon) $m^{-2}$ $s^{-1}$ (for detail see Wilhelmová *et al*. 1997). Continuously irradiated plants (CL plants) were grown under continuous irradiation 220 μmol $m^{-2}$ $s^{-1}$, temperature 24 °C and air humidity 60 %. All plants were watered daily by distilled water and twice a week by Hewitt nutrient solution. The cotyledons of C plants were harvested at plant age 8, 10, 13 and 16 d, cotyledons of CL plants were harvested at plant age 8, 10 and 13 d after sowing. Samples were deep frozen in liquid nitrogen immediately after harvesting and stored at -70 °C until analysed for content of proteins, pigments and antioxidants. Fresh samples were used for fluorescence measurements.

**Preparation of enzyme extracts:** For APOD, CAT and GR assay cotyledon extracts were prepared by homogenization in the extraction medium (0.1 M Tris, $10^{-3}$ M dithiothreitol (DTT), $10^{-3}$ M ethylenediamine-tetraacetic acid (EDTA)$Na_2$, 1 % *Triton X-100*, $5.10^{-3}$ M ascorbate, pH 7.8), 5 $cm^3$ $g^{-1}$(f.m.). Then the samples were processed for 2 min by ultrasound in an ice bath, after which they were incubated in ice for 30 min, then they were centrifuged for 10 min, 20 000 *g* at 2 °C in (*Heraeus* 700, Osterode, Germany). For SOD assay cotyledons were homogenized in the extraction medium (0.01 M phosphate buffer, 0.03 M KCl, pH 7.4) 5 $cm^3$ $g^{-1}$(f.m.). The samples were processed by ultra-sound in an ice bath in the same manner as in the case of other enzymes. After centrifugation (10 min, 20 000 *g*, 2 °C) the supernatant was desalted (*Sephadex* G-25). The supernatant was frozen in liquid nitrogen and stored at -70 °C for further analysis.

**Measurements of antioxidant enzymes activity:** The activities of SOD, APOD and GR as well as the contents of AsA and GSH were measured spectrophotometrically (*Hitachi* U 3300, Tokyo, Japan) at 25 °C. Total SOD activity was measured at 550 nm with the cytochrome *c* (McCord and Fridovich 1969). One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50 % inhibition of the cytochrome *c* reduction rate. APOD activity was determined as a decrease in absorbance at 290 nm due to ascorbate oxidation (Nakano and Asada 1981). GR activity was determined as a decrease in absorbance at 340 nm due to oxidation of NADPH (Schaedle and Bassham 1977). CAT activity was estimated polarographically (del Río *et al*. 1977)

using a liquid-phase oxygen electrode (*Hansatech Instruments*, King′s Lynn, Great Britain).

**Non-enzymatic antioxidants content:** Ascorbate assay was based on the reduction of $Fe^{3+}$ by ascorbate in acid solution. $Fe^{2+}$ forms complexes with bipyridyl, giving a pink colour with the maximum absorbance at 525 nm (Law *et al*. 1983). Cotyledons (1 g) were homogenized with a pestle in ice-cold 5 % metaphosphoric acid (1/10, m/v) in a prechilled mortar. Total ascorbate (AsA + DHA) was measured after a prior reduction of dehydro-ascorbate to ascorbate carried out by dithiothreitol. The concentration of dehydroascorbate was calculated from the difference of total ascorbate and reduced ascorbate. A standard curve was prepared with the concentration 0 - 100 μM AsA. For glutathione assay cotyledons (1 g) were homogenized with a pestle in ice-cold 5 % sulfosalicylic acid (1/10, m/v) in a cold mortar. Total glutathione (GSH + GSSG) was determined in the supernatant by 5,5′-dithio-bis-nitrobenzoic acid – GR recycling procedure (Griffith 1980). The reaction was monitored as the rate in absorbance change at 412 nm. Total glutathione was calculated from a standard curve, which was based on GSH in the range of 0 - 50 μM. GSSG was determined after removal of GSH from the sample extract by 2-vinylpyridine derivatisation. GSH was calculated by subtracting the amount of GSSG from total glutathione.

**Fluorescence measurements:** Photochemical efficiency ($F_v/F_m$) of electron transport through photosystem 2 (PS 2) was specified from chlorophyll fluorescence induction kinetic. It was measured after 15 min dark period with the *PAM Chlorophyll Fluorometer* (*Walz*, Effeltrich, Germany) on detached fresh cotyledons at room temperature. Several cotyledons (3 - 5) were stuck on a tape to cover the whole area. Measuring irradiance was 0.35 μmol $m^{-2}s^{-1}$, actinic irradiation 200 μmol $m^{-2}s^{-1}$, 700 ms saturated flash of 2 500 μmol $m^{-2}s^{-1}$. The *DA 100 Data Acquisition System* (*Walz*, Effeltrich, Germany) was used for sampling, control and calculation.

**Content of pigments:** The content of photosynthetic pigments was determined in acetone extract of cotyledons by HPLC (*Spectra-Physics*, San Jose, USA) using a reverse phase column *Sepharon* SGX C18 (*Tessek*, Praha, Czech Republic). The solvent system was acetonitrile: methanol:water (80:12:6) followed by 100 % methanol. The gradient run was 25 min, flow rate 1 cm $min^{-1}$, the detection wavelength 445 nm.

**Protein content** was determined by the Bradford (1976) method, with standard curves prepared using bovine serum albumin.

**Statistical evaluation:** All experiments were repeated four times; in the case of each experiment measurements were replicated 3 times. Similar results and identical trends were obtained in all experiments. The data reported here are from a single experiment with three replications. Data and statistical significance of difference were evaluated with analysis of variance (*ANOVA*) using program *NCSS* 6.0 (Kaysville, USA).

## Results

Cotyledons of C plants abscised on the 16$^{th}$ day, cotyledons of CL plants on the 13$^{th}$ day. Photochemical efficiency ($F_v/F_m$) decreased with increasing age (Fig. 1*A*). For the first 10 d there were no differences between both cultivations. On the 13$^{th}$ day photochemical efficiency in CL plants was significantly lower comparing both to 13- and to 16-d-old C plants.

For the first 13 d the content of total chlorophyll (Chl) increased in C plants, then sharply decreased (Fig. 1*B*). In CL plants there were no statistically significant changes during their whole life span. Chl content in 10- and 13-d-old CL plants was significantly lower than in the C plants of same age. Chl *a*/*b* ratio in C plants decreased between the 8$^{th}$ and 10$^{th}$ day, then it insignificantly increased (Fig. 1*C*). In CL plants the ratio increased during their whole life span.

The content of β-carotene increased more than twice during the first 13 d in C plants, then it decreased at the end of life span (Fig. 1*D*). Its content in CL plants slightly increased during their whole life span. For the first 10 d β-carotene content in CL plants was significantly higher but on the 13$^{th}$ d the content was practically the same as in C plants.

The ratio of total chlorophyll to total carotenoid contents increased up to 10$^{th}$ day in C plants, then it decreased. In CL plants the ratio slightly increased but the changes were not significant during whole life span (Fig. 1*E*). In CL plants, this ratio was always lower compared to controls.

SOD activity in both cultivations was practically the same on the 8$^{th}$ day, but on the 13$^{th}$ day it significantly dropped in CL plants: to less than 50 % (Fig. 2*A*). APOD activity was very similar in both cultivations (Fig. 2*B*). After a transient elevation, GR activity slightly decreased in both cultivations. Except the youngest plants, it was significantly lower in CL plants. CAT activity decreased during their life span in both cultivations. It was significantly lower in CL plants during their whole life span comparing to C plants (Fig. 2*D*).

Ascorbate (AsA)/dehydroascorbate (DHA) ratio

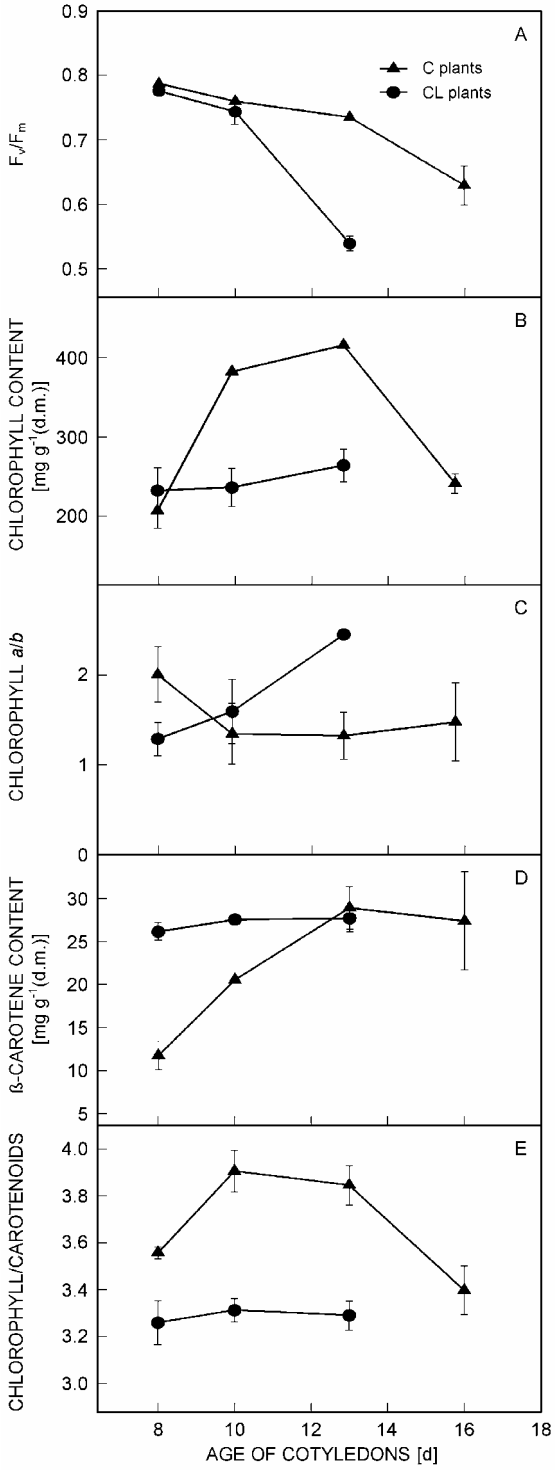


Fig. 1. Maximal efficiency of photosystem 2 ($F_v/F_m$) (*A*), content of total chlorophyll (*B*), chlorophyll *a*/*b* ratio (*C*), content of β-carotene (*D*) and chlorophyll/carotenoids ratio (*E*) in *Phaseolus vulgaris* cotyledons with natural life span (C plants) and with life span shortened by continuous irradiation (CL plants). Measurements were repeated three times. Means and standard deviations are given. Where *bars* are not shown, they are smaller than points in the plot.

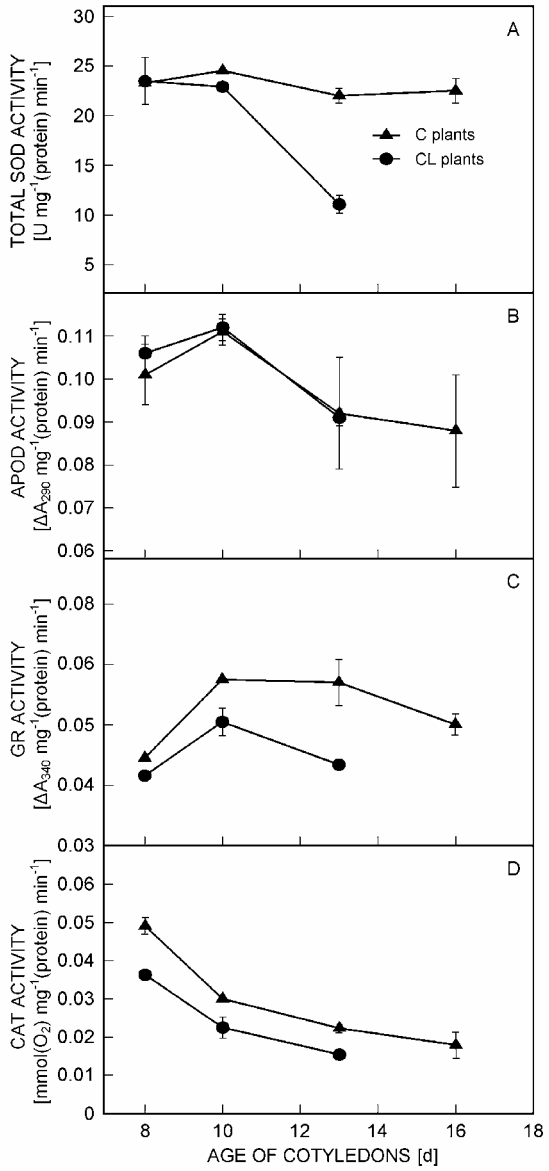


Fig. 2. Activity of total superoxide dismutase (*A*), ascorbate peroxidase (*B*), glutathione reductase (*C*) and catalase (*D*) in *Phaseolus vulgaris* cotyledons with natural life span (C plants) and with life span shortened by continuous irradiation (CL plants). One unit of SOD was defined as the amount of enzyme necessary to produce a 50 % inhibition of the cytochrome *c* reduction rate. Measurements were repeated three times. Means and standard deviations are given. Where *bars* are not shown, they are smaller than points in the plot.

decreased in both cultivations (Fig. 3*A*). In CL plants it was significantly lower on the 8$^{th}$ day comparing to C plants but the changes during the life span of CL plants were not statistically significant. On the 13$^{th}$ the ratio was lower in C plants.

GSH/GSSG ratio decreased in both cultivations (Fig. 3*B*). In the youngest cotyledons, it was approximately half in CL plants, otherwise it was almost identical.

## Discussion

The effect of irradiance on induction of senescence is complex, and many reports have been published describing both its senescence-inhibiting and senescence-promoting effects. The latter have been mostly reported in a context with relatively high irradiance, which induces leaves senescence (Biswal and Biswal 1984, Noodén *et al*. 1996, Weaver and Amasino 2001). In our experiments cotyledon senescence induced by continuous irradiation occurred earlier than natural senescence: the life span of cotyledons of C plants was 16 d, of CL plants only 13 d.

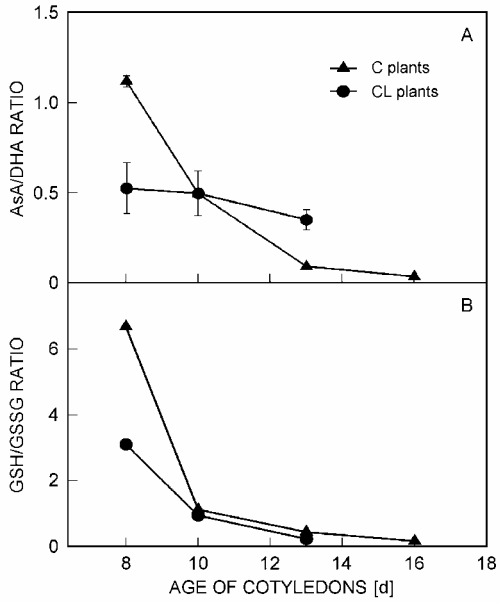


Fig. 3. Reduced/oxidised ascorbate ratio (*A*) and reduced/ oxidised glutathione ratio (*B*) in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with life span shortened by continuous irradiation (CL plants). Measurements were repeated three times. Means and standard deviations are given. Where *bars* are not shown, they are smaller than points in the plot.

Chlorophyll (Chl) content is a generally used marker of leaf senescence (Thimann 1980, Matile 1992, Hillman *et al*. 1994, Wilhelmová *et al*. 1997). It can be assumed that the onset of senescence occurred after the 13$^{th}$ day in C plants because from the 13$^{th}$ day both Chl content and photochemical efficiency (expressed as $F_v/F_m$ ratio) decreased. Surprisingly, no drop in Chl content was observed in CL plants during ageing, although a marked decrease of photochemical efficiency was observed. Chl content was significantly lower in CL compared to C plants. This could represent adaptative mechanism with consequence of lowering absorbed energy. It can be also deduced from changes in Chl *a*/*b* ratios, what gives evidence that light harvesting complex was reduced with age in CL cotyledons. As the light harvesting complex contains equimolar amounts of Chl *a* and *b*, reduction of its content results in a shift in the Ch *a*/*b* ratio in favor of Chl *a* (Hilditch 1986, Wilhelmová *et al*. 1997). The degradation of light harvesting complex could be another protective mechanism against continuous irradiation. It was observed earlier that increased light sensitivity and oxidative damage can be the reason why higher irradiance or longer duration can be detrimental for senescing leaves and enhance Chl breakdown (Goldthwaite and Laetsch 1967, Biswal and Biswal 1984, Kar 1993).

The sharp increase of Chl *a*/*b* ratio in CL plants indicated that during continuous irradiation the rapid transformation of Chl *b* to Chl *a* could also take place. In other words, the decrease of a green colour must be associated with the funneling of Chl *b* into the Chl *a*-form pool (Matile *et al*. 1999).

Most plants are able to adapt themselves to different light environments by changing the photosynthetic pigment composition of their green tissues (García-Plazaola *et al*. 2002). Carotenoids can also fulfil protective role as they lose excitation energy relatively rapidly in the form of heat. The significantly higher content of β-carotene in young CL plants indicated the higher demand for protecting against photodamage. The ratio of total Chl to total carotenoids content in CL plants suggests that these plants maintained very similar content of carotenoids during ageing in order to protect cells against the destructive effect of continuous irradiation.

Senescence of CL plants was probably accelerated by photodamage. Activities of all antioxidative enzymes declined either with senescence onset or during whole life span. As SOD activity sharply decreased in CL plants, it is possible that the production of hydrogen peroxide, which inhibites SOD, had to be very strong. Pastori and del Río (1994) observed that catalase activity decreases during natural senescence. We also found that CAT activity decreased in both cultivations but its activity was significantly lower in CL plants compared to C plants. It is in agreement with theory that light impairs the activity of CAT (Heinze and Gerhardt 2002). The photo-inactivation of is presumably due to irreversible photo-oxidation. With decreased CAT activity the redundant hydrogen peroxide could easily leak out of peroxisomes (Boveris *et al*. 1972, Pastori and del Río 1994). APOD activity was almost the same in both cultivations. Due to its decrease from the 10$^{th}$ day the protective mechanisms against hydrogen peroxide was weakened.

Since the reduction of GSSG is dependent on NADPH, which is mainly provided by the photosynthetic electron transport, a light-dependence of the reduced to oxidized glutathione ratio might be expected (Tausz 2001). When the capacity to reduce GSSG is below the capacity of GSH oxidation, it results in the decreased GSH/GSSG ratio, which is a strong indicator of oxidative

stress (Sies 1985, Albrecht and Wiedenroth 1994). However, except the youngest cotyledons, we did not observed marked difference between the two cultivations during ageing.

Reduced to oxidized ascorbate ratio did not decrease so sharply in CL plants compared to C plants. The biosynthesis of ascorbate is induced by light (Shigeoka *et al*. 1979). Although the shift from reduced to oxidized ascorbate occurred in CL cultivation it is probable that the higher synthesis of reduced ascorbate could contribute to maintaining of ratio of reduced and oxidized ascorbate.

Continuous irradiation in all probability triggered earlier senescence in cotyledons of CL plants. Their antioxidative capacity decreased with ageing and was not sufficient for protecting their cells from photodamage, although content of β-carotene and ratio of AsA/DHA were higher compared to C plants. APOD activity and its time course was practically the same in both cultivations. CAT activity in CL plants was lower because of its photooxidation, rapid decrease of SOD activity after the $10^{th}$ day could be caused by damage due to increased hydrogen peroxide content. APOD activity was practically the same in both cultivations. The important protection against photodamage caused by CL was represented by adaptative mechanism with consequence of lowering absorbed energy. While it was possible to determine the onset of senescence in C plants by generally used marker of senescence – decrease of chlorophyll content – such a sharp decrease not found in CL plants. The senescence onset in CL plants was estimated by the decrease of photochemical efficiency.

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